CHEMICAL MODIFICATION OF LYSOZYME

by

Karl Joseph Kramer

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CHEMISTRY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 7 1

THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Karl Joseph Kramer

entitled Chemical Modification of Lysozyme

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

Dissertation Director — Date: 1/4/71

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

	1/4/71
	1/4/71
	1/4/71
	1-4-71

*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Karl Joseph Kramer

To My Mother and Father

ACKNOWLEDGMENTS

The author would like to thank Professor John A. Rupley for his support and guidance throughout the course of this research. Dr. Rupley showed great patience waiting for this author to produce. His dedication to research is most inspiring.

The financial assistance of the National Institutes of Health, Public Health Service, is gratefully acknowledged.

The author also wishes to thank Pat Adams, Virginia Gates, and Douglas Rogers for technical assistance through portions of this work.

Finally, the author is particularly indebted to his wife, Virginia, and mother, Olivia, for their patience, encouragement, and understanding.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--Continued

LIST OF ILLUSTRATIONS--Continued

LIST OF TABLES

LIST OF TABLES--Continued

ABSTRACT

The carboxyl groups of lysozyme (there are ten total carboxyls) were reacted with a $1.7 \times 10^{+3}$ molar excess of sulfanilic acid (SA) using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) at pH 4.0. The product was chromatographically homogeneous on Biorex-70 and was shown by UV analysis to have 90% of the carboxyl groups sulfanilated. Peptide analysis showed that all ten groups reacted significantly and that the sulfanilation could be utilized for extensive modification.

The methylation of lysozyme (1% in methanol-0.1M HCl) was studied to determine the reactivity of each carboxyl group. It had been reported that one or two groups were slow to react. The overall rate of methylation, determined with radioactive methanol, showed that one or two groups reacted about ten times slower than the average. Peptide mapping analyses of sulfanilated methylated lysozymes on Sephadex SEC-25 determined quantities of unmethylated carboxyl groups as a function of methylation time and individual rates of methylation assuming first-order kinetics. Four rate classes were seen, with Asp 66 being the least reactive group. The reactivity in methanol-HCl was discussed with reference to the known crystal structure. The effect of methylation on

enzymic activity and the binding of inhibitors was interpreted in terms of essential carboxyl groups being methylated.

Previously the pH dependence of the solubility of lysozyme in 1.7M NaCl suggested that the conformation in the crystal may differ from that in solution. Since association of soluble protein can complicate interpretation of solubility measurements, molecular weight studies were conducted. No pH effect on the sedimentation rate of lysozyme under conditions of crystallization was shown, indicating that the solubility behavior was not a reflection of self-association.

Thyroxine has been reported to interact with lysozyme. Measurements of the lysozyme spectrum and of the binding and hydrolysis of the $\beta(1\rightarrow4)$-linked trimer and hexamer of N-acetyl-glucosamine, in the presence and absence of near saturating concentrations of thyroxine, showed that if thyroxine does bind, it binds to a region of lysozyme apart from the active site.

INTRODUCTION

Lysozyme from hen's-egg-white is a relatively small enzyme with an isoelectric molecular weight of 14307. It is a glycosidase which hydrolyzes N-acetylhexosaminidic linkages in bacterial cell walls, chitin, and various high and low molecular weight compounds derived from these polymers. These investigations were initiated to study the chemistry of the carboxylic acid residues, the state of aggregation in concentrated salt solution, and the site of binding with thyroxine. Lysozyme was chosen for this work because it is easily obtainable, well characterized, and its three-dimensional crystallographic structure has been determined (Blake et al., 1967a, 1967b).

Chemistry of Lysozyme

Lysozyme was discovered in nasal mucosa in 1922 by Alexander Fleming and was first crystallized by Abraham and Robinson (1937). Today, it is routinely prepared according to the method of Alderton and Fevold (1946) by crystallization from a 5% sodium chloride suspension of hen's-egg-white at pH 10.5, the isoelectric point (Jollés, 1960). It is acid and heat stable and hydrolyzed by pepsin in its native state but not by trypsin, chymotrypsin, or papain (Jollés, 1960).

In 1952 Salton showed that the substrate for lysozyme in the Gram-positive organism, Micrococcus lysodeikticus, is the cell wall mucopolysaccharide, a complex material containing both sugars and peptides. The first indication of the chemical linkage attacked by lysozyme came in 1957 when Berger and Weiser showed that the HEW enzyme hydrolyzes chitin, a linear $\beta(1\rightarrow 4)$ polymer of N-acetyl-D-glucosamine (NAG). Salton and Ghuysen (1960) showed that in bacterial cell walls lysozyme hydrolyzes $\beta(1\rightarrow 4)$ glycosidic bonds of alternating copolymers of N-acetylglucosamine and N-acetylmuramic acid.

In 1963 Jollés et al. and Canfield independently elucidated the complete primary structure of HEW lysozyme, which is comprised of a single polypeptide chain of 129 amino acids. The positions of the four disulfide bonds were later established (Jollés, Jauregui-Adell, and Jollés, 1964; Brown, 1964; Canfield and Lui, 1965). The complete formula is indicated in Figure 1. The only correction to the sequence is that residue 103 is now understood to be an asparagine (Brown, 1970). It is of particular interest that lysozyme has two glutamic acid residues (Glu), seven aspartic acid residues (Asp), and leucine (Leu) as the carboxyl terminal, a total of ten carboxyl groups.

In 1965 Phillips and his co-workers described the three-dimensional structure of lysozyme, based on X-ray crystallographic studies which had been initiated in 1960

Tryptic Peptide No.	Sequence	Carboxyl Group Residue
	1	
1	H_2N-Lys-	
2	Val-Phe-Gly-Arg-	
	7 10	
3	Cys-Glu-Leu-Ala-Ala-Ala-Met-Lys-	Glu 7
4	Arg-	
	18 20	
5	His-Gly-Leu-Asp-Asn-Tyr-Arg-	Asp 18
6	Gly-Tyr-Ser-Leu-Gly-Asn-Try-Val-	
	30	
	Cys-Ala-Ala-Lys-	
	35 40	
7	Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-	
	Thr-Asn-Arg-	Glu 35
	48 50 52	
8	Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-	Asp 48,
	60	
	Ilu-Leu-Gln-Ilu-Asn-Ser-Arg-	Asp 52
	66	
9	Try-Try-Cys-Asn-Asp-Gly-Arg-	Asp 66
	70	
10	Thr-Pro-Gly-Ser-Arg-	
	80	
11	Asn-Leu-Cys-Asn-Ilu-Pro-Cys-Ser-Ala-	
	87 90	
	Leu-Leu-Ser-Ser-Asp-Ilu-Thr-Ala-Ser-	
	Val-Asn-Cys-Ala-Lys-	Asp 87
12	Lys-	
	100 101	
13	Ilu-Val-Ser-Asp-Gly-Asn-Gly-Met-Asn-	
	110	
	Ala-Try-Val-Ala-Try-Arg-	Asp 101
14	Asn-Arg-	
15	Cys-Lys-	
	119 120	
16	Gly-Thr-Asp-Val-Gln-Ala-Try-Ilu-Arg-	Asp 119

Figure 1. Amino acid sequence and tryptic peptides of HEW lysozyme

17	Gly-Cys-Arg- 129	
18	Leu-COOH	α-COOH

Disulfide bond pairings: 6-127, 30-115, 64-80, 76-94.

Figure 1.--Continued

(Blake et al., 1965). A Fourier map of electron distribution at 2 A resolution was produced which showed clearly the structure of lysozyme, making it the first enzyme whose three-dimensional structure was established (Blake et al., 1967a, 1967b). Analysis of this structure and that of the complex formed from the enzyme and its inhibitors, NAG or its trimer, showed the location of each amino acid residue and interactions with the inhibitors. The binding site, diagramed in Figure 2, is a cleft in the surface of the molecule which contains three carboxyl groups, Glu 35, Asp 52, and Asp 101. The non-reducing terminal of trimer is bound at the top of the cleft, in a region or subsite designated A. There is a hydrogen bond between its NH group and the side chain of Asp 101. The second residue (B) makes a hydrogen bond between O_6 and Asp 101. Three more residues of NAG can be added to the tri-NAG in such a way that the entire binding site is filled and satisfactory interactions occur (subsites D-F). Rupley (1967) showed that the enzyme cleaves hexa-NAG into a tetramer and a dimer, the cleavage occurring between the carbon atom 1 and the bridge oxygen of the residue in the D subsite. The most reactive groups in the region of bond cleavage are the carboxyl groups of Glu 35 and Asp 52. A mechanism of action has been proposed involving these residues and the distortion of the sugar ring occupying subsite D (Figure 3).

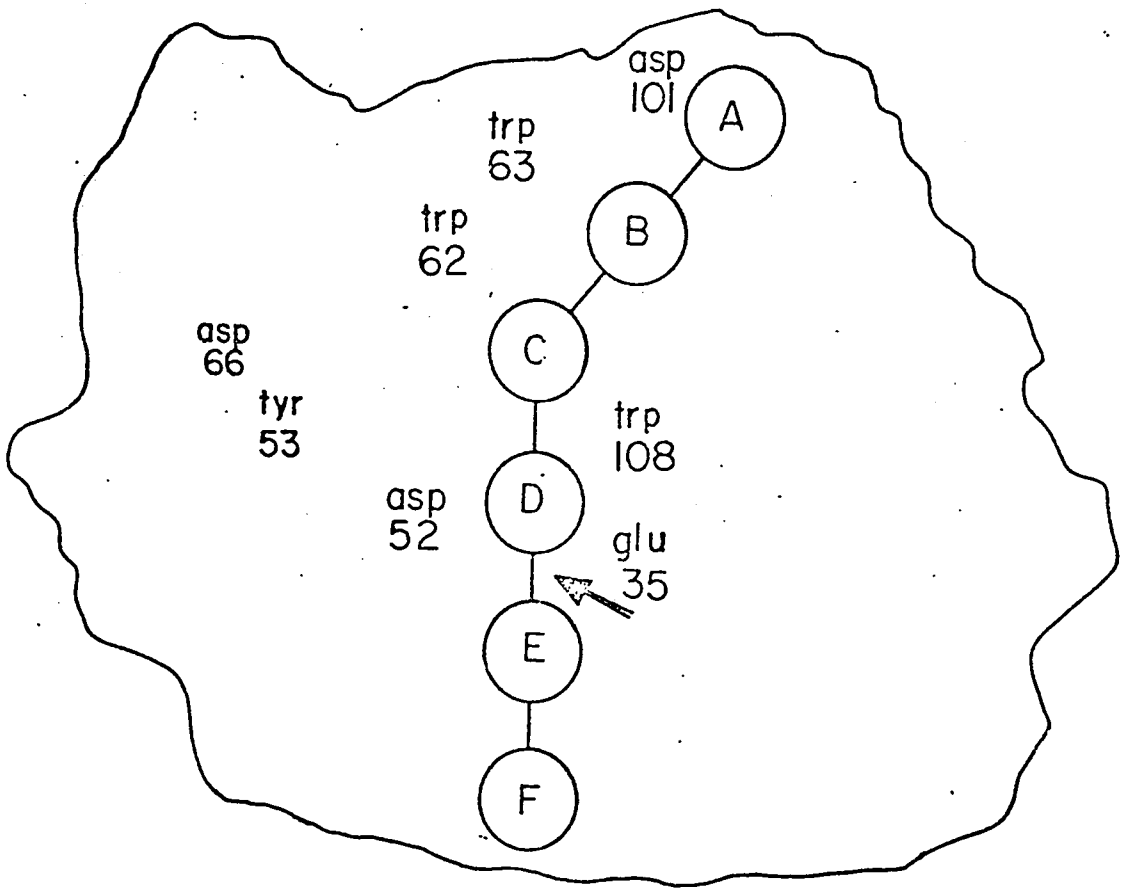
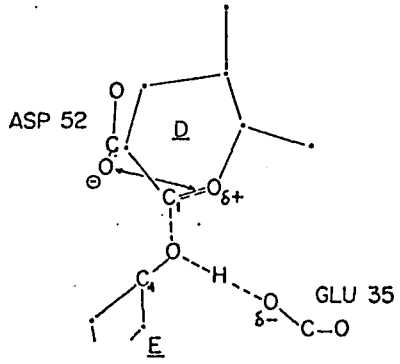


Figure 2. Sketch of the active site of lysozyme.

THE MECHANISM OF LYSOZYME CATALYSIS, PROPOSED BY PHILLIPS & COWORKERS



TRANSITION STATE FOR CLEAVAGE OR FORMATION OF A GLYCOSIDIC BOND

1. DISTORTION OF RING D INTO HALF-CHAIR CONFORMATION THROUGH BINDING
2. BREAKING OF C_1-O BOND, AIDED BY:
   GLU 35 – GENERAL ACID CATALYST
   ASP 52 – ELECTROSTATIC CATALYST
   DISTORTION OF RING D
3. C_1-CARBONIUM ION STABILIZED AS ION PAIR FORMED WITH ASP 52
4. DIFFUSION AWAY OF FIRST PRODUCT
5. REACTION OF CARBONIUM ION WITH AN ACCEPTOR, IN REVERSAL OF CLEAVAGE PROCESS

Figure 3. Mechanism of lysozyme catalysis.

The carboxyl group of Glu 35 appears in good position to protonate the D-E bridge oxygen, that is, to act as a general acid catalyst for the cleavage of the glycosidic bond. Distortion of the pyranose ring at subsite D toward the half chair conformation by means of binding interactions weakens the carbon 1 bridge oxygen bond and is the conformation expected in which a carbonium ion on carbon 1 would be stabilized by sharing its charge with the ring oxygen atom (Lemieux and Huber, 1955). The carboxylate group of Asp 52 would presumably be in the form of an anion to act as a nucleophile or a general base, or simply stabilize the carbonium ion by means of its negative charge.

Justification of Carboxyl Groups Study

At the beginning of this research it was known that a number of other laboratories were also investigating the chemistry of the carboxyl groups of lysozyme. Groups headed by Kravchenko at the Zelinski Institute in Moscow, Hayashi at Kyushu University in Japan, and Raftery at California Institute of Technology were studying the effects of mild alcohol-HCl esterification. Also, Koshland at Berkeley and Hayashi were looking at heavy and light carbodiimide modifications, respectively. In order not to overlap with these investigators, a study of the exhaustive methylation of lysozyme was undertaken.

Donovan, Laskowski, and Scheraga (1960) had observed that one carboxyl residue was resistant to esterification in methanol-HCl. The intent of this present study was to correlate individual reaction rates of carboxyl groups with loss of structure and of hydrolytic and substrate binding functions, suggesting the assignment of roles to specific side chains. To determine reaction rates, a relatively easy method of detection and quantitation of individual carboxyl groups was developed utilizing carbodiimide coupling and peptide mapping procedures. Also, this study indicated whether reactivity of lysozyme in methanol-HCl was consistent with that expected from the crystal structure.

Chemistry of the Carboxyl Groups of Lysozyme

As stated above, carboxyl groups are most likely participants in the enzymic mechanism of lysozyme. Proton and saccharide binding data demonstrated that several carboxyl groups are perturbed upon formation of the enzyme-trimer and tetramer complexes (Rupley, 1967; Banerjee and Rupley, 1971). pH rate profiles for tri- and hexasaccharide hydrolyses also indicated carboxyl participation (Rupley, 1967; Rand-Meir, Dahlquist, and Raftery, 1969; Rupley, Kregar, and Turk, 1970). Acid titration studies showed carboxyl groups with abnormally low and high pK's which normalized under denaturing conditions

(Tanford and Wagner, 1954; Donovan et al., 1960; Sakakibara and Hamaguchi, 1968). A pKa of 6.1 was attributed to Glu 35 because of its non-polar environment (Blake et al., 1967a), effect on tryptophan absorption since Glu 35 is three angstroms away from Try 108 (Donovan, Laskowski, and Scheraga, 1961; Imoto and Rupley, 1970), and saccharide binding (Dahlquist and Raftery, 1968; Banerjee and Rupley, 1971). A pH difference titration of the β-ethyl ester derivative of Asp 52 versus native lysozyme indicated a pKa of 5.9 for Glu 35 and 4.5 for Asp 52 (Parsons and Raftery, 1970).

Chemical modification of lysozyme has also implicated carboxyl groups in enzymic mechanism. Ethylation of Asp 52 with triethyloxonium fluoroborate abolished enzymic activity (Parsons and Raftery, 1969). Lin and Koshland (1969) coupled all the carboxyl groups with aminomethanesulfonic acid except those of Glu 35 and Asp 52 using carbodiimide and activity toward M. lysodeikticus was not abolished. Modification of Asp 52 did abolish activity. Hartdegan, Imoto, and Rupley (1970) inactivated lysozyme by reaction with iodine which formed an intramolecular ester linkage between the γ-carboxyl group of Glu 35 and the indole 2 carbon of Try 108. This ester derivative possessed essentially the same conformation of the active enzyme (Blake, 1970) and exhibited identical binding, but was inactive toward both cells and hexa-NAG. This

modification clearly indicated Glu 35 to be catalytically important.

Chemical Modification of Carboxyl Groups in Proteins

A number of methods have been used to modify carboxyl groups in proteins (Glazer, 1970; Spande et al., 1970; Stark, 1970). The most popular of these include esterifications with alcohol-HCl, triethyloxonium fluoroborate, isoxazolium salts, and water-soluble diazo compounds, and coupling with a nucleophile through the use of water-soluble carbodiimides.

Treatment of a protein with methanol-HCl has the advantage that all or most of the carboxyl groups can be esterified, but the effect of the organic solvent on the conformation of the protein may interfere with the investigation of particular properties of interest (Wilcox, 1967). The only side reactions that occur appear to be the methanolysis of amide groups, and the N-to-O acyl shift in peptide bonds which involve linkages to serine and threonine (Figure 4).

The most thorough investigation of a protein methanol-HCl esterification was that done on ribonuclease by Broomfield, Riehm, and Scheraga (1965a, 1965b). Eight of the eleven carboxyl groups were methylated while maintaining many physiochemical properties similar to the native molecule. Proteolytic digestion and peptide

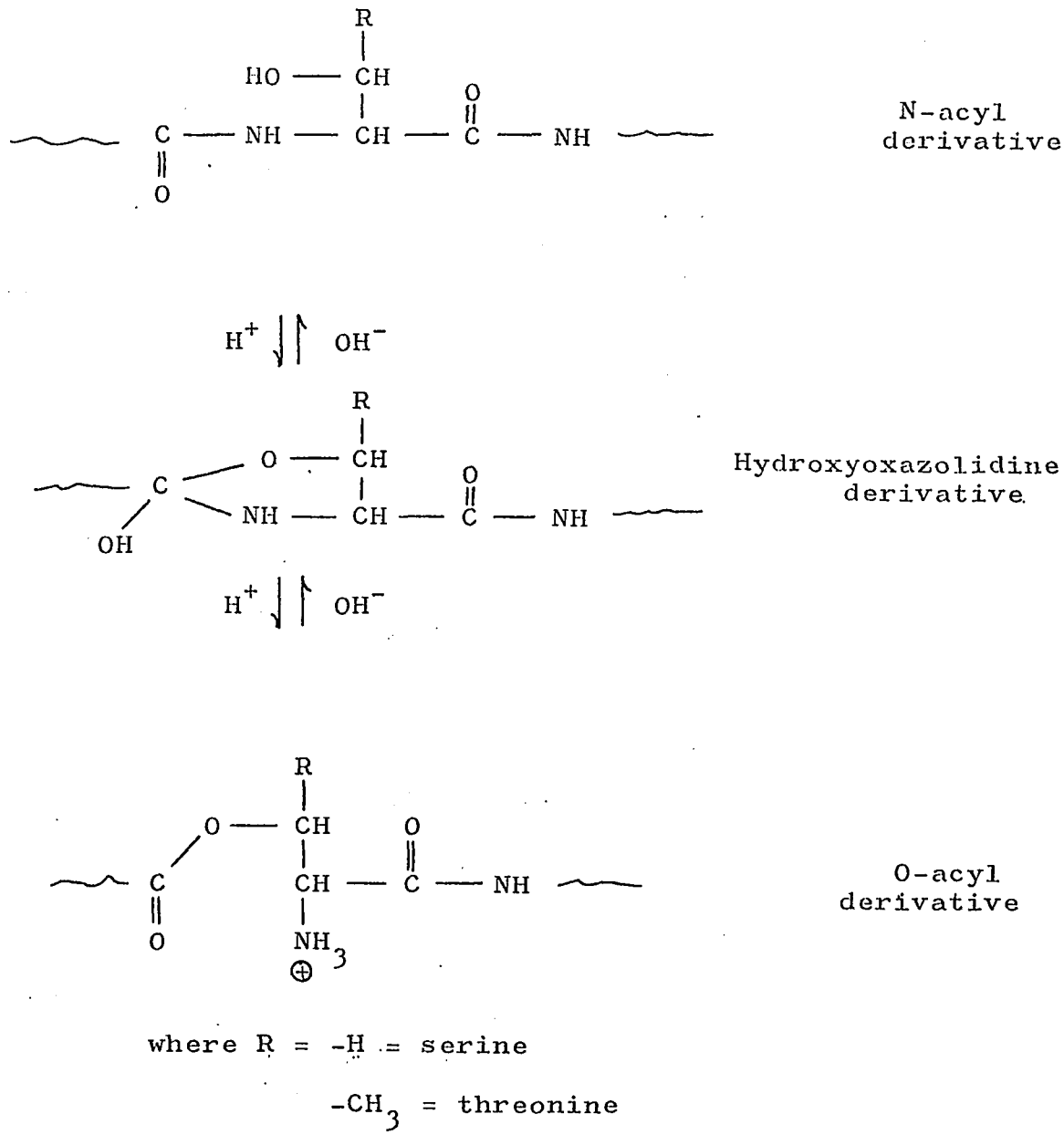


Figure 4. N-to-O acyl rearrangement of the peptide bond -- Iwai and Ando (1967).

analysis demonstrated that the unmethylated residues were Asp 14, Asp 38, and Asp 83. Crystallographic analysis (Wyckoff et al., 1970) showed that two of the three abnormal tyrosines (Woody, Friedman, and Scheraga, 1966) are hydrogen bonded to two of these unmethylated aspartic acids, Asp 38 → Tyr 92 and Asp 14 → Tyr 25. Thus, it appeared that even though the conformation in methanol-HCl was probably different from that in aqueous solution, some structural similarities remained so that residues expected to be unreactive in aqueous solution were also unreactive in methanol-HCl.

The N→O acyl shift side reaction was best documented in a study of insulin methyl ester by Levy and Carpenter (1970). Two methylated products were isolated, each with all six carboxyl groups reacted. Derivative I irreversibly converted into II at pH values above 2.2. Deamination of II with nitrous acid produced an unexpected loss of threonine which occurred only at position 27 of the B chain. Conversion of a peptide to an ester bond (N→O shift) at threonine had introduced a new amino group that was susceptible to deamination.

The methylation of lysozyme has been studied by several workers. Fraenkel-Conrat (1950) was the first to report that esterification produces an inactive enzyme. Frieden (1956) and Fujio et al. (1959) reported that lysozyme methyl ester also inhibited native lysozyme

hydrolysis of bacterial cell walls. The forty-eight hour methylated derivative of Donovan et al. (1960), in which most of the carboxyl groups were esterified, showed a single titratable group in the carboxyl region to remain, suggesting that one carboxyl group was resistant to methylation. Kravchenko, Chentsova, and Kaverzneva (1967) showed that four hour methylated lysozyme resulted in a mixture of products with some fractions showing signs of denaturation. Parsons et al. (1969) esterified 5-6 carboxyl groups with ethanol-HCl. This derivative was 3-5% active against M. lysodeikticus cells and bound chitotriose at 5% of the binding strength of unmodified lysozyme.

The present work was concerned with the exhaustive chemical modification of the carboxyl groups of lysozyme, effecting esterification with methanolic hydrogen chloride, and the subsequent characterization of products using physical, chemical, and enzymatic techniques. Most particular, this work investigated the observation of Donovan et al. (1960) that one or two carboxyl groups of lysozyme reacted very slowly in methanol-HCl.

Perhaps the most popular specific method for modifying carboxyl groups in proteins involves the use of water-soluble carbodiimides. These carbodiimides react with carboxyl groups at slightly acidic pH to give an o-acylisourea (4), an activated intermediate that can in

turn either rearrange, condense internally, or react with a nucleophile, as shown in Figure 5 (Khorana, 1953; Hegarty and Bruice, 1970). Riehm and Scheraga (1966) used 1-cyclohexyl-3-(2-morpholiny 2-[4]-ethyl)-carbodiimide (CMC) in the absence of nucleophile to modify ribonuclease and suggested that imides and acylureas formed in the several partially reacted derivatives isolated. Riordan and Hayashida (1970) recently reported a similar modification with carboxypeptidase A, suggesting that an active center carboxyl, Glu 270, was modified. Hoare and Koshland (1966) modified proteins, one of which was lysozyme, with 1-benzyl- or 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) at pH 4.75 in the presence of high concentrations of nucleophiles. Since rearrangement was slow relative to nucleophilic reaction, the latter dominated with good nucleophiles such as glycinamide or aminomethane-sulfonate (each 1M). Quantitative modification was obtained only in the presence of denaturants such as urea or guanidinium hydrochloride. Lower yields were obtained with other nucleophiles employed, implying that steric or electrostatic factors may play an important role in reactivity.

Sulfhydryl, phenolic, and hydroxyl side chain groups were also reactive toward carbodiimides. Carraway and Triplett (1970) reported that 85% of the sulfhydryl groups of reduced bovine albumin or β-lactoglobulin react at pH 5.0. Lysozyme contains no cysteine residues for this

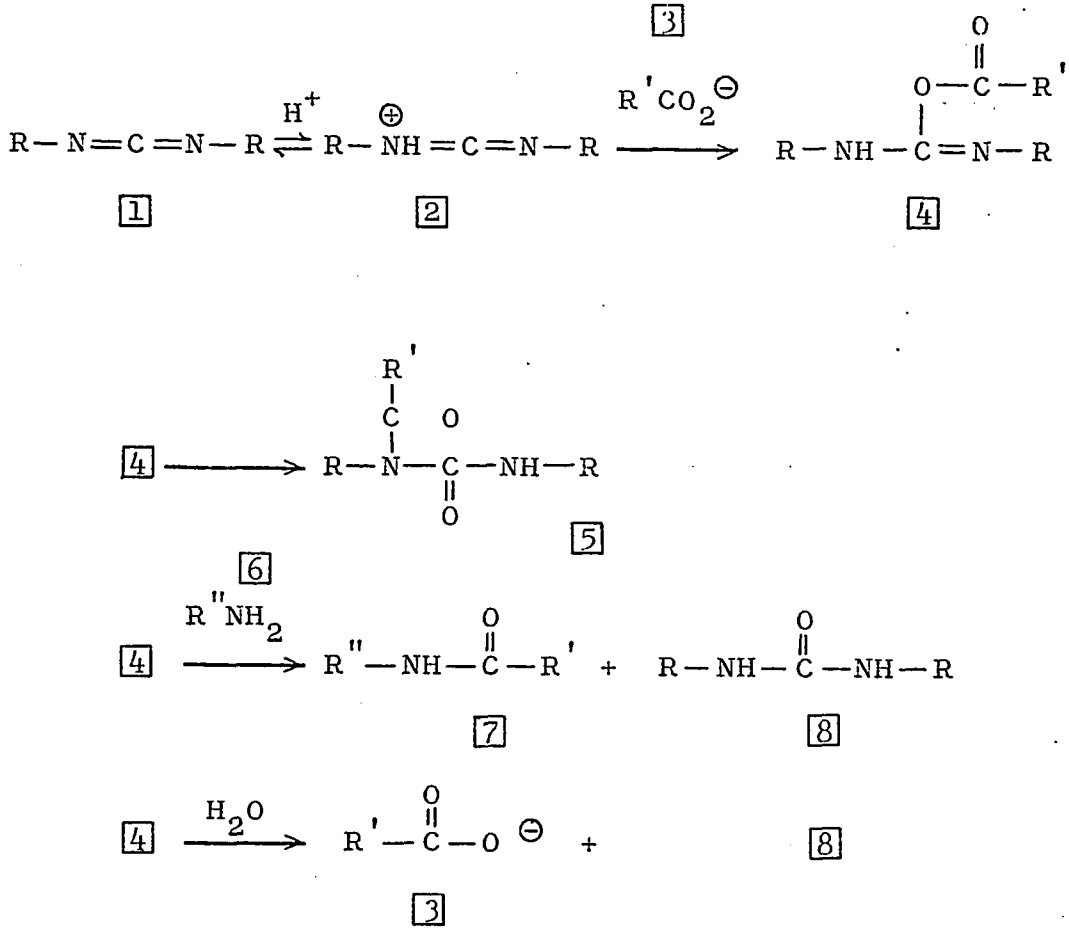
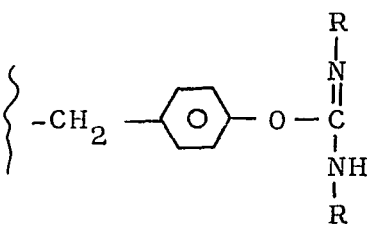


Figure 5. Reaction of carbodiimide with the carboxyl group -- Hegarty and Bruice (1970).

to occur. Carraway and Koshland (1968) found that one of four tyrosines in chymotrypsinogen reacts with a carbodiimide at pH 4.75 to probably form the O-arylisourea (see below), but that tyrosine could be regenerated by treatment with a suitable powerful nucleophilic reagent like hydroxylamine. Saponification treatment at pH 10.5 which should regenerate any modified tyrosine was used in the present study. Banks, Blossey, and Shafer (1969) have shown that CMC inactivates α-chymotrypsin by reaction with the active site serine residue.



O-aryl isourea

The most detailed studies of a carbodiimide catalyzed nucleophilic incorporation with a protein were those by Lin and Koshland (1969), Lin (1970), and Osawa (1970), all of which used EDC. The first of these has been discussed in a preceding section. The second (Lin, 1970) examined specific carboxyl group reactivity of lysozyme. Reaction with glycinamide at pH 4.75 led to modification of 8 out of 10 carboxyl groups with a loss of cell-wall lytic activity. Further treatment of the modified protein with ^{14}C-glycinamide in 4M guanidine hydrochloride incorporated 2.1 extra residues of nucleophile. Analysis of peptides from

tryptic hydrolysates showed that Glu 35 was essentially unreactive and that Glu 7 and Asp 66 were partially unreactive in the native conformation. Osawa (1970) reacted insulin with ethyl glycinate and found that all carboxyl groups can react but that some specificity is introduced at higher pH and shorter reaction times.

The present work was concerned with the development of a method for exhaustive modification of lysozyme carboxyl residues with a nucleophilic reporter group using EDC. Previously, quantitative reaction and identification of carboxyl groups have required laborious techniques such as denaturing solvent conditions and amino acid or radioactive tracer nucleophile analyses.

Sedimentation of Lysozyme in Concentrated Salt Solution

Cole, Bryan, and Bryan (1969) have shown that the solubility of crystalline lysozyme increases between pH 2 and 5. Solubility data as a function of pH can be used to evaluate the difference between the number of protons bound by a lysozyme molecule in the crystal and in solution. The data obtained indicated that a group titrating in this range has a higher pK in the crystal. Their examination of the crystal structure (Blake *et al.*, 1967a) showed no such group in a lattice contact to account for the perturbed pK. It was suggested that the conformation of lysozyme in the

crystal might differ from that in solution, specifically in the environment about one or more carboxyls.

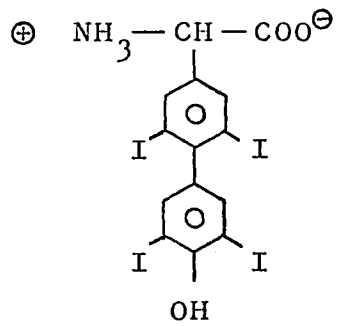
This suggestion conflicted with previous results that showed identical chemistry and presumably structure for crystal and solution. Butler and Rupley (1967) showed that NAG and di-NAG bind with equal strength to both crystalline and soluble lysozyme. Praissman and Rupley (1968) found that solution and crystalline lysozyme exhibited identical tritium-hydrogen exchange. These experiments reflected chemistry of groups distributed over a considerable portion of the lysozyme molecule and it was difficult to imagine them not being sensitive to a conformational change sufficient to perturb a carboxyl group.

Association of soluble protein can complicate interpretation of solubility measurements (Rupley, 1968). Differences in protons bound are related to degree of polymerization of soluble protein as well as to intrinsic effects under conditions of crystallization. To determine the degree of association of soluble protein under conditions of crystallization, gel filtration and sedimentation studies of lysozyme in 1.7M NaCl were undertaken in this study.

The Weak Interaction of Thyroxine With Lysozyme

Litwack (1963) reported that lysozyme was inhibited *in vivo* and *in vitro* by L-thyroxine, the amino acid

component of the thyroid hormone, thyroglobulin. Precipitation of a lysozyme-hormone complex was also presented as evidence for binding by Litwack and Sears (1965). Direct binding was observed using density-gradient centrifugation and fluorescence quenching by Viscidi, Consiglio, and Roche (1966). These results indicated that thyroxine might bind at the active site of lysozyme, i.e., in the region of the active site tryptophans (Blake et al., 1967a). Previously, several small molecules (Biebrich Scarlet, Rossi et al., 1969; cobalt ion, McDonald and Phillips, 1969) have served as useful probes of the lysozyme active site. The interaction of thyroxine with lysozyme was studied further to see whether thyroxine might be another.



thyroxine

EXPERIMENTAL

Materials

Twice crystallized lysozyme (lots LY 8AA and LY 9FA) and dried Micrococcus lysodeikticus cell-walls (lot ML 0606-23) were purchased from Worthington Biochemical Corporation; PTCK-treated trypsin (lot 73325), L-thyroxine and mercaptoethanol were from California Corporation for Biochemical Research; DL-thyroxine, mercaptoacetic acid, iodoacetic acid, and norleucine were from Mann Research Laboratories. The iodoacetic acid was further purified by recrystallization twice from ethyl ether-petroleum ether (30-60°) mixtures. Sulfanilic acid and deuterium oxide were products of Mallinckrodt Chemical Company and Stohler Isotope respectively. The β(1→4)-linked trimer, pentamer and hexamer of N-acetylglucosamine were prepared as previously described (Rupley, 1964).

1-Ethyl-3-dimethylaminopropylcarbodiimide and 1-cyclohexyl-3-(2-morpholinyl)-4-ethylcarbodiimide were purchased from the Ott Chemical Company and the Aldrich Company respectively. 1-Benzyl-3-dimethylaminopropyl-carbodiimide was synthesized from benzyl isocyanate (K and K Laboratories) and N,N-dimethyl-1,3-propanediamine (Aldrich) by the method of Sheehan, Cruikshank, and Boshert (1961). It was recrystallized from methylene

dichloride-ether as its p-toluenesulfonate salt with a melting point of 115°; lit. mp 118° (Hoare and Koshland, 1966).

The N-acetyl derivative of sulfanilic acid was prepared by warming an alkaline ethanolic solution of sulfanilic acid with acetic anhydride, cooling, and recrystallizing twice from ethanol-ethyl ether mixtures (Scheline and Longberg, 1967). The sodium salt decomposed at 270°. The nuclear magnetic resonance spectrum of N-acetylsulfanilic acid is shown in Figure 6. The measurement was performed in a Varian Model A-60 Spectrometer at 60 MHz in deuterium oxide with 5% H_2O as internal reference. Integration was done by area tracing using a Gelman Planimeter Model 39231. The spectrum is presented in tau (τ) units with peak description and integration in parentheses. Integration gives a ratio of 1.35 for the areas obtained from the aromatic protons relative to the methyl protons. The theoretical ratio is 1.33.

$^{14}CH_3OH$ and C^3H_3OH were purchased from New England Nuclear Corporation. These were diluted with nonradioactive methanol, refluxed with an alkaline furfural solution and distilled to remove an impurity(s) that reacted with lysozyme (Vogel, 1959). For scintillation counting, Omnifluor and 25 ml glass counting vials were purchased from New England Nuclear Corporation. The solvent for scintillation counting was prepared as follows: 8 grams of

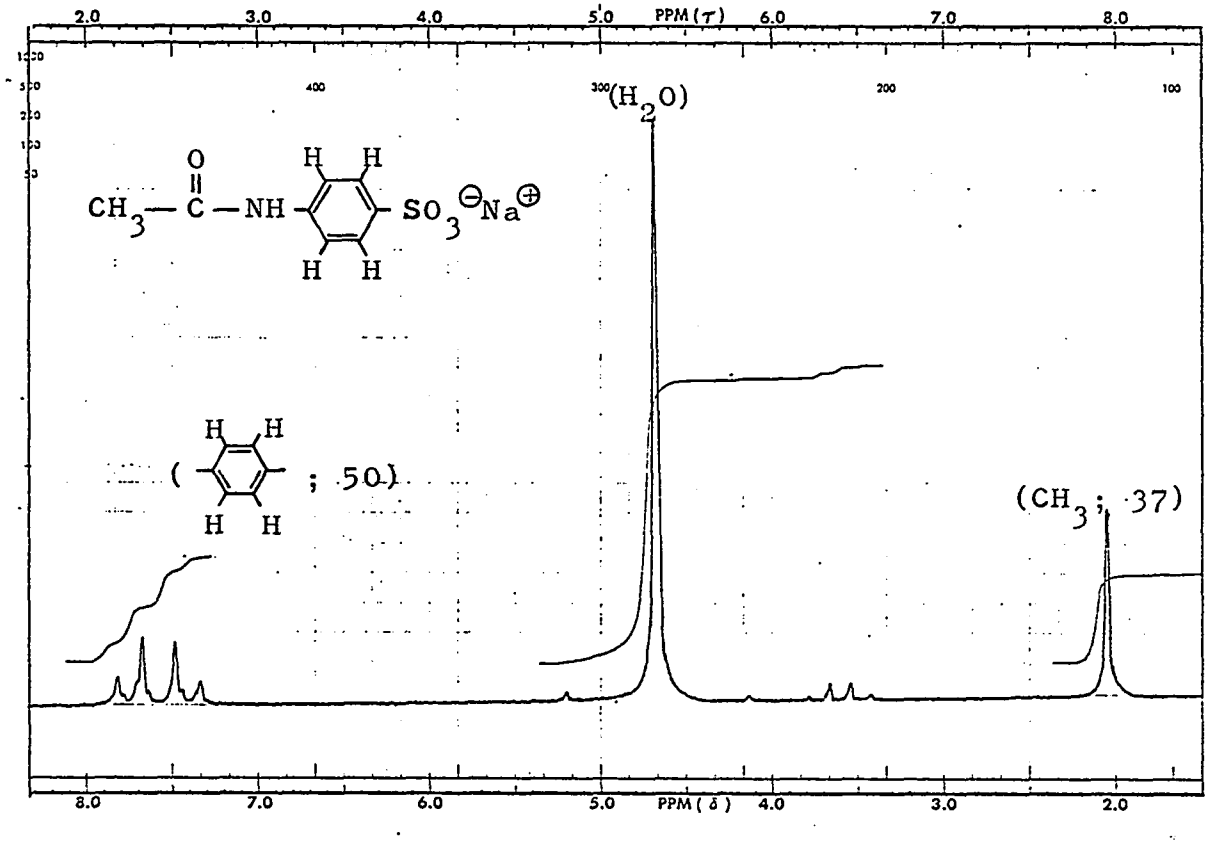


Figure 6. Proton magnetic resonance spectrum of N-acetylsulfanilic acid.

Omnifluor and 60 grams of napthalene (practical, Eastman Organic Chemicals) were dissolved in p-dioxane (Matheson, Coleman, and Bell, twice refluxed over sodium metal and stored in the dark). One hundred ml of anhydrous methanol (Mallinckrodt) and 20 ml of ethylene glycol (Matheson, Coleman, and Bell) were added and the solution was made up to 1-liter with p-dioxane and stored in the dark (Bray, 1960).

For amino acid and peptide analyses, ninhydrin, hydrindantoin, thiodiglycol, Brij-35, and methyl cellosolve were purchased from Pierce Chemical Company. ARW-7 alkaline wetting agent was purchased from Technicon Corporation.

For polyacrylamide electrophoresis, cyanogum-41, tetramethylethylenadiamine, ammonium persulfate and amido black were purchased from the E. C. Apparatus Company.

Sephadex G-25 and SEC-25 were purchased from Pharmacia; Biogel P-2, P-20, Biorex-70, Dowex-1x2 and 50x2 were from Bio Rad Laboratories.

All other chemicals were of highest purity commercially available. Water was deionized and freed of organics using a Continental system.

pH

pH was measured with a Beckmann 76, Radiometer TTTIC or Radiometer 26 meter standardized using pH 4.01

(0.05M phthalate) and 6.50 (Radiometer type S1001 concentrate) buffers. For difference spectra, a universal buffer of 0.1 ionic strength (phosphate-acetate-borate-KCl; Frugoni, 1957) was used to control pH.

Concentrations and Spectra

Absorbance measurements were made at fixed wavelengths with a Zeiss PMQII spectrophotometer. Absorption spectra were obtained with a Cary 15 recording spectrophotometer. Cylindrical and rectangular quartz cells used in spectrophotometric measurements were obtained from Pyrocell. Sample concentrations were determined by optical density measurements using the molar extinction coefficients listed in Table 1. Difference spectra were measured using a Cary 15 instrument on the 0-0.1 slide wire. Saccharide binding was measured by the difference spectra method of Banerjee and Rupley (1971), where the 293-289 nm peak-trough difference was the measure of association.

Lytic Activity

Rates of *M. lysodeikticus* cell-wall lysis were determined in 0.05M phosphate buffer, pH 7.1, using a Zeiss PMQII spectrophotometer equipped with a Varicord Photovolt Model 43 Linear-Log recorder. A stock suspension of cells was diluted into buffer to give a final volume of 3.1 ml and an absorbance at 450 nm of 1.0. Enzyme (0.05 ml) was added to a final concentration of 1-10 μg/ml (10^{-6}-10^{-7}M).

Table 1. Extinction coefficients for concentration measurements.

Sample	λnm	ϵ_M	Reference
1. Lysozyme	280	36500	a
	250	16200	
2. Sulfanilic acid (SA)	250	16200	b
	280	2014	
3. N-acetylsulfanilic acid (NASA)	250	15200	b
	280	1138	
4. Thyroxine	326.5	6450	c
5. Chymotripsinogen A	280	40000	d

[a]Hartdegan (1967).

[b]This work, pH 5.3, universal buffer (Frugoni, 1957).

[c]Sadtler Standard Spectra (1967).

[d]Wilcox, Cohen, and Tan (1957).

Initial rates were determined from the absorbance trace of turbidity decrease over the first minute of lysis and were expressed as a percentage of the rate obtained with an equal weight concentration of native lysozyme.

Oligosaccharide Hydrolytic Activity

Penta- and hexa-N-acetylglucosamine hydrolyses by lysozyme were followed during the initial 30% of reaction using a Technicon-Gilford system to measure the increase in ferro-ferricyanide color associated with the increase in

reducing group concentration (Rupley and Gates, 1967; Rupley et al., 1970). Hydrolyses were performed in sodium phosphate buffer (0.1M Na^+), pH 7.1.

Preparation of Lysozyme Methyl Esters

The methyl ester of lysozyme was prepared according to the procedure of Fraenkel-Conrat and Olcott (1945). Two grams of lysozyme were added with stirring to 200 ml of absolute methanol at room temperature. Two ml of concentrated HCl were then added dropwise to the suspension. The protein dissolved momentarily, but subsequently reprecipitated to give a very fine gel-like suspension. Aliquots were centrifuged and the precipitate dissolved in water, adjusted to pH 5 with 1N NaOH, frozen, and lyophilized.

Radioactive methyl ester of lysozyme was prepared using furfural refluxed radioactive methanol. In earlier work rate studies had failed because of impurities in commercial samples of radioactive methanol which resulted in radioactivity associated with the protein in unexpected high amounts. The gel-like solid remaining after centrifugation (about 2 mg) was dissolved in 0.05M ammonium acetate, pH 5.0, frozen or immediately chromatographed on a 1 x 50 cm column of Sephadex G-25 equilibrated with the pH 5 buffer.

Scintillation Counting

The ^{14}C or ^{3}H content of the lysozyme methyl ester was determined as follows: 1 ml of the solution of radioactive protein obtained from G-25 chromatography was transferred to 15 ml of scintillation solvent with stirring. The samples were counted in a Nuclear Chicago Liquid Scintillation Spectrophotometer Mark I (4-10 minute count times). A sample blank was also counted and subtracted. A quenching correction was determined by external standardization counting using barium-133 (ca. 200,000 cpm).

Reaction of Lysozyme With Sulfanilic Acid Catalyzed by Carbodiimide

The carboxyl groups of lysozyme were reacted with sulfanilic acid using the carbodiimides and solvents listed (Table 2) and utilizing a procedure similar to that of Hoare and Koshland (1967).

The sulfanilated lysozyme was purified by Sephedex G-25 chromatography in 0.05M ammonium acetate, pH 6.8. The incorporation of sulfanilic acid was followed by the increase in optical density at 250 nm relative to the optical density at 280 nm. Highest incorporation was obtained with EDC and 1.2M SA solution and the following conditions were used throughout this work: 1% lysozyme (10 mg/ml) was dissolved in 1.2M SA solution and the pH adjusted to 4.9-5.0 with a Radiometer pH stat. Solid EDC (0.05M) was added to the solution with stirring and the pH

Table 2. Reagents and solvents for carbodiimide reactions.

Carbodiimides:
1-ethyl-3-dimethylaminopropylcarbodiimide (EDC)
1-benzyl-3-dimethylaminopropylcarbodiimide (BDC)
1-cyclohexyl-3-(3-morpholinyl)-(4)-ethylcarbodiimide (CMC)
Solvent Composition:
a. 4M urea and 0.5M sulfanilic acid (SA)
b. 2M guanidine hydrochloride and 0.5M SA
c. Formamide and 0.5M SA
d. 1.2M SA (near saturation)

was maintained at 4.9 with 4N HCl. The tip of the acid delivery tube was located directly above the solution since sulfanilic acid tended to precipitate and block the tubing if the tip was located below the surface. The EDC addition was repeated after one hour. At the end of six hours total reaction time, the mixture was quenched in five volumes of 1M sodium acetate which produced a precipitate. The heterogeneous solution was dialyzed versus nine changes of 10^{-3}M HCl, frozen, and lyophilized. A soluble product resulted after the dialysis treatment. Lysozyme methyl esters subjected to the sulfanilic acid modification produced precipitates during sulfanilation. These also solubilized when dialyzed against dilute HCl.

Saponification of Lysozyme and Sulfanilated Lysozyme Methyl Esters

The methyl ester lysozyme derivatives were saponified to generate unmodified carboxyl groups at pH 10.5 as a 1% heterogeneous solution for 24 hours on a pH stat with 1N NaOH. Native lysozyme was similarly treated to further ensure that the only difference in treatment between native and lysozyme methyl ester was the methylation.

Gel Electrophoresis

Vertical gel electrophoreses of native, methylated, and sulfanilated lysozymes were performed at pH 5.0, 0.05M acetate, with 5% acylamide gel at 300 volts for three hours (E. C. Apparatus Technical Bulletin #128, 1966).

Biorex-70 Chromatography of Sulfanilated Lysozyme and Lysozyme Methyl Ester

Sulfanilated lysozyme was chromatographed on a 1 x 50 cm column of Biorex-70 at pH 7.5 in 5M urea utilizing a three chambered continuous gradient. Lysozyme methyl ester was chromatographed at pH 6.5. The gradient buffers are listed in Table 3.

Reduction and S-Carboxymethylation

The procedure of Canfield and Anfinsen (1963) was used. One hundred mg of saponified lysozyme and 3.61 grams of urea were dissolved in 3 ml of pH 8.6 tris buffer

Table 3. Gradient conditions for Biorex-70 chromatography.

Chamber 1	50 ml 0.02M Na_3PO_4
Chamber 2	50 ml 0.02M Na_3PO_4
Chamber 3	50 ml 0.02M Na_3PO_4, 1M NaCl

(5.23 grams tris + 9 ml 1N HCl + H_2O to give 30 ml of buffer with 0.2% EDTA). Water was added to give a volume of 12 ml. One-tenth of a ml of mercaptoethanol was reacted with stirring under N_2 for four hours at room temperature. Then 0.270 g of 2X recrystallized iodoacetic acid dissolved in 1 ml of 1N NaOH was added and reacted for fifteen minutes with the reaction vessel wrapped in aluminum foil to protect the solution from light. The sample was dialyzed against eight changes of distilled water in a one gallon container also protected from light, centrifuged, and the insoluble material dried under vacuum. Typical yield was 85 mg of reduced and S-carboxymethylated (RCM) lysozyme or lysozyme methyl ester.

Tryptic Hydrolysis

Eighty-five mg sample of RCM protein in 9.0 ml H_2O was reacted at 39° with PTCK treated trypsin (2% lysozyme weight) for four hours at pH 8, maintained by the addition of 1N NaOH with a pH stat. The trypsin was added 0.85 mg at a time, at the initiation of the reaction and one hour

later. In a typical experiment 11 moles of base were used per mole of protein. At the end of four hours, the reaction product was adjusted to pH 5 with 30% acetic acid and centrifuged. The supernatant was immediately applied to a Sephadex SEC-25 cation exchange chromatographic column. The soluble peptide yields for native and sulfanilated lysozymes were 95 and 75% respectively.

Peptide Separation in Sephadex SEC-25

Peptide samples (60-70 mg) were separated in jacketed 1.5 x 7.5 cm columns of Sephadex SEC-25 at 30°. The resin was prepared by washing with 0.5N HCl, H_2O, 0.5N NaOH, water, and then equilibrated with 0.1M acetic acid. The resin was degassed using a water aspirator immediately before pouring the column. The column was further equilibrated for four hours before sample application. The sample was eluted by pumping at 1.6 ml/min a gradient obtained by using an old nine chamber Technicon Varigrad. The gradient is listed in Table 4.

Fractions of 5.5 ml were collected and analyzed for optical density at 250 and 280 nm. Seven per cent of the eluent was collected for peptide analysis using the Technicon Autoanalyzer. The peptide analyzer automatically hydrolyzes the eluting peptides with NaOH to produce individual amino acids, neutralizes the hydrolysis mixture,

Table 4. Gradient conditions for Sephadex SEC-25 chromatography.

	ml 0.1N HOAC	ml 0.1N HOAC, 1N NaCl	M NaCl
Chamber 1	150	0	0.0
Chamber 2	135	15	0.10
Chamber 3	130	20	0.13
Chamber 4	120	30	0.20
Chamber 5	115	35	0.23
Chamber 6	110	40	0.27
Chamber 7	100	50	0.33
Chamber 8	80	70	0.27
Chamber 9	0	150	1.0

reacts with ninhydrin, and records at 570 nm (Technicon Technical Bulletin #T-67-101, 1967).

Dowex-1 and DEAE-cellulose absorbed sulfanilic acid peptides irreversibly. Dowex-50 gave no separation of sulfanilic acid peptides and low yields of tryptophan peptides.

Peptide Desalting on Biogel P-2 and Sephadex G-25

The peptide fractions were evaporated to dryness in a National Appliance Company Vacuum oven. For desalting, the sample was dissolved completely in either dilute acetic acid, ammonium hydroxide, or triethylamine and

applied to a Biogel P-2 column equilibrated with 0.1M acetic acid. Fractions of 0.5 ml were collected and tested with silver nitrate to locate salt-containing fractions which precipitated silver chloride. The salt-free and salt-containing fractions were combined to give fractions of 2.5-3 ml for absorbance analysis at 250 and 280 nm.

Most peptides would not desalt on Sephadex G-25 except for the tryptic peptide containing residues 62-68, which elutes well resolved from salt (Hartdegan, 1967; Hayashi et al., 1965).

Peptide Purification by Paper Chromatography

Certain salt-free peptide fractions were purified using Whatman #1 chromatography paper that had been treated by washing alternately with 0.1N HCl, H_2O, 30% HOAC, H_2O, and methanol. The two solvent systems used were

1. Butanol: pyridine: acetic acid: water (BPAW)
   15 10 3 12
2. Butanol: acetic acid: water (BAW, upper phase-chromatography; lower-chamber equilibration)
   4 1 5

Quantities applied never exceeded 0.5 mg/cm and phenol red was used as a reference marker. Chromatograms were developed in a Warner-Chilcott Chromatocab for 20 hours. An Ultraviolet Products Mineralight was used to detect fluorescent peptides. To detect ninhydrin positive

peptides, a guide strip was sprayed with the cadmium-ninhydrin reagent: 0.1 grams cadmium acetate, 5 ml acetic acid, 10 ml H_2O mixed with 1 gram ninhydrin dissolved in 100 ml acetone (Heilmann, Barollier, and Watzke, 1957). The color was developed at room temperature and observed at various times to determine the colors and locations of any bands. Peptide areas were eluted with dilute acetic acid or ammonium hydroxide. To obtain a UV analysis of eluted material, an equal area of paper which did not contain sample was cut out and treated similarly for a blank. Eluted peptide solutions were dried in a vacuum oven.

Peptide Purification by Paper Electrophoresis

Salt-free peptide fractions were purified using Whatman #1 paper treated as described in the previous section. The solvent systems used were:

1. pH 1.9, 8% acetic acid, 2% formic acid by volume.
2. pH 3.6, 100 ml acetic acid, 10 ml pyridine diluted to 3 liters.
3. pH 5.5, 0.025M triethylamine, acetic acid to desired pH.
4. pH 8.5, 0.025M triethylamine, CO_2 (dry ice) to desired pH.

Electrophoreses were carried out using 2000 volts for 45-90 minutes in a Servonuclear ET-48 tank. Arginine was used as a reference marker for those peptides migrating

toward the anode. Peptides were detected as described in the preceding section except for the fluorescence analysis which could not be utilized with papers that exhibited a high fluorescent background after being placed in the electrophoresis tank.

Peptide Purification by Combined Paper Chromatography and Electrophoresis

The procedure of Canfield (1963a) was used: chromatography with butanol: acetic acid: water followed by electrophoresis at pH 3.6. The papers were lightly sprayed with cadmium-ninhydrin and the spots themselves cut out and eluted as described previously. No UV analysis was possible because of interference from the ninhydrin spray.

Quantitative Amino Acid Analysis

Amino acid analyses were performed using the Technicon Autoanalyzer 75 cm column, 6-1/2 hour gradient system (Technicon Technical Bulletin #AAA-1, 1967). Peptide samples containing an internal norleucine standard were hydrolyzed in 6N HCl at 108° for 20 hours in vacuo. For quantitative determination of tyrosine, 1% phenol-6N HCl solution was used (Sanger and Thompson, 1963); for tryptophan, 2% mercaptoacetic acid-6N HCl solution was used (Matsubara and Sasaki, 1969). Hydrolysates were cooled, evaporated to dryness under vacuum, and dissolved in 0.1N HCl immediately before application to the ion exchange

column. The resin was used for 20 separations before regeneration and repacking. For better resolution of valine and cysteine, pH 3.5 buffer was used in chamber four. A quantitative determination of carboxymethyl-cysteine, aspartic acid, threonine, serine, glutamic acid, and proline was impossible with 2% mercaptoacetic acid hydrolysates because of interferring components with high absorbances. Analyses are reported as micromoles amino acid per sample, and as residue ratios calculated by a least squares fit to integer values (Olivetti Programma 101). Micromoles are determined by comparison to a known amount of norleucine added as an internal standard to the peptide before hydrolysis. The absolute recoveries (moles of norleucine recovered per mole of norleucine added) ranged from 85-90%.

Sedimentation Coefficients

Sedimentation coefficients of lysozyme in 0.15 and 1.7M NaCl at 20° and 37° were determined at pH 2.0, 5.0, and 8.0 using a Spinco Model E Ultracentrifuge. Solvent densities and viscosities were determined using Svedberg and Pedersen (1940) and Handbook of Chemistry and Physics (1963). The $S^{c}_{20,w}$ was calculated using the equation of Schachman (1957).

Biogel P-20 Column Procedures

Proteins were dissolved in water and diluted with 0.2 or 3.4M (20%) NaCl to give solutions of 0.1 or 1.7M NaCl respectively. pH was adjusted with 0.1-1N HCl and NaOH. The sometimes slightly turbid solutions were clarified by centrifugation and placed carefully on a 1 x 46 cm column of Biogel P-20, thermostated at 30°. Sufficient solution (approximately 15 ml) was applied to the column to ensure a plateau in the optical density of the effluent. The eluting buffers (for pH 5, 0.05M NaOAc, 0.1 or 1.7M NaCl; for pH 2.5 or 2, $10^{-2.5}$ or 10^{-2}N HCl, 0.1 or 1.7M NaCl) were pumped (Technicon proportioning pump) at 30 ml/hr. The effluent was collected in 0.45 $\pm$ .05 ml fractions, using a Gilson Collector. These were diluted with water for spectrophotometric analysis at 280 nm. The elution volume was determined as that at which the protein concentration was half of the plateau value. Blue dextran 2000 and $Cu(NO_3)_2$ were used to determine exclusion and retention volumes. Distribution coefficients were calculated as

$$K_d = \frac{V_e - V_o}{V_i - V_o}$$

where V_e = elution volume

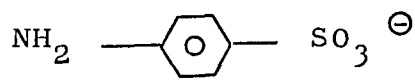
V_o = void or exclusion volume

V_i = retention volume or volume of solution within and out of gel matrix.

RESULTS

Preparation of Sulfanilated Lysozyme

Sulfanilic acid was found to be a reactive nucleophile for incorporation into the carboxyl groups of lysozyme using EDC. Sulfanilated lysozyme was generated with an absorption maximum at 250 nm. The spectra of lysozyme, N-acetylsulfanilic acid, and tryptophan are shown in Figure 7. NASA is seen to absorb most strongly at 252 nm, minima of the lysozyme and tryptophan spectra.



sulfanilic acid

The extent of incorporation was determined by comparing the relative increase in 250 and 280 nm absorbance of sulfanilated lysozyme. Figure 8 shows the increase of this ratio as a function of time when 0.05M EDC is added to a 1% lysozyme solution in 1.2M sulfanilic acid at pH 4.9.

The theoretical sulfanilic acid incorporation curve is shown in Figure 9 from the data of Table 5, the maximum ratio expected from incorporation of ten groups being 3.5. Experimentally the maximum ratio obtained was 3.2, indicating that nine carboxyl groups react. Until the summer of 1970, it was believed that there were eleven carboxyl groups in lysozyme (Canfield, 1963a; Jollés *et al.*, 1963), which was quite perplexing since only nine groups could be

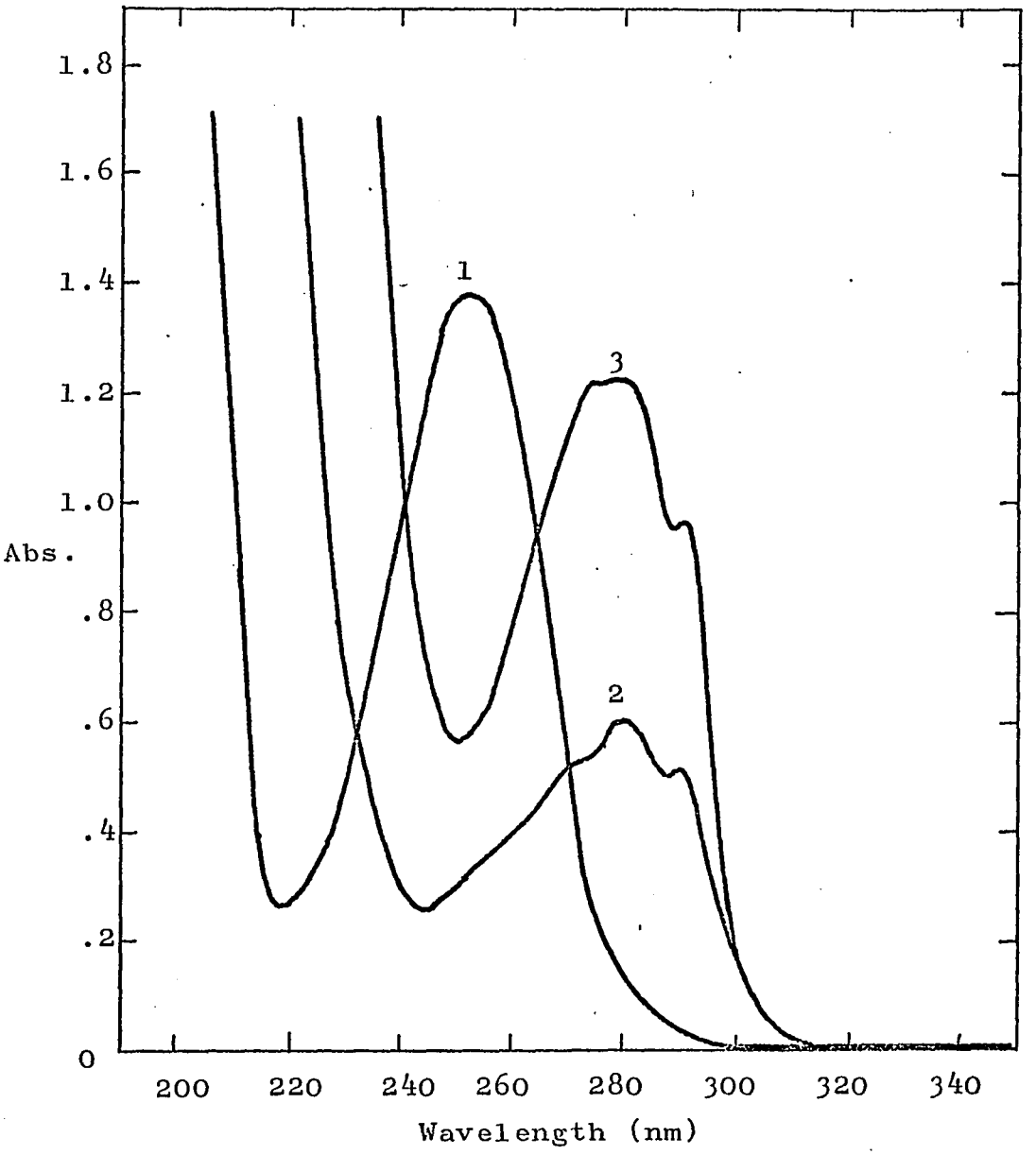


Figure 7. Ultraviolet spectra of lysozyme, N-acetylsulfanilic acid, and tryptophan.

(1) N-acetylsulfanilic acid 9 x 10^{-5}M, pH 5; (2) tryptophan 2.25 x 10^{-4}M, pH 5; (3) lysozyme 1.62 x 10^{-5}M, pH 5.

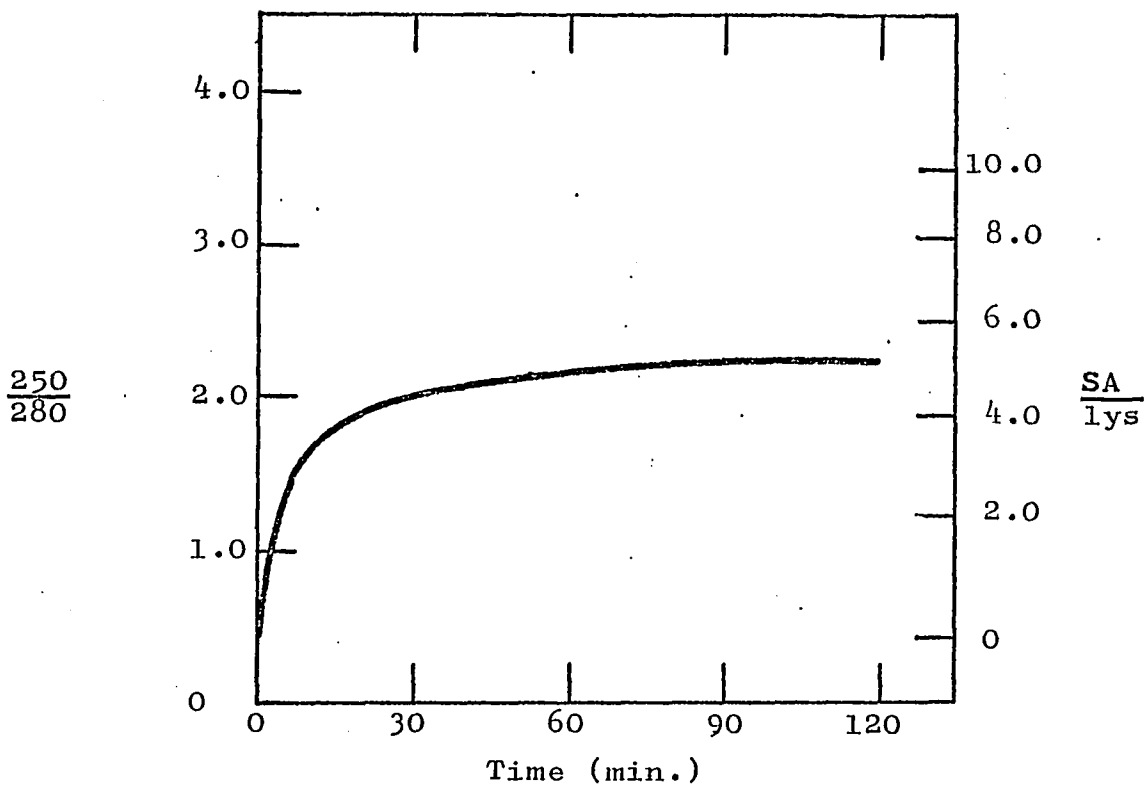


Figure 8. Incorporation of sulfanilic acid into lysozyme.

Conditions: 1% lysozyme, 1.2M sulfanilic acid, 0.05M EDC, pH 4.9.

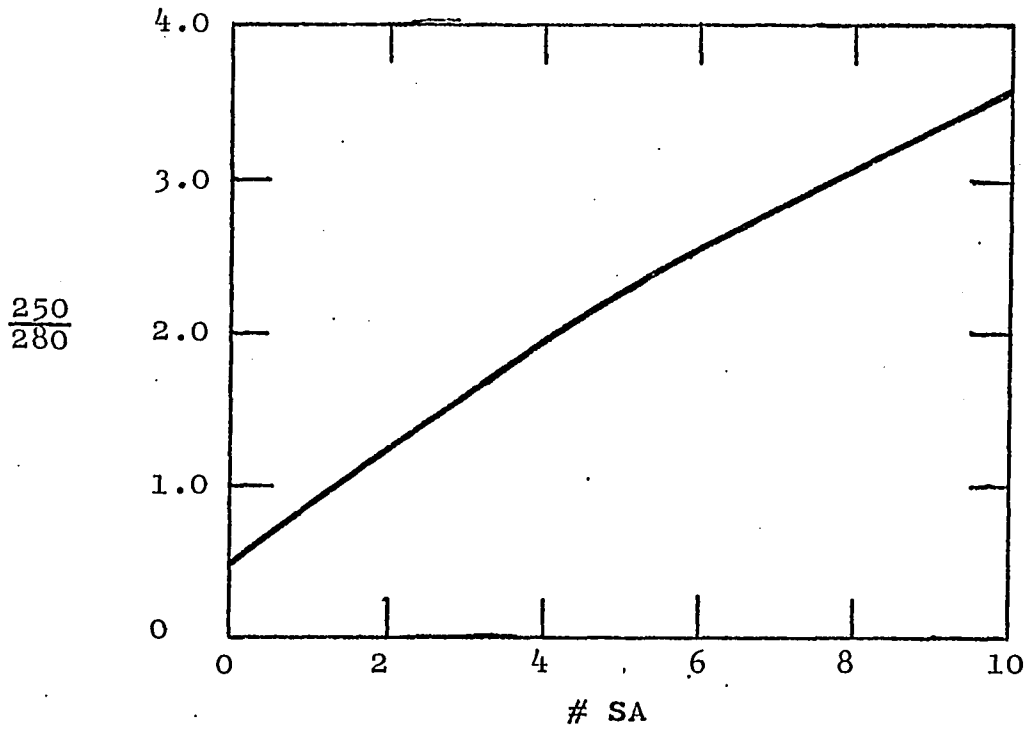


Figure 9. Theoretical sulfanilic acid incorporation into lysozyme.

Table 5. Sulfanilic acid incorporation into the ten carboxyl groups of lysozyme.

No. of SA Incorporated	ϵ_M, 250nm	ϵ_M, 280nm	250/280
0	16200	36500	0.44
1	31450	37638	0.84
2	46700	38776	1.23
3	61950	39914	1.55
4	77200	41052	1.88
5	92450	42190	2.19
6	107700	43328	2.49
7	122950	44466	2.76
8	138200	45604	3.03
9	153450	46742	3.28
10	168700	47880	3.52

N-acetylsulfanilic acid used as reference extinction coefficient.

sulfanilated. Brown (1970), however, partially corrected this discrepancy by determining that residue 103 is actually an amide. Peptide mapping analyses (to be discussed later) showed that all ten carboxyl groups incorporated sulfanilic acid significantly and that the one group which appeared not to react was most likely a small percentage of each of the ten carboxyls not sulfanilating.

The EDC concentration dependence on the sulfanilation reaction is shown in Figure 10a; 0.15M EDC was necessary for 85% incorporation. When multiple additions were employed, two additions of 0.05M EDC gave 85% incorporation (Figure 10b). This multiple addition procedure was used routinely in the present study.

The sulfanilic acid concentration dependence is shown in Figure 10c. Four carboxyl groups reacted at 0.05M SA. Increasing the SA concentration to 1.2M caused 8.5 sulfanilic acid groups to incorporate. No lysozyme concentration dependence was seen with 0.1-1% protein solutions.

Eighty-five per cent sulfanilated lysozyme was electrophoresed at pH 5 in 5% polyacrylamide as shown in Figure 11. The mobility and homogeneity relative to native lysozyme decreased slightly. Figure 12 shows the chromatography of sulfanilated lysozyme on Biorex-70 at pH 7.5 in 5M urea. The derivative appeared homogeneous and chromatographed in 85% yield.

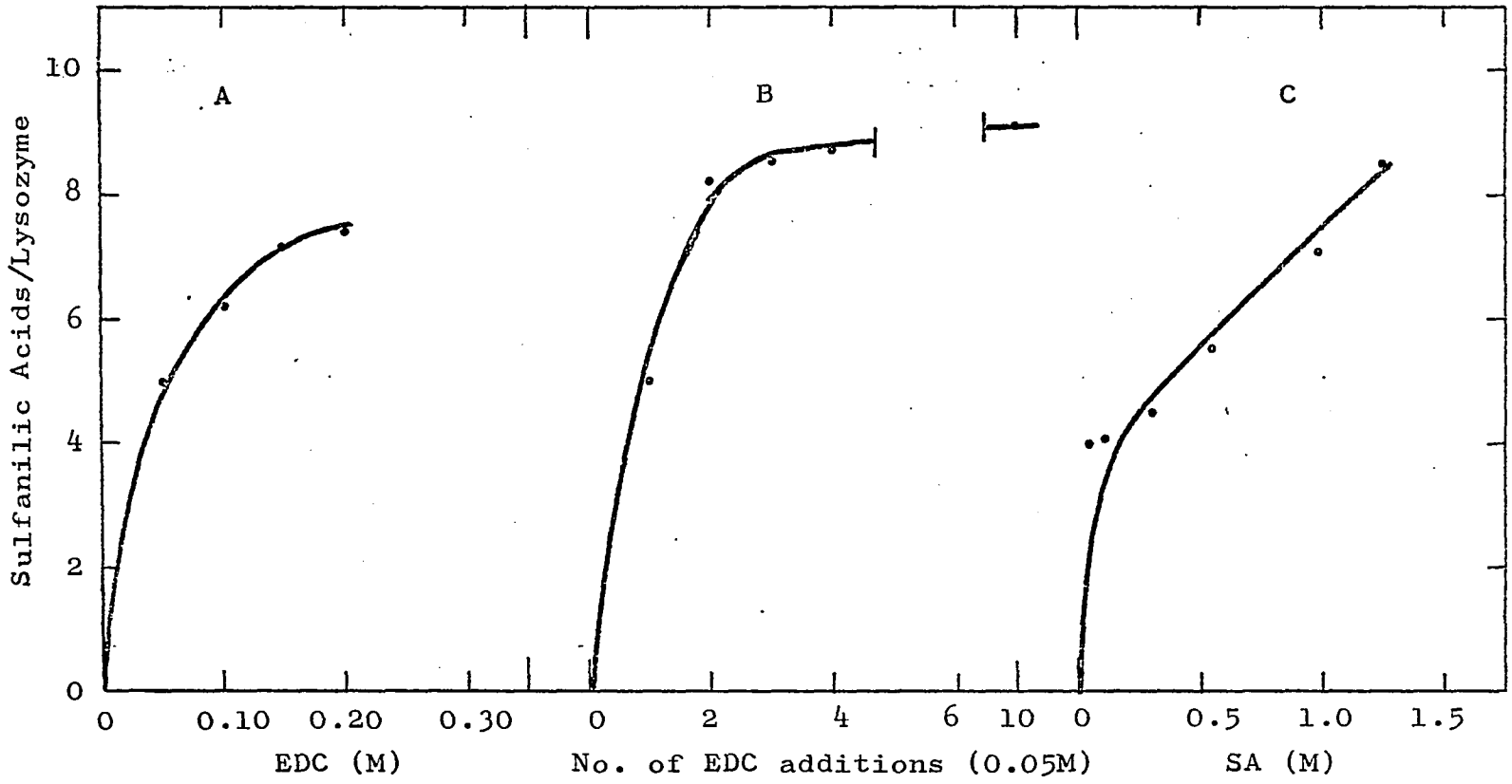


Figure 10. Concentration dependence of nucleophile and carbodiimide on sulfanilic acid incorporation into lysozyme.

(A) EDC concentration dependence of sulfanilation reaction (single addition) in 1.2M sulfanilic acid, (B) EDC concentration dependence of sulfanilation reaction (multiple additions) in 1.2M sulfanilic acid, (C) sulfanilic acid concentration dependence (two additions 0.05 MEDC). Conditions: 1% lysozyme, pH 4.9.

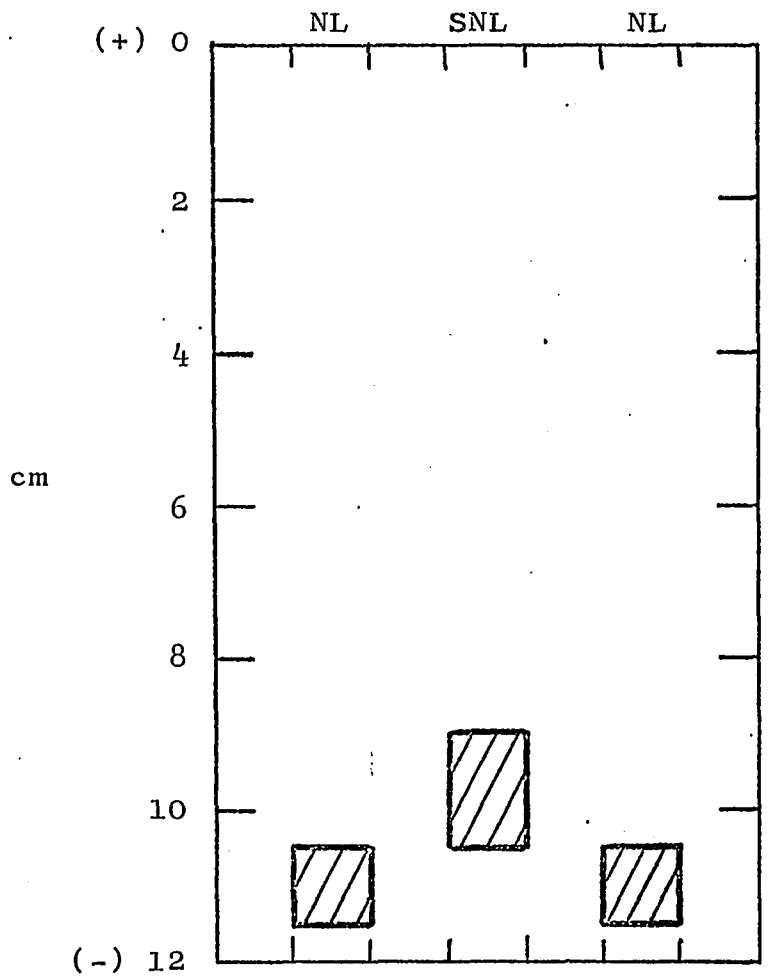


Figure 11. Polyacrylamide gel electrophoresis of native and sulfanilated lysozyme.

Conditions: 1% protein, 5% polyacrylamide, pH 5, 300 volts, three hours.

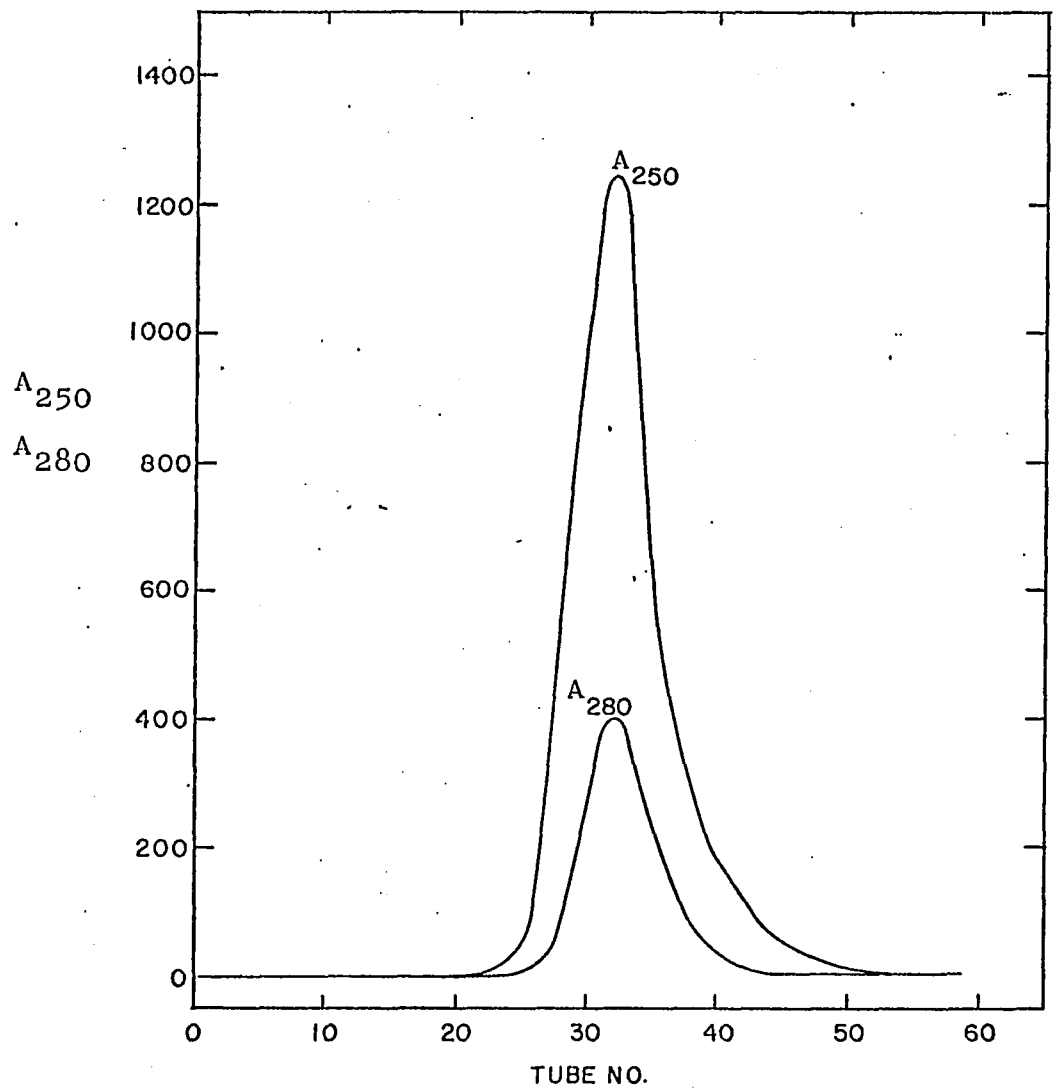


Figure 12. Biorex-70 chromatography of sulfanilated lysozyme.

Esterification of Lysozyme With Methanol-Hydrochloric Acid

A 1% lysozyme solution was reacted as a fine suspension with 0.12N hydrochloric acid in methanol at room temperature. The rate of methylation was determined using tritiated methanol and is shown in Table 6 and Figure 13. Samples of methylated lysozyme (1-2 mg) were taken at various times and purified by centrifugation and Sephadex G-25 chromatography, as shown in Figure 14. Five of the ten carboxyl groups methylated in 8-12 hours and about eight in 48 hours. Overall, one or two groups appeared to react with a rate ca. ten times slower than the average. The methanol content exceeded ten at extended reaction time (> 150 hours) which probably indicated methanolysis of peptide and/or amide bonds. These observations will be discussed in greater detail in later sections.

Various methylated lysozymes were chromatographed on Biorex-70 and carboxymethyl-cellulose. Complex mixtures and low yields of esterified products were obtained (30% yield at 48 hours methylation). Kravchenko et al. (1967) also reported low yields with light methylation products. DEAE-cellulose gave no separation at all. Methylated lysozymes were electrophoresed on 5% polyacrylamide gel at pH 5 (Figure 15) and mobility and homogeneity decreased with extent of methylation.

Table 6. Tritiated methoxyl incorporation into lysozyme.

Sample	Reaction Time (Hours)	Micromole ^{3}H-MeOH[a]	Micromole Lysozyme	MeOH/ Lysozyme
1	0.05	0.032	0.059	0.53
2	1.25	0.115	0.065	1.76
3	2.75	0.154	0.057	2.69
4	5.67	0.169	0.148	3.52
5	7.25	0.290	0.061	4.74
6	8.58	0.372	0.076	4.91
7	10.67	0.260	0.048	5.67
8	22.25	0.540	0.079	6.80
9	24.58	0.430	0.059	7.24
10	51.42	0.580	0.066	8.72
11	72.25	0.633	0.064	9.87
12	97.00	0.361	0.035	10.32
13	123.25	0.795	0.076	10.43
14	145.10	0.651	0.063	10.39
15	169.20	0.810	0.076	10.73

[a]Specific activity of ^{3}H-MeOH = 2.05×10^{4} DPM/ micromole.

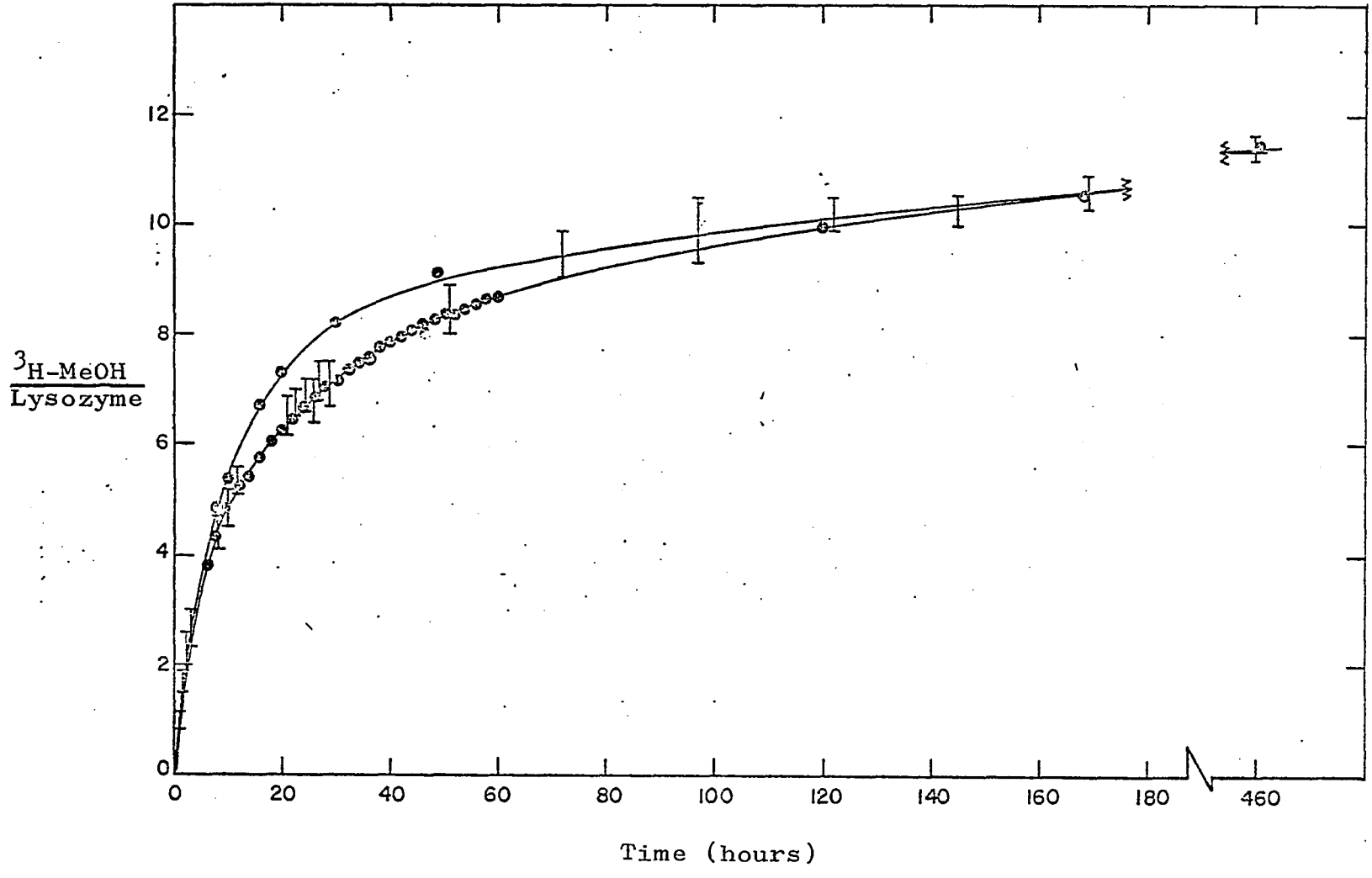


Figure 13. Incorporation of tritiated methanol into lysozyme and fitting of methylation rate constants -- Tritiated methoxyl data: I. Upper curve (A): peptide mapping rate constants. Lower curve (B): grouped rate constants (Fit B).

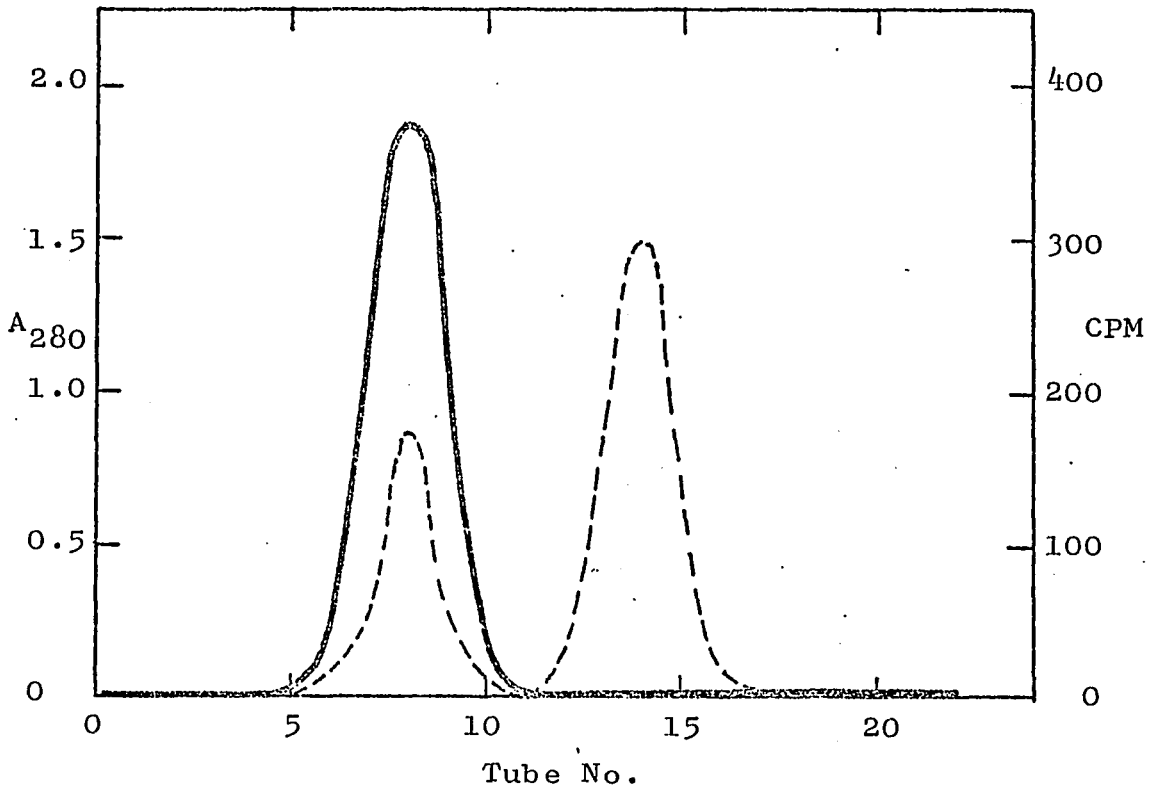


Figure 14. Sephadex G-25 chromatography of tritiated lysozyme methyl ester.

——— A_{280}

----- CPM

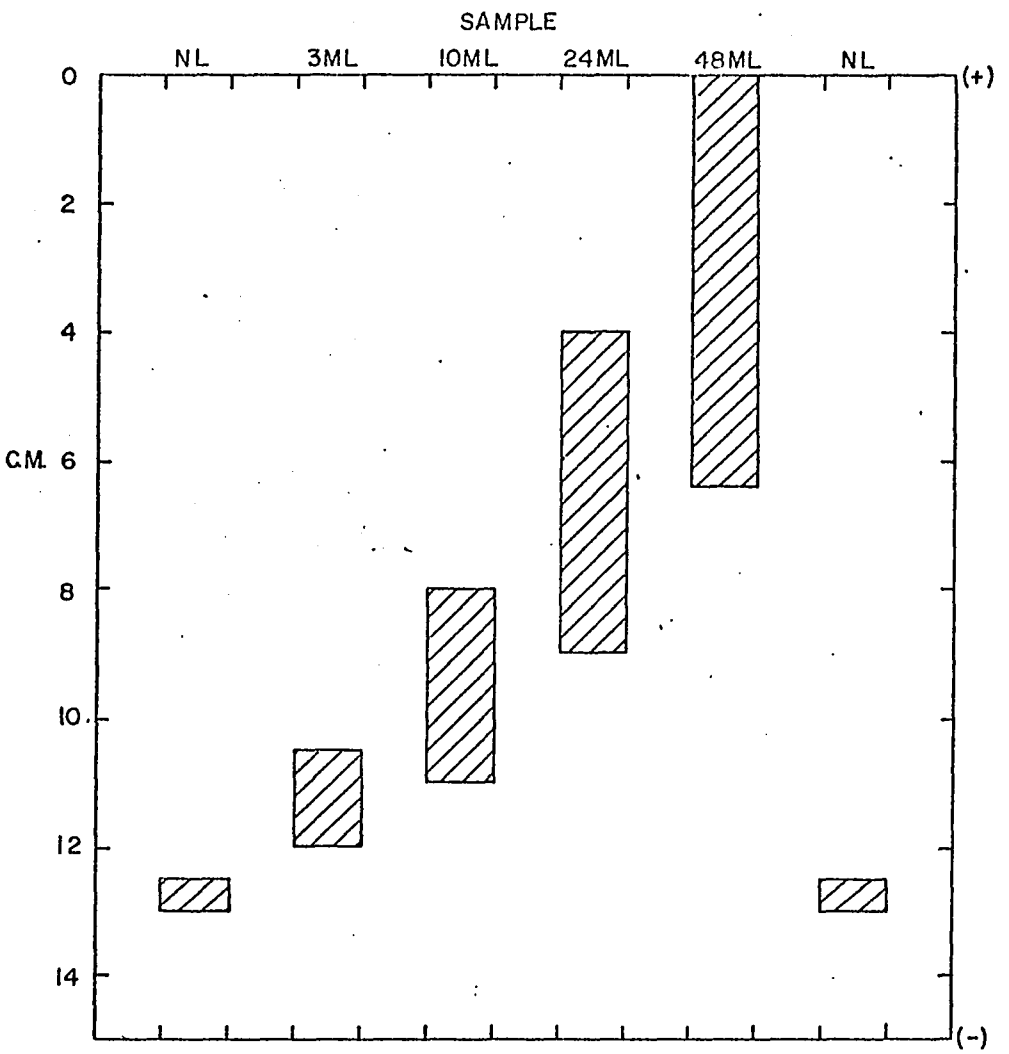


Figure 15. Polyacrylamide gel electrophoresis of lysozyme methyl esters

Conditions: 1% protein, 5% polyacrylamide, pH 5, 300 volts, three hours.

Sulfanilation of Lysozyme Methyl Esters

Lysozyme methyl esters from various methylation times were reacted with sulfanilic acid to determine the amount of unmethylated carboxyl groups. The data, shown in Table 7, indicated that ten groups were modified by methylation and/or sulfanilation except at extended reaction time.

Table 7. Sulfanilic acid incorporation into lysozyme and methyl ester derivatives[c].

Sample	SA/molecule[a]	MeOH/molecule[b]	Total Modified Carboxyls[d]
Native	0.0	0.0	0.0
Sulfanilated Native	9.1	0.0	9.1
Sulfanilated 3 Hour Methylated	6.8	3.0	9.8
Sulfanilated 10 Hour Methylated	4.2	5.3	9.5
Sulfanilated 24 Hour Methylated	2.9	7.0	9.9
Sulfanilated 48 Hour Methylated	1.9	7.7	9.6
Sulfanilated Two Week Methylated	0.6	11.0	11.6

[a]Calculated from 250/280 ratio.

[b]Calculated from ^{3}H-MeOH incorporation.

[c]There are ten carboxyl groups in lysozyme.

[d]Calculated from SA and MeOH incorporation.

Sequence Location of the Sulfanilated Carboxyl Groups and Determination of Native Lysozyme Methylation Rates. General Comments

The determination of which carboxyl groups sulfanilate and the individual methylation rates, particularly the ones which sulfanilate or methylate abnormally slow, is important to help understand the unique chemistry of each particular carboxyl residue. To this end, peptide mapping techniques have been developed using ion-exchange chromatography. Figure 16 summarizes the reactions carried out with lysozyme. Native and methylated lysozymes were reacted with sulfanilic acid to label the free carboxyl groups. Next, the sulfanilated derivatives were saponified at pH 10.5 for 24 hours. This treatment produces free carboxyl groups from methylated ones and therefore native peptides for map comparisons. These saponified derivatives were then reacted with mercaptoethanol in urea to unfold and generate sulfhydryl groups which were subsequently carboxymethylated with iodoacetic acid to prevent air oxidation. The products were hydrolyzed by trypsin, a proteolytic enzyme which cleaves at the C-terminal end of lysine and arginine residues. The tryptic peptides were separated on Sephadex SEC-25 and purified by paper chromatography and electrophoresis. Peptides containing carboxyl groups labeled with sulfanilic acid were identified by UV spectrum and amino acid composition. The amount of each sulfanilic acid peptide as a function of

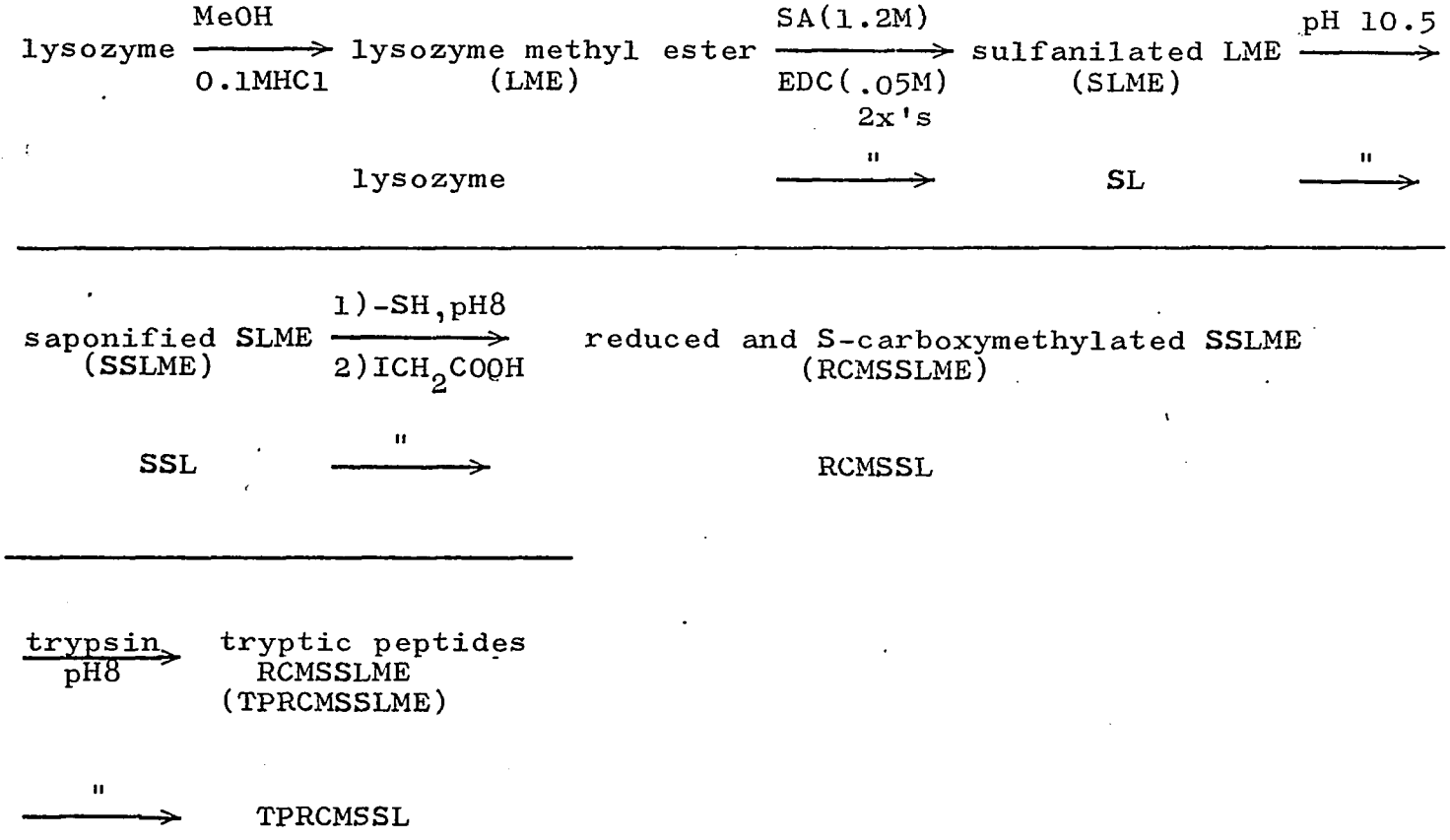


Figure 16. Peptide mapping scheme of native and methylated lysozymes.

methylation was determined using the tryptic peptide maps. The rate constants of the individual carboxyl groups for the methylation reaction were then calculated assuming pseudo first order kinetics. Further details are described in subsequent sections.

The tryptic peptides of native lysozyme are shown in Figure 1, using the nomenclature of Canfield (1963a). The positions of the 10 carboxyl groups are Glu 7, Asp 18, Glu 35, Asp 48, Asp 52, Asp 66, Asp 87, Asp 101, Asp 119, and C-term Leu 129. Hydrolysis by trypsin ideally results in eighteen peptides, each having a unique amino acid composition, nine of which contain carboxyl groups. Peptides T-3, 5, 7, 9, 11, 13, 16, and 18 all contain one carboxyl residue each; peptide T-8 contains two. In all chromatographic separations, each fraction was measured for optical density at wavelengths of 250 nm (N-acetylsulfanilic acid maximum), 280 nm (tryptophan and tyrosine), and 570 nm (after alkaline hydrolysis, neutralization and reaction with ninhydrin).

Separation of the Tryptic Peptides of Lysozyme

The tryptic peptides produced from sulfanilated lysozyme at pH 8 resulted in 80% soluble material at pH 5 based on the original amount of reduced and S-carboxymethylated derivatives. Since peptides containing all acid residues were isolated, it is believed that all sulfanilated

peptides were soluble to some extent and utilizable for peptide mapping procedures. In accord with this, the insoluble 20%, when solubilized in urea and chromatographed on Sephadex G-25, eluted as a mixture of several components and most likely was composed of nondigested protein and a mixture similar to the soluble peptides.

The tryptic peptides of native, sulfanilated native, and sulfanilated methylated lysozymes were separated on Sephadex SEC-25 at pH 3 using an ionic strength gradient from zero salt to 1N NaCl; the optical densities at 250 nm and 280 nm and 570 nm of each fraction are shown in Figures 17-21. Figure 17 shows the native peptides with the aromatic peptides labeled N-1 to N-10. Figure 18 shows the sulfanilated native peptides (SN-1 to SN-15). Figures 19-21 show sulfanilated three hour, ten hour, forty-eight hour methylated lysozymes. All peptides of native lysozyme had higher optical density at 280 than 250 nm. All but one of the peptides from sulfanilated derivatives had higher 250 nm, indicating the presence of sulfanilic acid. Peptide SN-13 of sulfanilated lysozyme corresponded to peptide N-5 of native lysozyme and amino acid analysis (see amino acid analysis of peptides) showed that this was peptide T-6, which contains tryptophan 28 and tyrosine 23, the only tryptophan-containing peptide not containing a free carboxyl group for sulfanilation. This peptide was used as an internal standard for peptide map

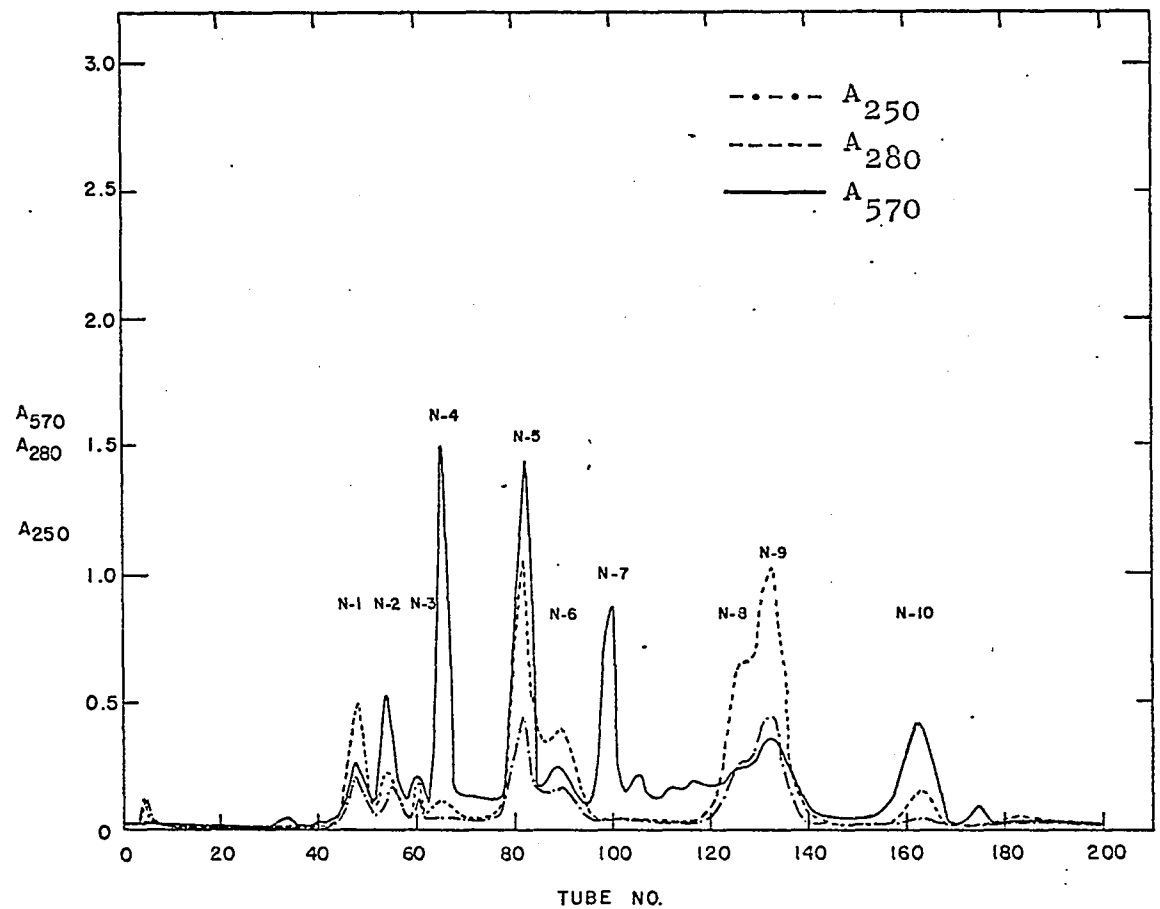


Figure 17. Sephadex SEC-25 chromatography of the tryptic peptides of reduced and S-carboxylmethylated native lysozyme.

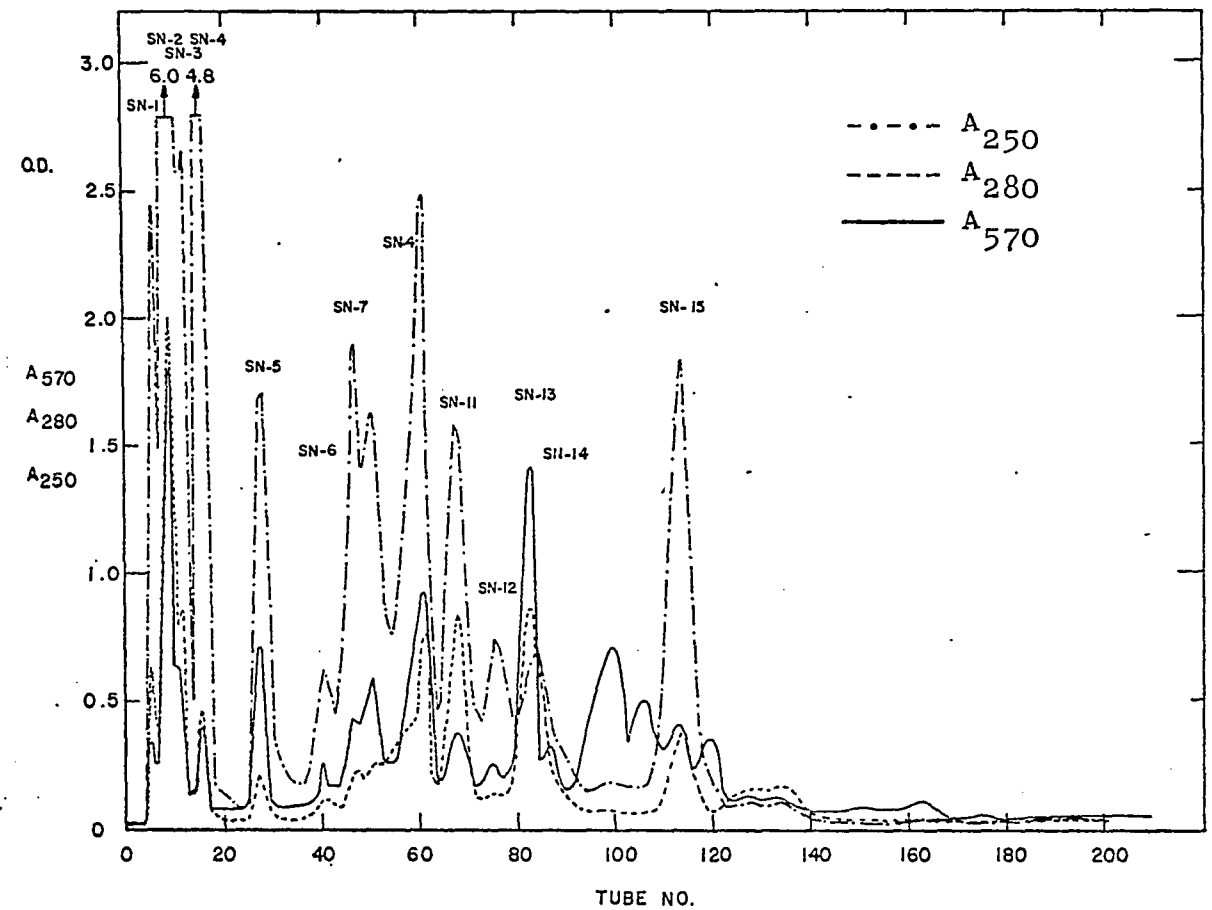


Figure 18. Sephadex SEC-25 chromatography of the tryptic peptides of reduced and S-carboxylmethylated sulfanilated native lysozyme.

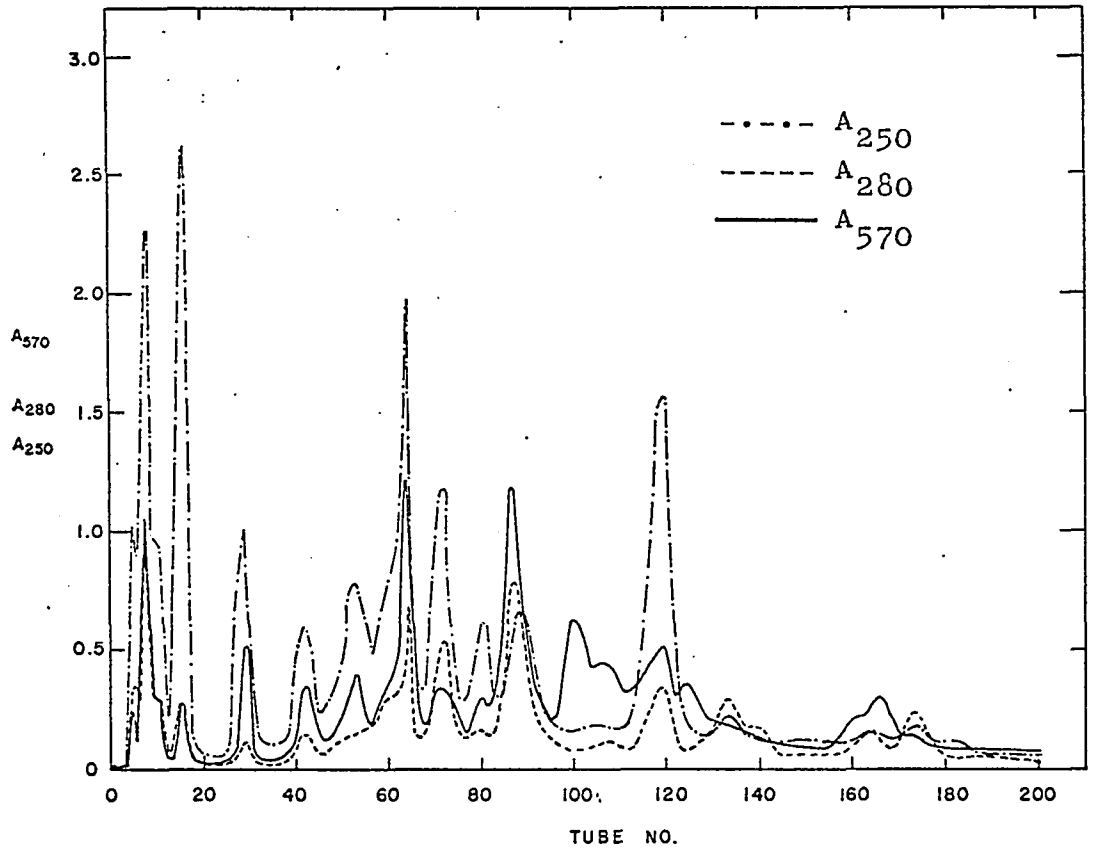


Figure 19. Sephadex SEC-25 chromatography of the tryptic peptides of reduced and S-carboxylmethylated sulfanilated three hour methylated lysozyme.

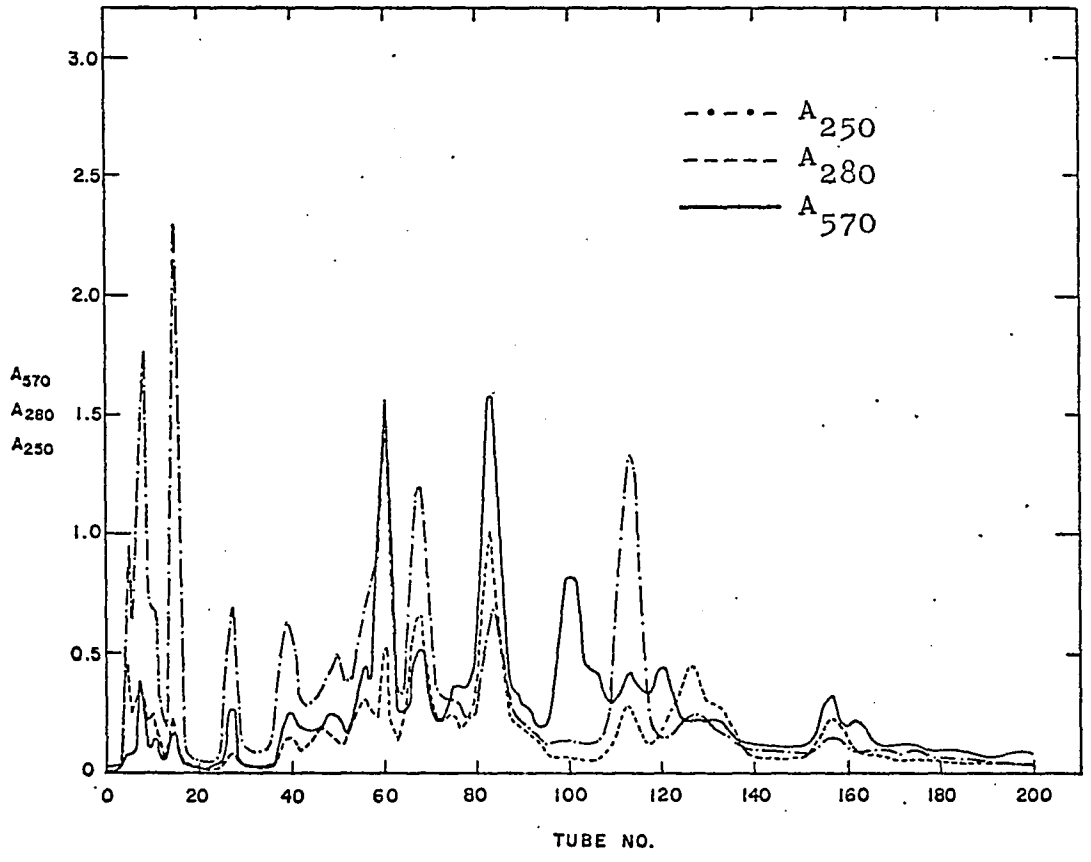


Figure 20. Sephadex SEC-25 chromatography of the tryptic peptides of reduced and S-carboxylmethylated sulfanilated ten hour methylated lysozyme.

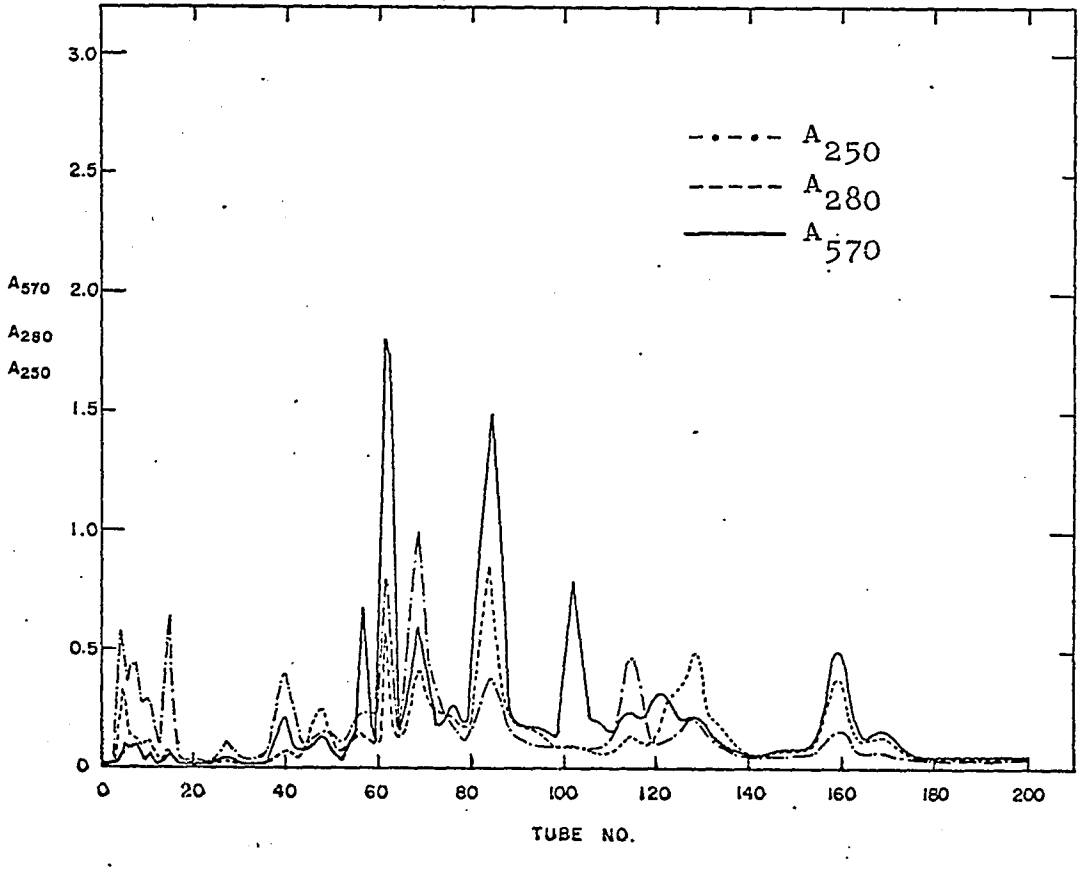


Figure 21. Sephadex SEC-25 chromatography of the tryptic peptides of reduced and S-carboxylmethylated sulfanilated forty-eight hour methylated lysozyme.

comparisons. The minimal number of sulfanilic acid peptides present was 15 in native and methylated lysozymes.

Comparison of the sulfanilated native with the sulfanilated methylated peptide separations showed that they were remarkably similar and that the methylation treatment introduced no sulfanilated peptides that eluted in new positions. This result suggested that methanol-HCl does not significantly hydrolyze peptide and/or amide bonds except at long reaction times.

Purification and Identification of Tryptic Peptides

The identification of peptides containing sulfanilic acid and/or tryptophan was based upon their spectra, as shown in Figure 22. Figure 7 shows the spectrum of NASA and tryptophan; each spectrum is readily distinguishable from the other. Tryptophan is evidenced in a sulfanilic acid containing peptide by a shoulder around 290 nm. Amino acid analyses of the peptides are listed in Table 8. All peptides except SN-10 were desalted on Biogel P-2. SN-10 was desalted on Sephadex G-25. All yields were quantitative except for SN-2 and SN-6 whose yields were 50-65%.

Peptide SN-1 was purified using paper chromatography (BuOH:HoAc:H_2O) followed by pH 8.5 electrophoresis. UV analysis showed sulfanilic acid and tryptophan content

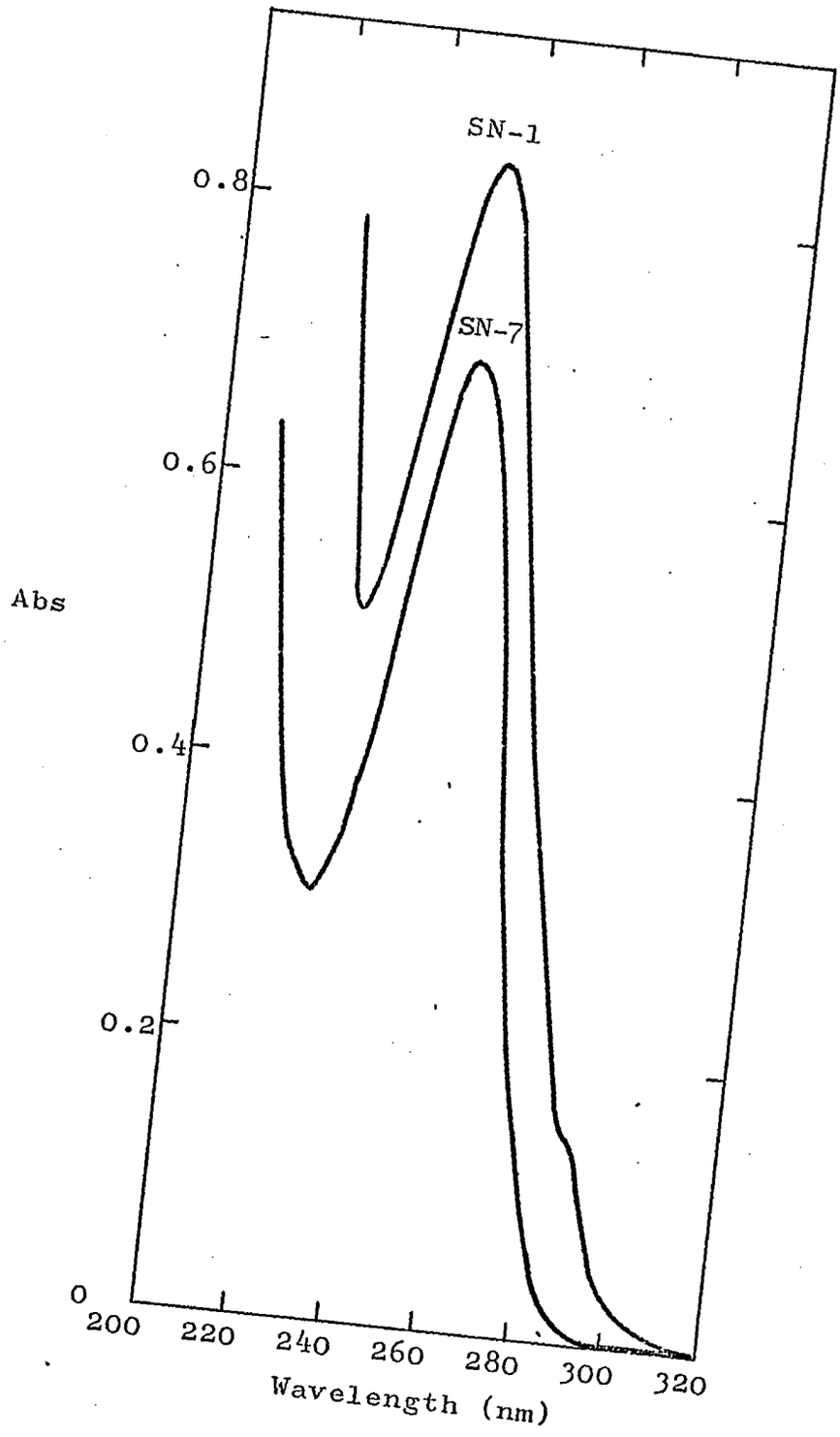


Figure 22. Ultraviolet spectra of sulfanilated peptides SN-1 and SN-7.

Conditions: 0.05 M ammonium acetate, pH 5.

Table 8. Amino acid analyses of sulfanilated peptides.

	Peptide Fractions				
	SN-1			SN-2	
Amino Acid	Found	Expected T-13	T-13'	Found	Expected T-8
Asp	2.93±.12(.10)	3	3	4.00±.16(.91)	4
Thr				2.12±.22(.50)	2
Ser	0.73±.12(.03)	1	1	2.08±.24(.50)	2
Glu				1.25±.12(.30)	1
Pro					
Gly	2.16±.30(.08)	2	2	2.00±.30(.46)	2
Ala	1.05±.02(.04)	2	1	(.07)	
Val	0.42±.14(.02)[c]	2	1	(.05)	
CM Cys					
Met	0.28±.14(.01)[c]	1	1		
Ilu	0.49±.09(.02)[c]	1	1	1.72±.18(.39)	2
Leu				0.93±.08(.21)	1
Tyr[c]				0.69±.28(.14)	1
Phe				(.03)	
Lys				(.05)	
His				(.05)	
Try		2	1		
Arg		1		0.78±.09(.17)	1
NASA[c]					
1/2 Cys					

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

Amino Acid	Peptide Fractions: SN-3 Found	SN-3 Expected T-16	SN-3 Expected T-16'	SN-4 Found	SN-4 Expected T-18
Asp	0.93±.04(.23)	1	1		
Thr	0.91±.11(.21)	1	1		
Ser	(.07)				
Glu	0.91±.12(.21)	1	1		
Pro					
Gly	1.15±.15(.27)	1	1		
Ala	1.08±.08(.25)	1	1		
Val	0.91±.14(.21)	1	1		
CM Cys					
Met	(.01)				
Ilu	(.01)	1			
Leu	(.01)			1.00±.09(.14)	1
Tyr[c]					
Phe	(.04)				
Lys					
His	(.01)				
Try		2	1		
Arg	(.01)	1			
NASA[c]				1.20 (.17)	
1/2 Cys					

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

Amino Acid	Peptide Fractions			
	SN-5		SN-6	
	Found	Expected T-11	Found	Expected T-8
Asp	4.23±.16(.28)	4	4.14±.16(.09)	4
Thr	0.97±.11(.06)	1	1.67±.22(.04)	2
Ser	3.90±.48(.22)	4	2.11±.24(.05)	2
Glu			1.08±.12(.02)	1
Pro	0.93±.12(.06)	1		
Gly			2.03±.30(.05)	2
Ala	2.92±.24(.17)	3	(.01)	
Val	0.79±.14(.05)	1		
CM Cys	2.78±.30(.16)	3		
Met				
Ilu	1.80±.18(.11)	2	1.91±.18(.04)	2
Leu	3.23±.27(.19)	3	1.30±.08(.03)	1
Tyr[c]			0.58±.28(.01)	1
Phe	1.31±.14(.08)	1		
Lys				
His				
Try				
Arg			0.95±.09(.02)	1
NASA[c]	1.15 (.07)			
1/2 Cys				

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

Amino Acid	Peptide Fractions			
	SN-7		SN-8	
	Found	Expected T-3	Found	Expected T-7
Asp	(.08)		3.00±.12(.43)	3
Thr	(.04)		1.84±.22(.27)	2
Ser	(.04)		0.84±.12(.12)	1
Glu	0.92±.12(.19)	1	2.02±.24(.29)	2
Pro				
Gly	(.05)			
Ala	3.03±.24(.62)	3	1.30±.08(.19)	1
Val	(.01)			
CM Cys	0.95±.10(.20)	1		
Met	0.95±.14(.20)	1		
Ilu	(.03)			
Leu	1.07±.09(.22)	1		
Tyr[c]				
Phe			2.03±.05(.29)	2
Lys	0.92±.14(.19)	1		
His				
Try				
Arg	(.03)		1.08±.09(.16)	1
NASA[c]	1.10 (.21)		0.91 (.13)	
1/2 Cys				

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

	Peptide Fractions			
	SN-9		SN-10	
Amino Acid	Found	Expected	Found	Expected T-11
Asp	(See text)		4.00±.16(.14)	4
Thr			0.74±.11(.03)	1
Ser			3.60±.48(.12)	4
Glu			(.01)	
Pro			0.84±.12(.03)	1
Gly			(.01)	
Ala			2.99±.24(.10)	3
Val			0.82±.14(.03)	1
CM Cys			(.04)	3
Met				
Ilu			2.12±.18(.07)	2
Leu			2.94±.27(.10)	3
Tyr[c]				
Phe				
Lys			1.19±.14(.04)	1
His			(.01)	
Try				
Arg			(.04)	
NASA[c]			1.17±.09(.04)	
1/2 Cys				

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

Amino Acid	Peptide Fractions			
	SN-11		SN-12	
	Found	Expected T-9	Found	Expected T-3,4
Asp	2.07±.08(.29)	2		
Thr				
Ser				
Glu			1.20±.12(.13)	1
Pro				
Gly	0.83±.15(.12)	1		
Ala			2.96±.24(.32)	3
Val				
CM Cys	1.08±.1-(.14)	1	1.20±.10(.13)	1
Met			0.77±.14(.08)	1
Ilu				
Leu			1.09±.09(.12)	1
Tyr[c]		2		
Phe				
Lys			0.92±.14(.11)	1
His				
Try	1.37 (.19)			
Arg	0.98±.09(.14)	1	0.88±.09(.09)	1
NASA[c]	1.14 (.16)		1.40 (.16)	
1/2 Cys				

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

	Peptide Fractions			
	SN-13 or N-5		SN-14	
Amino Acid	Found	Expected T-6	Found	Expected
Asp	1.08±.04(.14)	1	(See text)	
Thr				
Ser	1.10±.12(.15)	1		
Glu				
Pro				
Gly	2.08±.30(.27)	2		
Ala	2.00±.16(.26)	2		
Val	0.93±.14(.12)	1		
CM Cys		1		
Met				
Ilu				
Leu	0.93±.18(.12)	1		
Tyr[c]	0.65±.28(.07)	1		
Phe				
Lys	1.08±.14(.14)	1		
His				
Try		1		
Arg				
NASA[c]				
1/2 Cys				

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

	Peptide Fractions	
	SN-15	
Amino Acid	Found	Expected T-5
Asp	1.92±.08(.09)	2
Thr		
Ser		
Glu		
Pro		
Gly	1.25±.15(.06)	1
Ala		
Val		
CM Cys		
Met		
Ilu		
Leu	1.03±.09(.05)	1
Tyr[c]	0.90±.28(.04)	1
Phe		
Lys		
His	0.79±.21(.04)	1
Try		
Arg	0.96±.09(.05)	1

Table 8.--Continued Amino acid analyses of sulfanilated peptides.

	Peptide Fractions	
	SN-15	
Amino Acid	Found	Expected T-5
NASA[c]	1.07 (.05)	
1/2 Cys		

[a]Values are integer ratios fitted by least squares analysis.

[b]Micromoles analyzed are given in parentheses.

[c]Not included in the least squares fitting.

[d]Average deviation from theoretical values.

[e]T-13' is the T-13 tryptic peptide minus the C-terminal residues Val-Ala-Try-Arg.

[f]T-16' is the T-16 tryptic peptide minus the C-terminal residues Ilu-Arg.

[g]Mercaptoacetic acid added to protect tryptophan.

(Figure 22). Amino acid analysis indicated that it was derived from the tryptic peptide T-13 with a cleavage C-terminal to tryptophan 108. The low molar values of isoleucine and valine occurred because the N-terminal valyl-isoleucyl bond is difficult to cleave; Canfield (1963a) and Hartdegan (1967) reported low values for these residues also. Cleavage after Try 108 was unexpected, but has been observed previously with iodinated lysozyme by Hartdegan (1967). This peptide eluted very rapidly on Sephadex SEC-25 because it lacked an N-terminal basic amino acid. The peptide was most likely sulfanilated on the carboxyl group side chain of aspartic acid 101. Sulfanilic acid could not be quantitatively determined because this peptide was isolated from a ninhydrin stained spot.

Peptide SN-2 was purified using paper chromatography (BuOH:HoAc:H_2O) followed by pH 3.6 electrophoresis (Katz, Dreyer, and Anfinsen, 1959). It contained more sulfanilic acid than any other peptide. Amino acid content showed that it was derived from tryptic peptide T-8. It eluted very rapidly off Sephadex SEC-25 because it probably contained two moles of sulfanilic acid per mole of peptide, both Asp 48 and Asp 52 being modified. This peptide showed no mobility under the purification conditions and remained at the origin. The T-8 peptide which was isolated from reduced and S-carboxymethylated native lysozyme also exhibited this lack of mobility.

Peptide SN-3 was purified by the same procedure as SN-1. Spectrum showed tryptophan present. It was derived from T-16 with a cleavage C-terminal to Try-123, also unexpected and similar to SN-1 above. It was probably sulfanilated at Asp 119.

Peptide SN-4 was pure enough for analysis after Biogel P-2 desalting. It was derived from T-18, the C-terminal residue, Leu 129. The α-carboxyl was sulfanilated and contained one mole of sulfanilic acid per mole of leucine.

SN-5 was purified by pH 2 electrophoresis (R_f arg = 0.11, orange). It was derived from T-11, the largest tryptic peptide. It contained one mole of sulfanilic acid per four moles of aspartic acid, Asp 87 being modified (this peptide also contained Asn 74, Asn 77, Asn 93).

Peptide SN-6 was purified by the same procedure as SN-2. It also was derived from T-8 like SN-2. The yield was 20% relative to SN-2, however, and it probably had only one of the two carboxyl side chains (Asp 48 and Asp 52) sulfanilated, which explained the greater retention on Sephadex SEC-25.

Peptide SN-7 was purified by pH 5.5 electrophoresis (R_f arg = -0.18, brown). It was derived from T-3 and contained one mole sulfanilic acid per mole of peptide, Glu 7 being sulfanilated.

Peptide SN-8 was purified using pH 5.5 electrophoresis (R_f arg = 0.11, red). It was derived from peptide T-7 which contained two of the three phenylalanine residues in lysozyme. The peptide contained two moles of glutamic acid per mole of sulfanilic acid and was sulfanilated at Glu 35 (this peptide also contained glutamine 41).

SN-9 had not been purified sufficiently for positive identification. It contained tryptophan and the amino acid analysis resembled a multiple peptide derived from T-11, 12, 13.

Peptide SN-10 was purified using the same procedure as SN-2. Like SN-5, it was derived from T-11, and was sulfanilated at Asp 87. However, this peptide contained one cysteine which was not carboxymethylated. SN-10 eluted later off Sephadex SEC-25 because it was less acidic.

Peptide SN-11 was pure enough for analysis after Sephadex G-25 desalting. The spectrum showed tryptophan content. It was derived from tryptic peptide T-9 and was virtually identified before amino acid analysis by its behavior on Sephadex. Hayashi et al. (1965) and Hartdegan (1967) have shown that only T-9 has an unusually high affinity for Sephadex G-25 and eluted much after salt retention volume. This peptide contained 2 moles of aspartic acid per mole of sulfanilic acid and was sulfanilated at Asp 66 (this peptide also contained asparagine 65).

Peptide SN-12 was purified using pH 2 electrophoresis (R_f arg = 0.45, pink). It was derived from peptide T-3,4, the same as SN-7 except for the uncleaved lysine 13-arginine 14 N-terminal bond. Canfield (1963a) also reported isolation of this peptide in the native. It contained one Glu per SA and was sulfanilated at Glu 7. SN-12 eluted later than SN-7 because of the basicity contributed by the added arginine.

Peptides SN-13 and N-5 were purified using pH 2.0 electrophoresis. UV analysis indicated tryptophan and no sulfanilic acid. This peptide was the native tryptic peptide T-6 which contained tyrosine 23 and tryptophan 28. It was the only tryptophan peptide without a carboxyl group side chain and was present unmodified in all maps.

SN-14 had not been purified sufficiently for positive identification. It contained tryptophan and amino acid analysis suggested that it was derived from either T-15,16 or T-16.

Peptide SN-15 was purified using pH 2 electrophoresis (R_f arg = 0.67, orange). It was derived from T-5 which contained the only histidine in lysozyme, residue 15. This was the last sulfanilic peptide to elute from Sephadex SEC-25 because of the added charge on histidine at pH 3. It contained two aspartic acids per sulfanilic acid and was sulfanilated at Asp 18 (this peptide also contained asparagine 19).

Recovery and Yields of Peptides

The total recovery of peptides from Sephadex SEC-25 chromatography was 85-90% for all derivatives based on absorption at 250 and 280 nm. Although the missing 10-15% could have represented a substantial loss of one sulfanilic acid peptide and complete recovery of all others, this was unlikely since all peptides containing carboxyl groups were obtained and losses of this magnitude were expected with Sephadex SEC-25 separations. The per cent of the recovered optical density represented by each peptide is shown in Table 9.

The yields of the sulfanilated native lysozyme peptides obtained from tryptic hydrolyates are listed in Table 9. These were calculated by comparing the optical density at 250 nm with the amount theoretically expected. The peptides contained either one sulfanilic acid (T-3, 5, 7, 11, 18), two sulfanilic acids (T-8), one tryptophan plus one sulfanilic acid (T-13', 16'), or two tryptophans plus one sulfanilic acid (T-9). The yields ranged from 20-95%. Peptides derived from T-6, 13, and 16 were generated in low yields, as also reported by Jollés *et al.* (1963) and Canfield (1963a).

Yields of Tryptic Peptides from Sulfanilated Methylated Lysozyme and Rates of Carboxyl Group Methylation

Table 10 shows the yields of sulfanilic acid peptides obtained from various sulfanilated methylated

Table 9. Recovery and yield of sulfanilated lysozyme peptides.

Peak	Tube No.	% Total OD_{250nm}	Sequence Identification	% Yield
SN-1	4	3.7	98-108	20
2	9	15.3	46-61	53
6	40	2.9	46-61	20
				73
3	12	6.2	117-123	32
4	20	9.9	129	69
5	28	7.0	74-96	44
10	61	5.0	74-96	31
				75
7	47	7.8	6-13	50
12	75	4.4	6-14	30
				84
8	50	6.5	34-45	46
9	56	8.5		
11	67	7.7	62-68	47
13	82		22-33	
14	84	2.5	117-125	
15	113	12.6	15-21	87

Table 10. Yields of sulfanilated peptides as function of methy

Peptide	SNL(OD_{250nm})	S3 hr ML(%SNL)	S10 hr ML	S24 hr ML
T-3	9.27±.8[a]	45±7	29±2	11
T-5	9.16±.9	93±6	62±3	38
T-7	3.73±.9	90±6	26±6	13
T-8	13.29±.5	73±4	50±6	35
T-9	5.20±.9	96±7	93±7	86
T-11	5.19±.7	59±3	33±2	17
T-13'	2.82±.2	64±16	30±5	22
T-16'	4.72±.2	34±3	20±10	13
T-18	7.27±.3	82±12	50±3	31
T-6	4.47±.9	105±10	103±4	95
No. of Experiments	5	2	2	1

[a]Average deviation.

[b]Standard deviation.

[c]In order of decreasing reactivity.

f methylation and carboxyl group rate constants for methylation.

hr ML	S48 hr ML	Carboxyl Group	$k_{m,COOH,P}(hr^{-1})$	Order of Reactivity[c]
11	0	Glu 7	0.179 ±.049[b]	2
38	27±12	Asp 18	0.036 ±.005	9
13	0	Glu 35	0.098 ±.023	5
35	20±2	Asp 48/52	0.052 ±.012	7,8
86	77±4	Asp 66	0.0058±.0004	10
17	8±4	Asp 87	0.118 ±.025	3
22	16±2	Asp 101	0.109 ±.030	4
13	9±2	Asp 119	0.304 ±.101	1
31	16±4	α-COOH	0.054 ±.007	6
95	96±15			
1	4			

lysozymes. Peptide yields decreased as methylation time increased. By comparing peptide yields of native with methylated lysozymes, the quantities of individual unmethylated carboxyl groups at 3, 10, 24, and 48 hours were determined (Table 10). Rate constants were calculated assuming first order kinetics according to

$$\frac{x}{a} = 1 - e^{-kt}$$

where a = initial concentration of reactant

x = amount reacted at time t

k = reactant rate constant

Constants were fit to the rate data using the nonlinear least squares program described by Banerjee and Rupley (1971). The methylation rate curves of the carboxyl groups are shown in Figure 23.

Rates of individual carboxyl group reactivity varied markedly, as shown in Table 10 and can be grouped into four classes of reactivity:

Class 1. Fast-Glu 7 and Asp 119

Class 2. Moderately Fast-Glu 35, Asp 87, Asp 101

Class 3. Moderate-Asp 18, Asp 48/52, α-COOH

Class 4. Slow-Asp 66.

Glu 7 and Asp 119 methylated quickly with half-times of 3.9 and 2.3 hours respectively. Glu 35, Asp 87, and Asp 101 reacted less rapidly with half-times of 7.1,

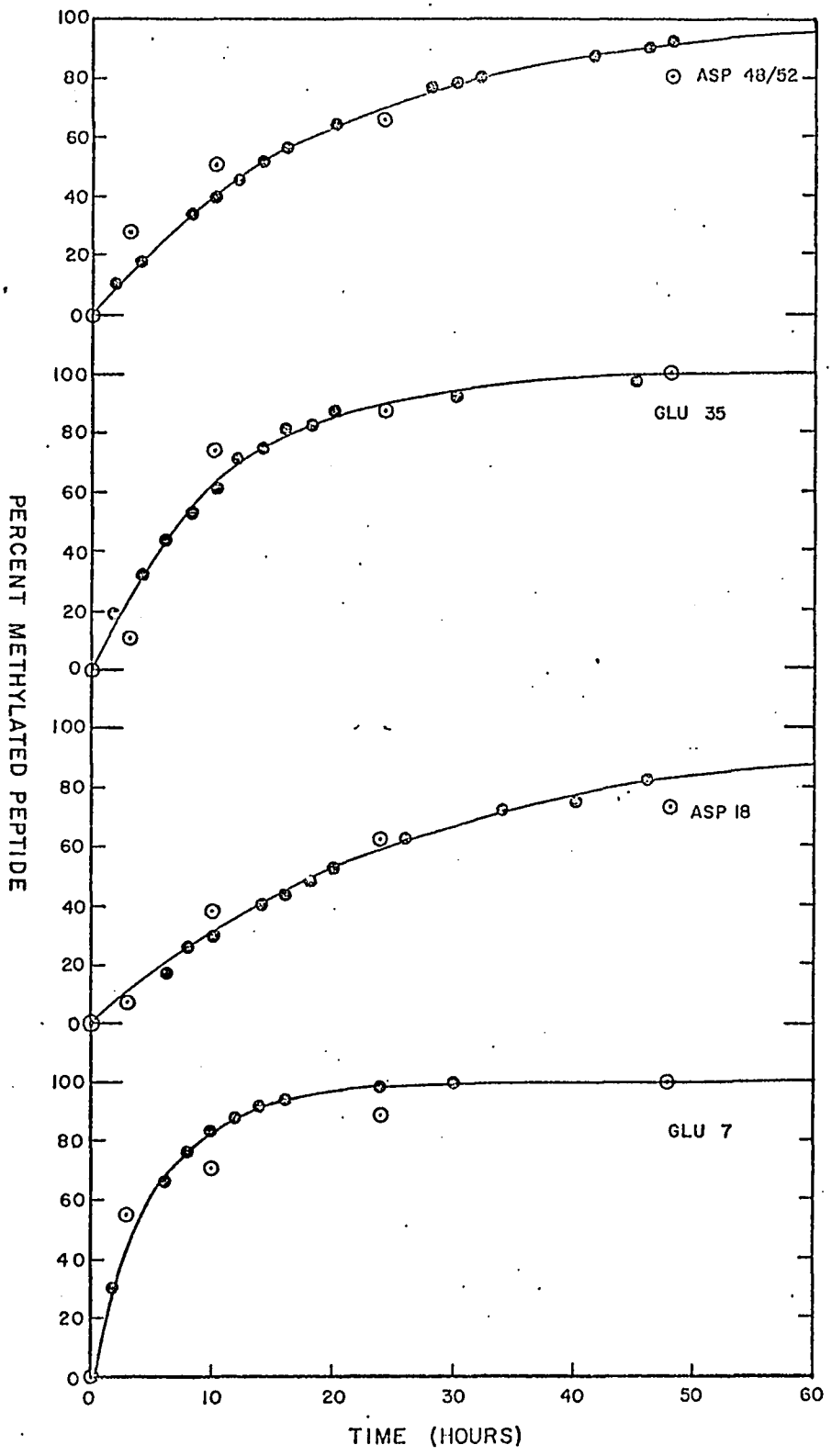


Figure 23. First order methylation rate curves of carboxyl groups of lysozyme.

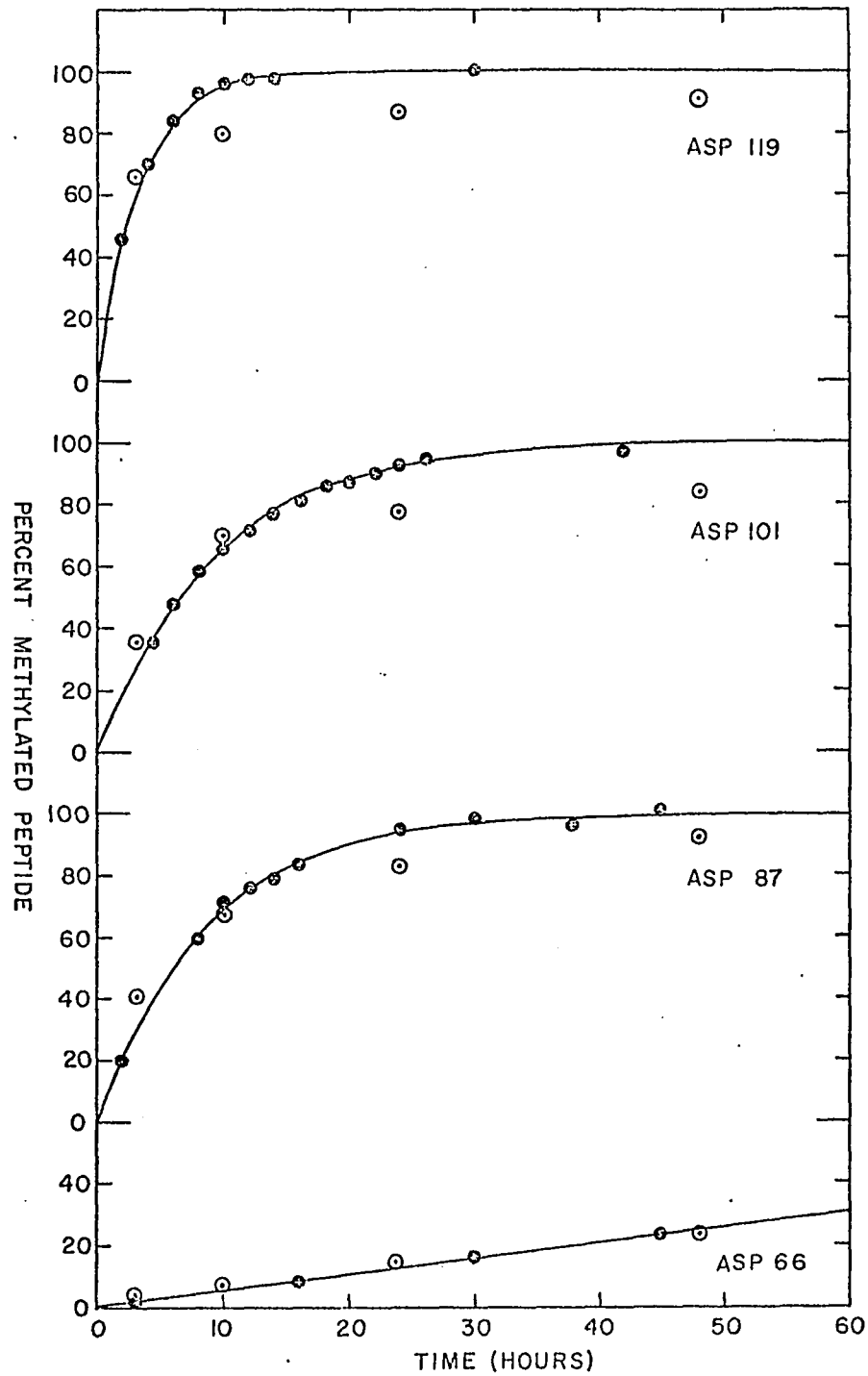


Figure 23.--Continued First order methylation rate curves of carboxyl groups of lysozyme.

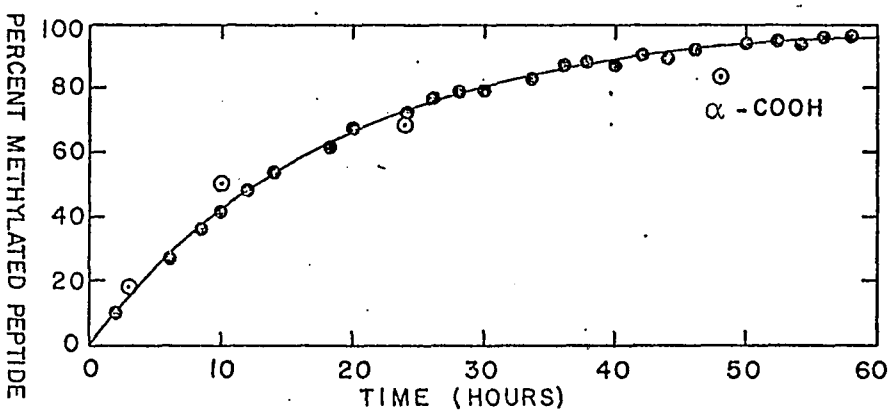


Figure 23.--Continued First order methylation rate curves of carboxyl groups of lysozyme.

5.9, and 6.4 hours. Reacting slower were Asp 18, Asp 48/52, and the α-carboxyl with half-times of 19.2, 13.3, and 12.8 hours. The slowest group was Asp 66 with a half-time of 119.5 hours. Relative active site carboxyl rates were as follows: Asp 48/52 reacted at one-half the rate of Glu 35 and Asp 101, and Asp 66 reacted at one-tenth the rate of Asp 48/52.

Fitting of Methylation Rates to Overall Methoxyl Incorporation

Figure 13 shows the acid catalyzed radioactive methanol incorporation data and several theoretical curves. Curve A is calculated from the peptide mapping rate constants and using the equation

$$G = k_B \cdot t + \sum_{i=1}^{10} (1 - e^{-k_i t})$$

where G = the number of groups reacted.

k_B = the background methylation rate, 0.003 groups per hour estimated from the slow, long time incorporation of methoxyl.

k_i = the rate constants for individual carboxyl groups.

Although the fit to the data is good for short and long methylation times, the intermediate time region (ca. 10-40 hours) showed approximately one group differences.

The methylation rate data of Figure 13 were insufficient to allow simultaneous refinement of ten separate group rate constants (the fitting diverged). The

rate constants could be grouped into classes, however, which allowed better fitting to the data. Table 11 shows the individual rate constants obtained by the various fits. Fit B (curve B) used the average constants of the four classes listed previously with the slowest and fastest groups held fixed and the intermediate classes allowed to vary. Fit C allowed all but the slowest group to vary. Fit D placed Asp 101, the most exposed carboxyl in the crystal structure, in the fastest class and allowed all except the slowest group to vary. Fits C and D deviated at most only 0.2 group from Fit B. All final adjustments gave good fits to the experimental methoxyl incorporation data.

Effect of Methylation On Enzymic Activity

Both bacterial cell wall and penta-N-acetylglucosanine hydrolytic activities decreased rapidly with methylation (Figure 24). Fifty per cent activity was lost in less than two hours and complete inactivation in ten hours. Attempts to regenerate enzymic activity by exposure to acid (pH 2) or alkali (pH 10.5) were unsuccessful as was also reported by Frieden (1956). Fraenkel-Conrat (1950) reported that acid treatment generated 46% and 10% activity from methylated lysozymes with 40% and 6% activity originally.

The effects caused by extensive modifications of proteins are difficult to interpret in terms of specific

Table 11. Methylation rate constants obtained by nonlinear least squares analysis of the individual rate constants to overall lysozyme methylation.

Residue	$k_{m,COOH,Peptides}(hr^{-1})$	$k_{m,COOH,Fit\ B}$	$k_{m,COOH,Fit\ C}$	$k_{m,COOH,Fit\ D}$
Glu 7	0.179 ±.049[a]	0.240	0.49 ±.066	0.336 ±.033
Asp 18	0.036 ±.005	0.028±.001	0.032±.0024	0.029 ±.001
Glu 35	0.098 ±.023	0.120±.011	0.077±.0096	0.056 ±.010
Asp 48/52	0.052 ±.012	0.028±.001	0.032±.0024	0.029 ±.001
Asp 66	0.0058±.0004	0.0058	0.0058	0.0058
Asp 87	0.118 ±.025	0.120±.011	0.077±.0096	0.056 ±.010
Asp 101	0.109 ±.030	0.120±.011	0.077±.0096	0.336 ±.033
Asp 119	0.304 ±.101	0.240	0.49 ±.066	0.336 ±.033
α-COOH	0.054 ±.007	0.028±.001	0.032±.0024	0.029 ±.001
Background	--	0.003	0.003	0.0032±.0004

[a]Standard deviation.

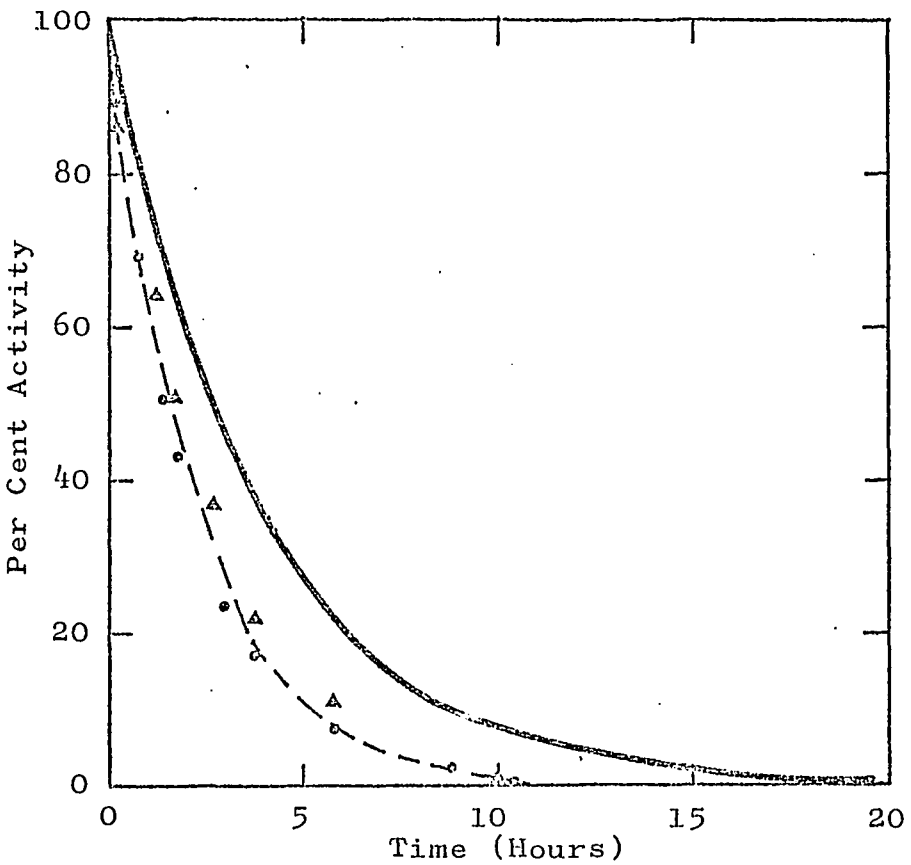


Figure 24. Effect of methylation on enzymic activity and carboxyl group modification fits.

• Penta-NAG hydrolysis

Δ cell wall lysis

——— active site carboxyl group rate constants

----- essential carboxyl group rate constants

residues since all side chains of a certain type are reacted. Also, the effects of a denaturing solvent like methanol-HCl must be considered. Kurono and Hamaguchi (1964) concluded from optical rotatory dispersion studies that 80% methanol-HCl (0.005M) increased the helical content of lysozyme. The circular dichroism spectrum of β-lactoglobulin in methanol-HCl (0.01M) was typical of a predominately α-helical structure (70%) with some anti-parallel β and unordered conformations (Timasheff et al., 1968). A similar experiment by the present author suggested even more α-helical structure with methanol-HCl (0.1M).

Evidence suggested that there was no solvent-induced irreversible denaturation of lysozyme in methanol-HCl. The protein is a small stable molecule that reverts to native structure after being unfolded (Goldberger and Epstein, 1963). It can be exposed momentarily to methanol-0.1M HCl with no loss of enzymic activity. Also, the overall methoxyl incorporation and peptide analyses did not indicate any significant covalent bond cleavage (peptide or amide bond methanolysis) or rearrangement (N→O acyl shift). All effects on lysozyme caused by the methanol-HCl treatment were attributed to the carboxyl group methylations.

Since some groups are more likely to be involved in enzymic activity than others, i.e., they are in the active site, it seemed reasonable to determine whether their

modification paralleled any changes in enzymic properties. For fitting of carboxyl group modification rates to activity effects, it was assumed that the probability of an active enzyme is the product of the probability of each group in the active site not reacting, that is,

$$\text{Active enzyme} = \prod_i (1 - \frac{x_i}{a_i}) = \prod_i e^{-k_i t}$$

where symbols are defined as before. The active site rate constants obtained by peptide mapping techniques did not fit the activity curve (Figure 24). In order for a fit to occur it was necessary to include carboxyls outside the active site that would be essential for native conformation or to assume a faster rate for an active site carboxyl (Fit C). A rationale for the latter could be that the rate for Asp 101, the most exposed carboxyl in the crystal, is in error, since its rate was determined with a peptide isolated in low yield.

Effect of Methylation on the Binding of Tri-N-Acetylglucosamine

The effect of methylation on the non-productive binding of tri-NAG to lysozyme is shown in Table 12. The first order rate constant for the decrease in the association of tri-NAG with methylation was consistent with the Class 2 carboxyl rates. Active site carboxyls Glu 35 and Asp 101 are members of this class. Asp 101 is directly

Table 12. Effect of methylation on the binding of tri-NAG with lysozyme and 293-289 nm peak-trough difference.

Hours Methylation	Log $K_{obs}^{assoc,\ III}$	$\Delta A_{293-289}(10^{-3})$
0.0	4.94 (pK max)	113.6 (ΔA max)
2.25	4.66	98.5
2.92	4.60	83.6
8.75	3.92	71.9
24.75	3.47	57.2
48.17	3.43	39.2
	ΔpK=1.51	$\Delta\Delta$A=74.4
	$k_{m,III}=0.117\pm.049$	$k_{m,\Delta A}=0.142\pm.049$

involved in the trimer complex with two hydrogen bonds at subsite A and would be associated with this binding effect. The effect of methylation on the 293-288 nm peak-trough absorbance difference associated with tri-NAG binding is also shown in Table 12. The rate of absorbance decrease with methylation was consistent with Class 2. Glu 35 is the closest group to a tryptophan, residue 108, and would be associated with this spectral effect.

Sedimentation and Gel Filtration Behavior of Lysozyme Under Conditions of Crystallization

The sedimentation and gel permeation properties of lysozyme in concentrated salt solution were studied to determine whether the solubility behavior reported by Cole et al. (1969) was caused by association phenomena. Results of the sedimentation velocity experiments are shown in Table 13. In low salt concentration (0.1M NaCl), $S_{20,w}$ is seen to increase from 1.76 at pH 4.5 to 2.01 at pH 8.0, confirming the pH dependent association of lysozyme described by Sophianopoulos and Van Holde (1964) and Sophianopoulos (1969). No pH dependent association was observed in concentrated salt (1.7M NaCl). The solubility behavior, therefore, was not a reflection of association of the soluble protein.

Whitaker (1963) had shown that lysozyme behaves anomalously in dilute salt in Sephadex G-75 and G-100, and that it eluted much later than expected on the basis of molecular weight. This was explained by proposing a weak complex at the active center of lysozyme, a mucopolysaccharidase, with the dextran resin. Recently, however, Marzotto and Galzigna (1970) have used lysozyme as one of the standards for determining the calibration curve for molecular weight studies on Sephadex G-75. It was unclear whether the molecular weight of lysozyme and its associated species could be determined using Sephadex gels.

Table 13. Sedimentation of lysozyme.

Run No.	Lysozyme (mg/ml)	pH	NaCl(M)	T°C	$\rho_{t,s}$	η_{rel}	$s^c_{t,s}$	$s^c_{20,w}$
1A	12	4.5	0.1	20	1.0023	1.009	1.73	1.76
1B	12	8.0	0.1	20	1.0023	1.009	1.97	2.01
2A	4	2.0	1.7	20	1.0654	1.172	1.35	1.88
2B	4	5.0	1.7	20	1.0654	1.172	1.34	1.88
3A	4	2.5	1.7	37	1.0589	1.198	1.88	1.82
3B	4	5.4	1.7	37	1.0589	1.198	1.87	1.81
4A	4	5.0	1.7	20	1.0654	1.172	1.35	1.88
4B	4	8.0	1.7	20	1.0654	1.172	1.33	1.85
5A	9	5.0	1.7	37	1.0589	1.198	1.94	1.87
5B	9	8.5	1.7	37	1.0589	1.198	1.94	1.87

Studies with Sephadex G-50 showed lysozyme to behave anomalously as reported by Whitaker (1963), even in concentrated salt solution (1.7M NaCl). It behaved like a species of one-half its actual molecular weight. The suggestion of Whitaker (1963) that lysozyme forms a weak complex at its active center with the gel is probably correct. Sephadex G-25 chromatography of lysozyme tryptic peptides showed that an active site peptide, T-9, which contains residues 62-68, also gave anomalous adsorption behavior (Hayashi et al., 1965; Hartdegan, 1967, and this work). T-9 elution exceeded the retention volume

[determined with NaCl or $Cu(NO_3)_2$] by almost two times. This peptide contains Try 62 and Try 63, which interact with the substrate in the complex (Blake et al., 1967b).

Tryptophan itself has been shown to adsorb strongly to Sephadex (Gelotte and Porath, 1969). It is thus believed that the unusual behavior of lysozyme on Sephadex gels can be attributed, in part, to the active site tryptophan residues, 62 and 63. Sephadex gels cannot be used for molecular weight studies with lysozyme unless used in the presence of denaturants like urea or guanidine hydrochloride (Fish, Mann, and Tanford, 1969).

Attempts to study lysozyme association with Biogel P-20 gave anomalous results also, as shown in Table 14. The distribution coefficients in low and high salt concentrations were found to vary significantly with pH, where no variation should have occurred. In 0.1M NaCl, the distribution coefficient at pH 5.0 was 0.29 and at pH 2.5 was 0.56. Sophianoulos and Van Holde (1964) have shown that lysozyme is monomeric under these conditions using sedimentation equilibrium techniques. Bonilla (1970) has reported that lysozyme adsorbs to Biogel P-2, but was eluted with 1M NaCl. Perhaps this anomalous behavior can be explained on the basis of an electrostatic adsorption and retardation of basic protein by the polyacrylamide gel. There are acidic groups present in Biogel which cause this cationic ion exchange property.

Table 14. Elution of lysozyme on Biogel P-20.

Sample	Conc (mg/ml)	pH	NaCl(M)	V_e ($\pm$0.5 ml)	K_d
Blue dextran 2000	--	5.0	1.7	20.0 (V_o)	0.0
		2.5	1.7	20.0	0.0
Chymotrypsinogen	2.2	5.0	1.7	23.5	0.13
	2.0	2.5	1.7	24.0	0.15
Lysozyme	1.0	5.0	0.1	27.8	0.29
	1.0	2.5	0.1	34.9	0.56
	1.1	5.0	1.7	29.5	0.34
	1.4	2.5	1.7	34.0	0.53
	1.0	2.0	1.7	34.5	0.55
$Cu(NO_3)_2$	--	5.0	1.7	46.5 (V_i)	1.00
		2.5	1.7	46.5	1.00

The Interaction of Thyroxine With Lysozyme, Effect on Enzymic Activity

Litwack (1963) reported that thyroxine strongly inhibited cell wall lytic activity. At pH 7.1, 0.9×10^{-7}M lysozyme, 0.066M phosphate buffer, 25°, he found 28 per cent activity for enzyme preincubated with thyroxine (15 min at 37°). Preincubation was necessary for thyroxine inhibition; moderate changes in time and temperature of preincubation had no significant effect.

Because rates of cell wall solubilization are difficult to interpret, rates of hexamer hydrolysis catalyzed by thyroxine-incubated lysozyme were measured. Rates determined at higher thyroxine concentration than used in the lysis experiments showed no inhibition (Figure 25). Data obtained with oligosaccharide substrates, in particular the hexamer because it is cleaved at only one bond, should be more reliable than those with cell walls. Thyroxine was also shown not to inhibit cell wall lytic activity (Table 15). Thus, thyroxine, contrary to the previous report, does not inhibit lysozyme.

Absence of an Effect of Thyroxine on the Lysozyme Spectrum and on the Binding of Tri-NAG

Crystallographic analysis (Blake *et al.*, 1967a, 1967b) shows that the active site of lysozyme has three tryptophan side chains (residues 62, 63, 108). Binding of thyroxine at the active site would be expected to perturb

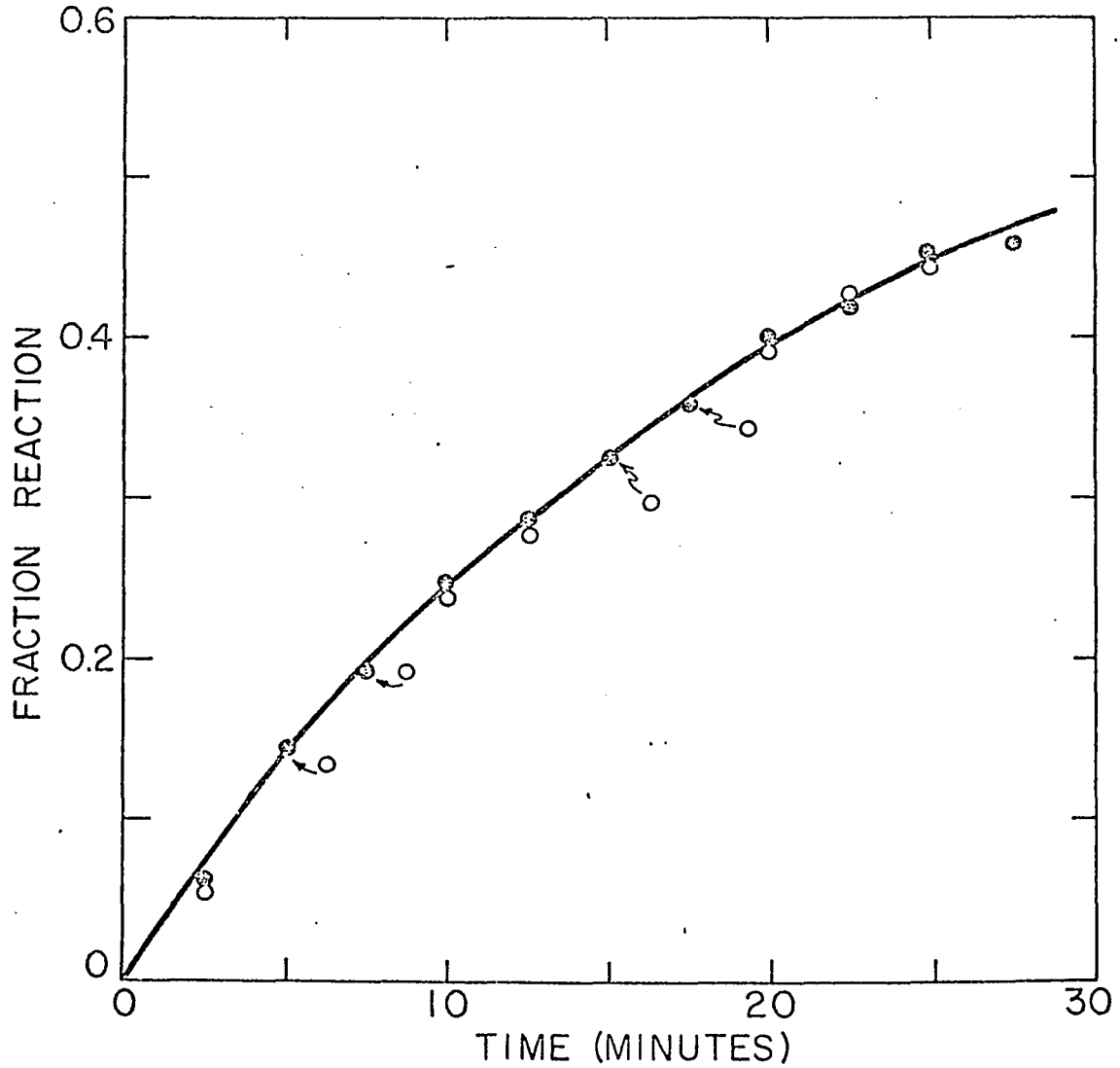


Figure 25. Effect of thyroxine on the lysozyme-catalyzed hydrolysis of hexa-NAG.

Presence (o), absence (•) of 8.6×10^{-6}M L-thyroxine. Conditions: 10^{-5}M hexamer, 0.005 mg/ml lysozyme (3.5×10^{-7}M), sodium phosphate buffer (0.1M Na^+) pH 7.1, 30°. L-thyroxine incubated 30 minutes at 30° with lysozyme before reaction.

Table 15. Effect of thyroxine on the rates of cell wall lysis by lysozyme.

Conditions: 0.05M phosphate buffer, pH 7.1, 25 or 30°.

Initial optical density of cell wall suspension: 1.0 at 450 nm.

Lysozyme	Thyroxine	Average Relative Lytic Activity
$5x10^{-7}$M	DL, $5x10^{-6}$M	97 ± 3%
$5x10^{-7}$	DL, $5x10^{-6}$[a]	96 ± 4
$6x10^{-7}$	DL, $3.4x10^{-6}$	100 ± 4
$6x10^{-7}$	DL, $3.4x10^{-6}$[a]	100 ± 5
$3x10^{-7}$	L, $8x10^{-6}$	96 ± 2
$3x10^{-7}$	L, $8x10^{-6}$[a]	95 ± 2

[a]Thirty minute incubation of thyroxine with lysozyme at temperature of assay.

the protein absorption spectrum. No absorbance perturbation was found at either neutral or alkaline pH, in the following experiments.

I. 0.1 ionic strength Universal buffer (Frugoni, 1957), pH 7.1: 0.1 mg/ml lysozyme (6.8×10^{-6}M) and 9.4×10^{-6}M L-thyroxine in 10 cm cell with buffer in tandem 1 cm cell read vs. same concentration of L-thyroxine in 10 cm cell and 10 times the concentration of lysozyme in tandem 1 cm cell. Result: $A_{293-289} < 0.002$, $A_{280} = 2.480$.

II. 0.1 ionic strength Universal buffer, pH 11.0, 1 cm cells: 1 mg/ml lysozyme (6.7×10^{-5}M) and 8×10^{-5}M L-thyroxine read vs. same concentrations in separate cells.

Result: $A_{293-289} < 0.002$, $A_{280} = 2.460$.

Binding of trimer should be sensitive to interaction of the enzyme with a ligand in the ABC region of the active site. The following experiment showed that ca. 10^{-4}M L-thyroxine did not displace trimer at a concentration that gave ca. 75% enzyme in the complex.

Conditions: 0.1 ionic strength Universal buffer, pH 11.0, 1 cm cells: 10^{-4}M trimer, 1 mg/ml lysozyme (7×10^{-5}M), 8.6×10^{-5}M L-thyroxine read vs. same concentrations of lysozyme and L-thyroxine in tandem separate cells; compared with similar set but L-thyroxine separate in sample also.

Results: L-thyroxine mixed, $A_{293-289} = 0.067$; L-thyroxine separated, $A_{293-287} = 0.063$.

Absence of an Effect of Lysozyme on the Thyroxine Spectrum

Figure 26 shows the thyroxine spectrum generated by 20% ethylene glycol, which produces the red shift expected for change in solvent polarizability. Addition of lysozyme to L-thyroxine solutions at pH 11 produced no such perturbation:

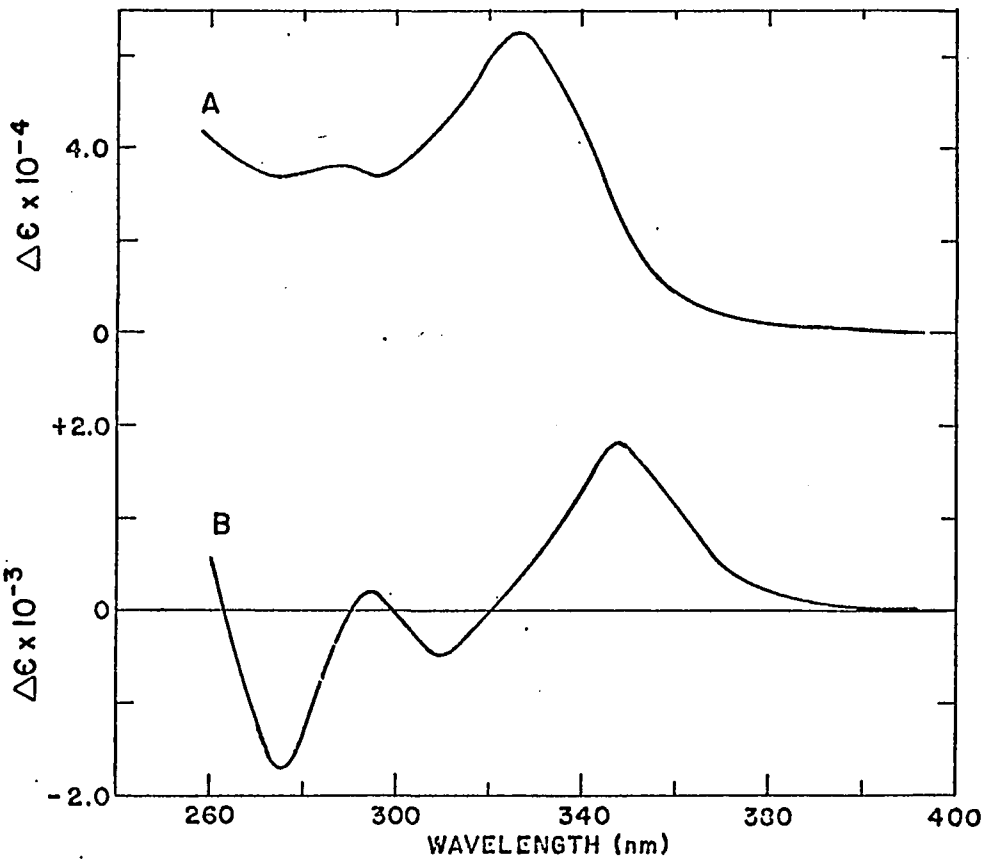


Figure 26. Thyroxine ultraviolet spectrum and difference spectrum produced by 20% ethylene glycol.

(A) L-thyroxine spectrum, (B) difference spectrum produced by 20% ethylene glycol. Conditions: 0.1 ionic strength Universal buffer, pH 11, 2.4×10^{-4}M L-thyroxine.

10 cm cells, under conditions like those of experiment II above; $A_{350}/A_{326} < 0.001$ compared with $A_{350}/A_{326} = 0.029$ for 20% ethylene glycol.

DISCUSSION AND CONCLUSIONS

Carbodiimide Catalyzed Lysozyme Carboxyl Group Reaction With Sulfanilic Acid

The first simple and general procedure for a well-characterized and quantitative modification of carboxyl groups was the reaction with a water soluble carbodiimide and nucleophile (Hoare and Koshland, 1966, 1967). This method has been used to study the carboxyl groups of several proteins for structure-reactivity correlations. Those not discussed previously include trypsin (Eyl and Inagami, 1970), trypsinogen (Radhakrishnan, Walsh, and Neurath, 1967), pancreatic trypsin inhibitor (Chauvet and Acher, 1968), chymotrypsin and chymotrypsinogen (Carraway, Spoerl, and Koshland, 1969; Abita et al., 1969), β-lactoglobulin A (Armstrong and McKenzie, 1967), bovine serum albumin (Mukerjee and Sri Ram, 1965), ribonuclease (Wilchek, Frensdorff, and Sela, 1967), staphylococcal enterotoxin B (Chu and Crary, 1969), and transferrin (Bezkorovainy and Grohlich, 1970). All have required use of urea or guanidine hydrochloride for complete reaction and tedious analyses of nucleophiles. One intent of this research was to develop an easier method for obtaining quantitative carboxyl group reaction and analyses of reaction products. Sulfanilic acid has proven to be a

sufficiently reactive nucleophile which incorporates into all carboxyl groups of lysozyme. Its unique ultraviolet absorption allowed easy detection and quantitation.

In 1.2M sulfanilic acid solution, 9.1 of the ten carboxyl groups of lysozyme sulfanilated. This extensively reacted product had 85% of the mobility of native lysozyme at pH 5 on polyacrylamide gel, the sulfanic acid group ($pK < 1$) rendering the protein less positive for migration toward the cathode. Some of the native protein carboxyl groups would be unionized at pH 5. The unreactive nine-tenths carboxyl group was most likely composed of a small amount of each side chain carboxyl group either not reacting or reacting to give products not involving sulfanilic acid. All tryptic peptides expected to contain sulfanilated carboxyl groups were identified in yields ranging from 25-90% which were similar to the yields obtained with the native peptides. This suggested significant incorporation of sulfanilic acid into all carboxyl groups. Several native carboxyl group peptides were present in low yield in the sulfanilated peptide maps. T-9 which contains aspartic acid 66 was present in 15% yield relative to a native digest. Also, a second sulfanilated T-8 peptide containing Asp 48 and Asp 52 was detected in 10% yield and chromatographed in a manner suggesting that only one of the carboxyl groups was sulfanilated.

The 9% of the carboxyl groups which did not react with sulfanilic acid could be made up of rearrangement products derived from the o-acylisourea activated carboxyl group (4) (Figure 5). This intermediate has several reaction pathways. It can react with nucleophiles like sulfanilic acid (6), water (9), or the protein itself, producing intra- or intermolecular crosslinks. Also, it can rearrange to give the N-acylurea (5), which would render the carboxyl group unavailable for reaction with a nucleophile. Whatever the cause of the unreactive 9%, it did not interfere sufficiently such that high nucleophilic incorporation of sulfanilic acid was not achieved.

Horinishi et al. (1968) had previously attempted to incorporate sulfanilic acid into carboxyl groups at pH 6.2 with 1-ethyl-3-(3-morpholinyl-[4]-propyl)-carbodiimide. They determined that the "aromatic amine could not be used for such a reaction" on the basis of titration experiments with acetylglycine. In the present study sulfanilic acid incorporated readily with EDC even at low concentrations. The pK of the amino group is 3.26 (Perrin, 1965) and at slightly acidic pH, sulfanilic acid is unprotonated for good nucleophilicity.

Previous studies of lysozyme carboxyl group reactions have shown that glutamic acid 35 would not react unless the reaction was carried out in urea or guanidine hydrochloride solutions. Lin (1970) has shown that Glu 35

is almost quantitatively unreactive and that Glu 7 and Asp 66 are partially unreactive toward EDC-glycinamide. He suggested that these properties were related to the unique environment involving the interaction between Try 108 and Glu 35 and also to the tight conformation which confines the region near -Cys 6 -Cys 27- and -Cys 64 -Cys 80-, shielding Glu 7 and Asp 66. In sulfanilic acid solution Glu 7, Glu 35, and Asp 66 all reacted. The environmental factors responsible for low reactivity with glycinamide were not present with sulfanilic acid. It may be that lysozyme is partially unfolded and all carboxyl groups exposed for reaction in 1.2M sulfanilic acid.

In some cases, unexpected peptides from the tryptic digest of sulfanilated lysozyme occurred. T-3,4 peptide resulted from the partially uncleaved Lys 13-Arg 14 bond. T-11 (free Cys) resulted from a partially nonreduced and S-carboxymethylated cysteine (either Cys 76, Cys 80, or Cys 94). Two T-8 peptides were also generated, one less acidic than the other as evidenced by its larger elution volume on Sephadex SEC-25. It is believed that this second peptide, which is at most present in 20% yield of the other T-8 peptide, was modified at only one of the two side chain carboxyl groups (Asp 48 and Asp 52). T-13' resulted from a cleavage of the Try 108-Val 109 bond and T-16' resulted from a cleavage of the Try 123-Ilu 124 bond, both not expected with trypsin. Several other tryptophan containing peptides

were produced intact without cleavage, i.e., T-6 (Try 28) and T-9 (Try 62-Try 63). The T-13 and 16 splits probably occurred during or subsequent to tryptic hydrolysis since dialysis of reduced and S-carboxylmethylated sulfanilated lysozyme produced 85% yield relative to starting material, a yield expected for lysozyme during dialysis treatment. The splits occurred for reasons not determined, but these bonds could have been the most susceptible to trace chymotrypsin impurity. Although the trypsin used was PTCK-treated to destroy chymotrypsin, trypsin does exhibit residual chymotryptic activity (Inagami and Sturtevant, 1960). Kravchenko and Lapuk (1969) reported that trypsin acts on an Asn-oxidized tryptophan bond (residues 27-28) bond in photooxidized lysozyme.

The Esterification of Lysozyme in Methanol-HCl

Exhaustive methylation of lysozyme led to a very heterogeneous product. The resultant electrophoretic mobility at pH 5 was less than native, which was unexpected in terms of overall charge since replacement of the ionized carboxyl with the methylated carboxyl should have rendered the derivative more basic. However, the carboxyl methylations have probably caused an unfolding of the molecule which could have led to the peculiar electrophoretic property.

Methanolysis of peptide and/or side chain amide groups was suggested to occur because the theoretical maximum of carboxyl groups methylated was exceeded at reaction times of ninety hours and longer (by 15% at 460 hours). However, no unusual peptides were obtained in peptide mapping experiments which indicated that amide methanolysis or the N to O acyl shift was not occurring to an appreciable extent at shorter methylation times.

Recently, Shimoda and Hayashi (1970) have suggested that mild methanol-HCl or carbodiimide reaction led to an intraester linkage in lysozyme. They isolated a tryptic peptide whose composition corresponded to peptides T-9 and T-10. The peptides were not separable unless subjected to saponification treatment. This result suggested an ester bond between the two peptides, perhaps Asp 66 (T-9) with Thr 69 (T-10). If such was the case in the present study, the saponification procedure employed would have liberated the individual peptides in high yield. The T-9 peptide was obtained as the sulfanilated form in high yield, indicating that Asp 66 was not involved in any intramolecular bond.

The carboxyl group methylation rate constants, determined by peptide mapping techniques, showed varied reactivity. The groups were categorized as follows:

Glu 7, Asp 119 > Glu 35, Asp 87, Asp 101 > Asp 18, Asp 48/52, α-COOH >> Asp 66

Table 16 and Figure 27 summarize the carboxyl group environments in the crystal (Blake et al., 1967a). It is of interest to compare the methylation reactivity with that expected in the crystal. Asp 66, the slowest group to methylate, is also the only completely buried carboxyl. Evidence suggests that it has an abnormally low pK. It lies near a positively charged group and participates in two hydrogen bonds, one of which involves Tyr 53, which is of abnormally high pK (> 12; Tojo et al., 1966). It is also unreactive with EDC-glycinamide (Lin, 1970).

The next most unreactive class of carboxyl groups included Asp 18, Asp 48, Asp 52, and the α-COOH. Aspartics 18, 48, and 52 are partially buried and participate in one or two hydrogen bonds. The α-carboxyl, although quite exposed, lies near a positively charged group and participates in a hydrogen bond.

Glu 35, Asp 87, and Asp 101 were the second fastest class to methylate. Glu 35 is 30% buried and 30% as reactive as the fastest group. Asp 87 is 64% exposed, but near to a positively charged group, His 15. Asp 101 is the most exposed carboxyl and should have been the most reactive, but it was the third fastest group to methylate. The peptide containing this carboxyl was one of two isolated in low yield (the other contained Asp 119) and perhaps was not a true measure of its reactivity.

Table 16. Carboxyl group envi

Group	Exposure	Rank in Exposure[a]	H-bonds	Neighboring Ch Groups and Dist
Glu 7	0.36	6	1(Lys 1)	Lys 1 (2.8-4.
Asp 18	0.33	5	1(Asn 19)	Lys 13 (5.9-7.
Glu 35	0.31	4		Asp 52 (5.9-8.
Asp 48	0.25	3	2(Ser 50, Arg 61)	Arg 61 (3.8-8.
Asp 52	0.19	2	2(Asn 46, Asn 58)	Glu 35 (5.9-8.
Asp 66	0.0	1	2(Tyr 53, Thr 69)	Tyr 53 (3.1-4. Arg 68 (4.7-7.
Asp 87	0.64	8		His 15 (4.3-7.
Asp 101	0.87	10		
Asp 119	0.48	7		
α-COOH	0.77	9	1(Lys 13)	Lys 13 (2.4-4.

[a]In increasing order.

[b]Relative to fastest carboxyl.

[c]The following estimates were made by J. A. Rupley in col that part of a surface drawn about the group, a distance equal to that of the solvent, that is not covered by similar spheres of ot fraction exposure of the same group in model tripeptides. In def closest approach less than 6A are included; the numbers are the s

'l group environments in lysozyme.

ighboring Charge ups and Distance (A)	Expected pK	Methylation Rate	Class[b]	Rank in Methylation[a]
ys 1 (2.8-4.1)	3-4	0.54	4	9
ys 13 (5.9-7.0)		0.11	2	2
sp 52 (5.9-8.3)	6	0.30	3	6
rg 61 (3.8-8.2)		0.16	2	3-4
lu 35 (5.9-8.3)	3-3.5	0.16	2	
yr 53 (3.1-4.7), rg 68 (4.7-7.9)	1.5-2.5	0.02	1	1
is 15 (4.3-7.2)	3-4	0.36	3	8
	4.3-4.5	0.33	3	7
		1.0	4	10
ys 13 (2.4-4.0)	3-4	0.16	2	5

Rupley in collaboration with Professor D. C. Phillips. Exposure is
ance equal to the van der Waals radius of each of its atoms plus
spheres of other atoms of the protein; it is expressed as the
ides. In defining distance between charges, only groups with
ers are the shortest and longest distances between heteroatoms.

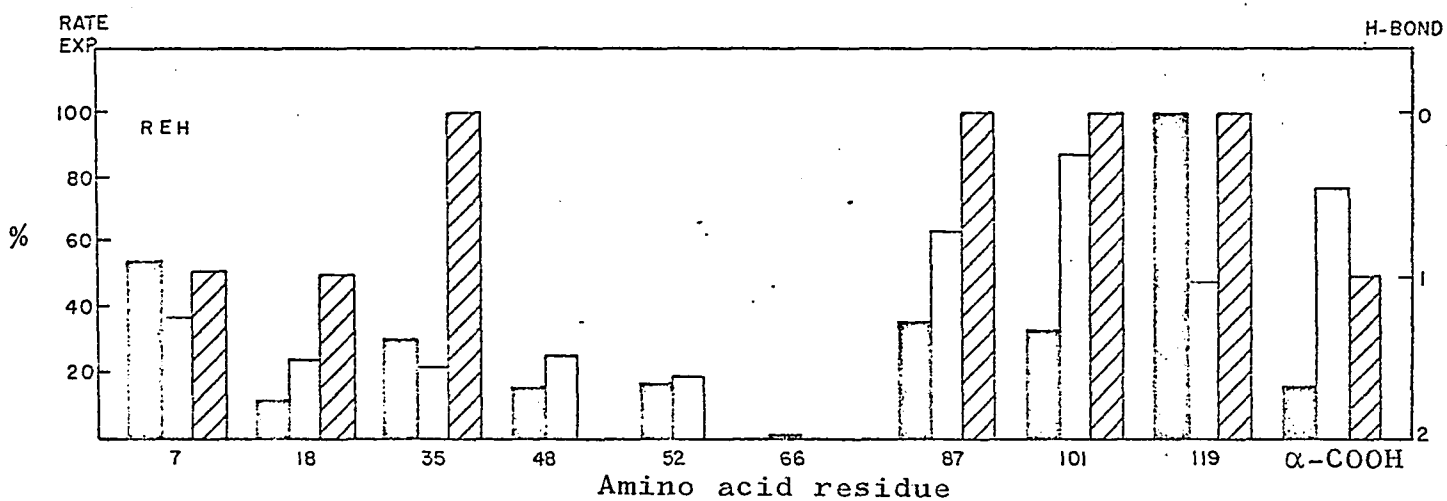


Figure 27. Reactivity in methanol-HCl, exposure and hydrogen-bonds in the crystal of the carboxyl groups of lysozyme.

The fastest groups to methylate were Glu 7 and Asp 119. Glu 7 is partially buried and hydrogen-bonded. Perhaps this bond was broken in methanol-HCl. Asp 119 is 50% exposed and not hydrogen bonded.

The reactivity of carboxyl groups did not follow perfectly the reactivity expected from the crystal structure. However, there does appear to be some correlation, especially with those carboxyl groups that are completely buried. Ribonuclease was shown to contain three nonmethylating carboxyl groups (Riehm and Scheraga, 1966) and at least two were buried in an environment similar to Asp 66 in lysozyme (Wyckoff et al., 1970). Even though methanol-HCl is a denaturant, it is interesting that carboxyl groups most likely to be unreactive in aqueous solution in terms of steric hindrance were also unreactive in methanol-HCl. This reactivity may reflect a similarity in local environment for Asp 66 in lysozyme, and Asp 14 and Asp 38 in ribonuclease when in water or methanol-HCl and suggests that esterification in methanol-HCl can be used to determine buried carboxylic acid groups in proteins. Asp 102 of chymotrypsin (Blow, Birktoft, and Hartley, 1969) is in an environment that resembles Asp 66 of lysozyme and would also be expected to be unreactive in MeOH-HCl. The carboxylate is withdrawn from solvent and forms hydrogen bonds to two groups.

Cell wall and oligosaccharide hydrolytic activities decreased rapidly with methylation. This result was not so easily interpretable since methylation was not a single group modification. It could not be explained in terms of active site carboxyl group modifications. Most likely, groups outside the active site that are essential for native conformation were involved. Effects on the binding of tri-NAG were interpretable in terms of active site carboxyl group modifications.

The inability of lysozyme to regenerate activity after being subjected to conditions which should have hydrolyzed the methyl esters is perplexing but can be explained. Alkaline or acid pH treatments might not have hydrolyzed the esters essential for activity. Also perhaps alkaline pH produced disulfide interchange or hydrolytic deamidation. Acid pH might have caused deamidation. Robinson, McKerrow, and Cary (1970) reported deamidation of asparaginyl residues in small peptides in acid solution, particularly when located near lysyl or arginyl residues. Several amides in lysozyme are close to basic residues, i.e., Asn 19 near Lys 13 and Asn 106 near Arg 112 and Lys 116.

Lysozyme Molecular Weight in Concentrated Salt Solutions

There has been considerable argument concerning the relationship of protein structures in the crystal and in solution. Since X-ray diffraction studies have produced the crystal structures of proteins, these structures have been used to explain solution chemistry. The crystal structure of lysozyme has been used to explain much of the solution behavior (Rupley, 1969). It is generally agreed that the solution structure is identical with the crystal and any difference in chemistry can be accounted for in terms of neighboring crystal lattice contacts.

Cole et al. (1969) suggested a conformational difference between the crystal and solution structures of lysozyme to explain a carboxyl group perturbation. Since association of soluble protein can also perturb carboxyl groups, the ultra-centrifuge was used to determine the degree of association of lysozyme under the conditions of the Cole et al. (1969) study. In 1.7M NaCl, no pH dependent association was seen between 2 and 8.5. Thus, the pH dependence of the solubility of lysozyme reported by Cole et al. (1969) cannot be attributed to self-association.

The suggestion of Cole et al. (1969) may be correct, in that a structural difference exists between lysozyme in the crystal and in solution. However, 25-30%

of the protein surface is affected by lattice contacts. These contacts are not so easily interpreted to suggest (as Cole *et al.* do) that no contact would render a carboxyl more basic. It is quite possible that one or several carboxyl groups could be affected in such a way as to increase their pK's, i.e., as hydrogen bond donors or being placed in a less polar environment in the crystal contacts. Figure 28 shows the groups whose exposure to bulk solvent are decreased by lattice contacts upon crystallization, the acidic groups involved being Glu 7, Asp 48, and the terminal carboxyl group.

Evidence That the Thyroxine-Lysozyme Interaction is Outside the Active Site

Thyroxine was shown not to inhibit cell wall or hexamer oligosaccharide hydrolytic activity. Also no inhibition of nonproductive trimer substrate binding was observable and lysozyme did not perturb the thyroxine UV spectrum. These results show that the association of thyroxine, if there is any, is outside the enzyme active site and that it cannot be used as an active site probe. The measurements of trimer binding and spectrum related to the upper half of the cleft and the rate studies to the entire active site.

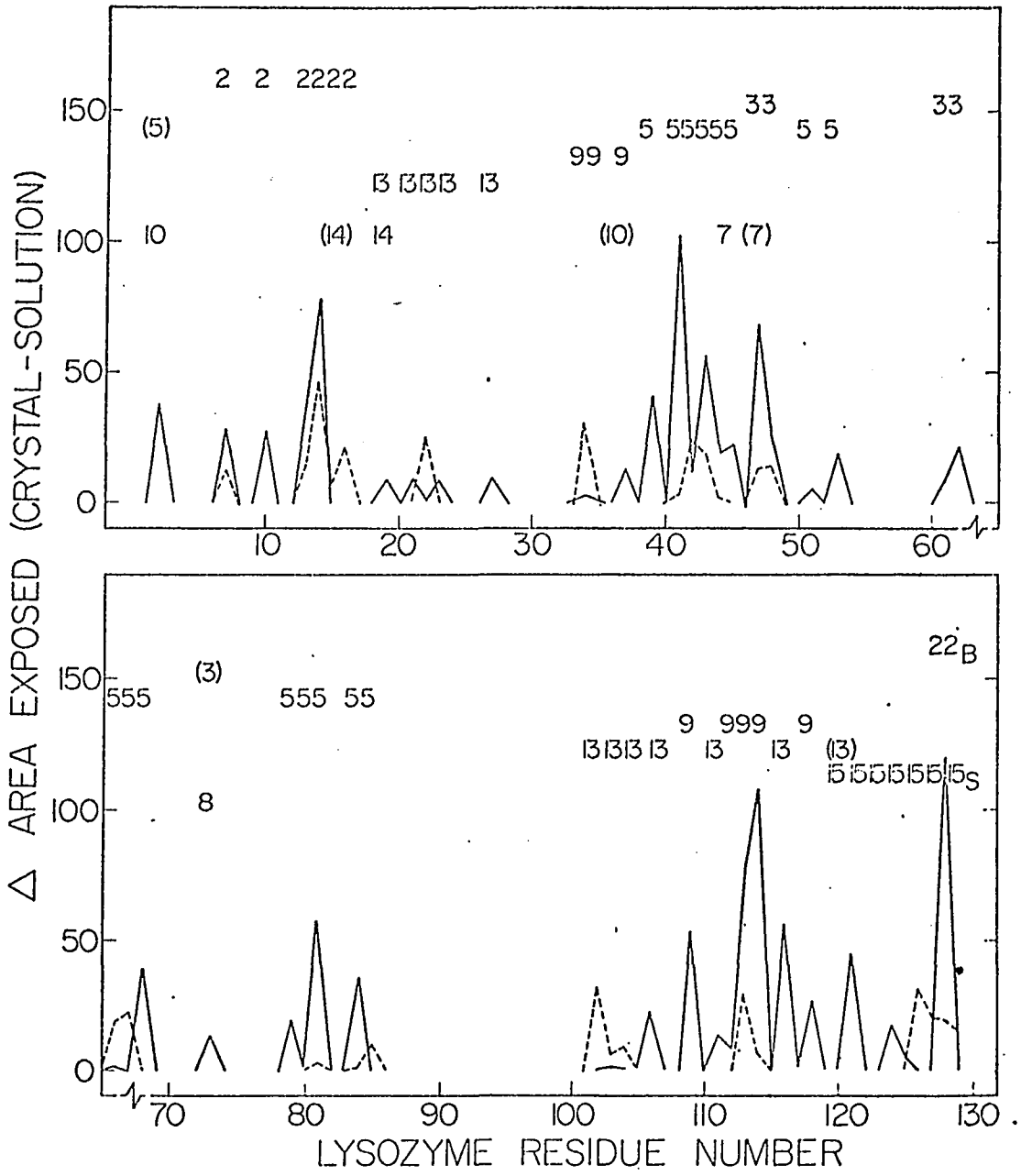


Figure 28. Effect of crystal lattice contacts on the exposure of the amino acid residues of lysozyme.

——— side chain atoms

----- backbone atoms

LIST OF REFERENCES

Abita, J. P., Maroux, S., Delaage, M., and Lazdunski, M. (1969), FEBS Letters 4, 203.

Abraham, E. P., and Robinson, R. (1937), Nature 140, 24.

Alderton, G., and Fevold, H. C. (1946), J. Biol. Chem. 164, 1.

Armstrong, J. M., and McKenzie, H. A. (1967), Biochim. Biophys. Acta 147, 93.

Banerjee, S. K., and Rupley, J. A. (1971), Biochem. in press.

Banks, T. E., Blossey, B. K., and Shafer, J. A. (1969), J. Biol. Chem. 244, 6323.

Berger, L. R., and Weiser, R. S. (1957), Biochim. Biophys. Acta 26, 517.

Bezkorovainy, A., and Grohlich, D. (1970), Biochim. Biophys. Acta 214, 37.

Blake, C. C. F. (1970), personal communication, University of Oxford.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sharma, V. R. (1965), Nature 206, 757.

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., and Sharma, V. R. (1967a), Proc. Roy. Soc. (London) Ser. B, 167, 378.

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sharma, V. R. (1967b), Proc. Roy. Soc. (London) Ser. B, 167, 365.

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), Nature 221, 337.

Bonilla, C. A. (1970), J. Chromatog. 47, 499.

Bray, G. A. (1960), Anal. Chem. 1, 279.

Broomfield, C. A., Riehm, J. P., and Scheraga, H. A. (1965a), Biochem. 4, 751.

Broomfield, C. A., Riehm, J. P., and Scheraga, H. A. (1965b), Biochem. 4, 760.

Brown, J. R. (1964), Biochem. J. 92, 13p.

Brown, J. R. (1970), personal communication, University of Texas.

Butler, L. G., and Rupley, J. A. (1967), J. Biol. Chem. 242, 1077.

Canfield, R. E. (1963a), J. Biol. Chem. 238, 2691.

Canfield, R. E. (1963b), J. Biol. Chem. 238, 2699.

Canfield, R. E., and Anfinsen, C. B. (1963), J. Biol. Chem. 238, 2684.

Canfield, R. E., and Lui, A. K. (1965), J. Biol. Chem. 240, 1997.

Carraway, K. L., and Koshland, D. E. (1968), Biochim. Biophys. Acta 160, 272.

Carraway, K. L., and Triplett, R. B. (1970), Biochim. Biophys. Acta 200, 564.

Carraway, K. L., Spoerl, P., and Koshland, D. E. (1969), J. Mol. Biol. 42, 133.

Chauvet, J., and Acher, R. (1968), FEBS Letters 2, 17.

Chu, F. S., and Crary, E. (1969), Biochim. Biophys. Acta 194, 287.

Cole, J. B., Bryan, M. L., and Bryan, W. P. (1969), Arch. Biochem. Biophys. 130, 86.

Dahlquist, F. W., and Raftery, M. A. (1968), Biochem. 7, 3277.

Donovan, J. W., Laskowski, M., and Scheraga, H. A. (1960), J. Amer. Chem. Soc. 82, 2154.

Donovan, J. W., Laskowski, M., and Scheraga, H. A. (1961), J. Amer. Chem. Soc. 83, 2686.

E. C. Apparatus Technical Bulletin #128 (1966), E. C. Apparatus Company, Philadelphia.

Eyl, A., and Inagami, T. (1970), Biochem. Biophys. Res. Comm. 38, 149.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), J. Biol. Chem. 244, 4989.

Fleming, A. (1922), Proc. Roy Soc. 93, 306.

Fraenkel-Conrat, H. (1950), Arch. Biochem. Biophys. 27, 109.

Fraenkel-Conrat, H., and Olcott, H. S. (1945), J. Biol. Chem. 161, 259.

Frieden, E. H. (1956), J. Amer. Chem. Soc. 78, 961.

Frugoni, J. A. C. (1957), Gazz. Chem. Ital. 87, 403.

Fujio, H., Kishiguchi, S., Shinka, S., Saiki, Y., and Amano, T. (1959), Biken's Journal 2, 56.

Gelotte, B., and Porath, J. (1969), Chromatography. Edited by E. Heftman, 2nd ed., Reinhold, New York.

Glazer, A. N. (1970), Ann. Rev. Biochem. 39, 101.

Goldberger, R. F., and Epstein, C. J. (1963), J. Biol. Chem. 238, 2988.

Handbook of Chemistry and Physics (1963), The Chemical Rubber Publishing Co., Cleveland, Ohio.

Hartdegan, F. J. (1967), Ph.D. Thesis, The University of Arizona.

Hartdegan, F. J., Imoto, T., and Rupley, J. A. (1970), personal communication, The University of Arizona.

Hayashi, K., Imoto, T., Funatsu, G., and Funatsu, M. (1965), J. Biochem. Tokyo 58, 227.

Hegarty, A. F., and Bruice, T. C. (1970), J. Amer. Chem. Soc. 92, 6568.

Heilmann, J., Barollier, J., and Watzke, E. (1957), Hoppe-Seyler's Z. physiol. Chem. 309, 219.

Hoare, D. G., and Koshland, D. E. (1966), J. Amer. Chem. Soc. 88, 2057.

Hoare, D. G., and Koshland, D. E. (1967), J. Biol. Chem. 242, 2447.

Horinishi, H., Nakaya, K., Tani, A., and Shibata, K. (1968), J. Biochem. Tokyo 63, 41.

Imoto, T., and Rupley, J. A. (1970), personal communication, The University of Arizona.

Inagami, T., and Sturtevant, J. M. (1960), J. Biol. Chem. 235, 1019.

Iwai, K., and Ando, T. (1967), Methods Enzymol. 11, 263.

Jollés, J., Jauregui-Adell, J., Bernier, I., and Jollés, P. (1963), Biochim. et Biophys. Acta 78, 668.

Jollés, P. (1960). In "The Enzymes," 2nd ed. (P. D. Boyer, H. Lardy, and K. Myrback, eds.), Vol. IV, Chapter 25. Academic Press, New York.

Jollés, P., Jaurequi-Adell, J., and Jollés, J. (1964), C. R. Acad. Sci. Paris 258, 3926.

Katz, A., Dreyer, W. J., and Anfinsen, C. B. (1959), J. Biol. Chem. 234, 2897.

Khorana, H. G. (1953), Chem. Revs. 53, 145.

Kravchenko, N. A., and Lapuk, Y. Kh. (1969), Biokhimiya 35, 53 (English).

Kravchenko, N. A., Chentsova, T. V., and Kaverzneva, E. D. (1967), Biokhimiya 33, 295 (English).

Kurono, A., and Hamaguchi, K. (1964), J. Biochem. Tokyo 56, 432.

Lemieux, R. U., and Huber, G. (1955), Can. J. Chem. 33, 128.

Levy, D., and Carpenter, F. H. (1970), Biochem. 9, 3215.

Lin, T. (1970), Biochem. 9, 984.

Lin, T., and Koshland, D. E. (1969), J. Biol. Chem. 244, 505.

Litwack, G. (1963), Biochim. Biophys. Acta 67, 501.

Litwack, G., and Scars, M. L. (1965), J. Biol. Chem. 240, 674.

Marzotto, A., and Galzigna, L. (1970), Arch. Biochem. Biophys. 137, 373.

Matsubara, H., and Sasaki, R. M. (1969), Biochem. Biophys. Res. Comm. 35, 125.

McDonald, C. C., and Phillips, W. D. (1969), Biochem. Biophys. Res. Comm. 35, 43.

Mukerjee, H., and Sri Ram, J. (1965), Federation Proc. 24, 418.

Osawa, H. (1970), Biochem. 9, 2158.

Parsons, S. M., and Raftery, M. A. (1969), Biochem. 8, 4199.

Parsons, S. M., and Raftery, M. A. (1970), Biochem. Biophys. Res. Comm. 41, 45.

Parsons, S. M., Jao, L., Dahlquist, F. W., Borders, C. L., Groff, T., Racs, J., and Raftery, M. A. (1969), Biochem. 8, 700 (69).

Perrin, D. D. (1965), "Dissociation Constants of Organic Bases in Aqueous Solution," p. 367. Butterworth, Inc., Washington, D. C.

Praissman, M., and Rupley, J. A. (1968), Biochem. 7, 2446.

Radhakrishnan, T. M., Walsh, K. A., and Neurath, H. (1967), J. Amer. Chem. Soc. 89, 3059.

Rand-Meir, T., Dahlquist, F. W., and Raftery, M. A. (1969), Biochem. 8, 4206.

Riehm, J. P., and Scheraga, H. A. (1966), Biochem. 5, 991.

Riordan, J. F., and Hayashida, H. (1970), Biochem. Biophys. Res. Comm. 41, 122 (70).

Robinson, A. B., McKerrow, J. H., and Cary, P. (1970), Proc. Nat'l. Acad. Sci. U.S. 66, 753.

Rossi, G. L., Holler, E., Kumar, S., Rupley, J. A., and Hess, G. P. (1969), Biochem. Biophys. Res. Comm. 37, 757.

Rupley, J. A. (1964), Biochim. Biophys. Acta 83, 245.

Rupley, J. A. (1967), Proc. Roy. Soc. (London) Ser. B, 167, 416.

Rupley, J. A. (1968), J. Mol. Biol. 35, 455.

Rupley, J. A. (1969), p. 291 in "Structure and Stability of Biological Macromolecules," edited by S. N. Tomasheff and G. D. Fasman. Marcel Dekker, Inc., New York.

Rupley, J. A., and Gates, V. (1967), Proc. Nat'l. Acad. Sci. U. S. 57, 496.

Rupley, J. A., Kregar, I., and Turk, V. (1970), personal communication, The University of Arizona.

Sadtler Standard Spectra (1967), Pharmaceutical Ultraviolet R625U, Sadtler Research Laboratories, Inc., Philadelphia.

Sakakibara, R., and Hamaguchi, K. (1968), J. Biochem. Tokyo 64, 613.

Salton, M. R. J. (1952), Nature 170, 746.

Salton, M. R. J., and Ghuysen, J. M. (1960), Biochim. et Biophys. Acta 45, 355.

Sanger, F., and Thompson, E. O. P. (1963), Biochim. Biophys. Acta 71, 468.

Schachman, H. K. (1957), Methods Enzymol. 4, 32.

Scheline, R. R., and Longberg, B. (1967), Acta. Pharmacol. et Toxicol. 23, 1.

Sheehan, J. C., Cruikshank, P. A., and Boshert, G. L. (1961), J. Org. Chem. 26, 2525.

Shimoda, T., and Hayashi, K. (1970), personal communication, Kyushu University, Japan.

Sophianopoulos, A. J. (1969), J. Biol. Chem. 244, 3188.

Sophianopoulos, A. J., and Van Holde, K. E. (1964), J. Biol. Chem. 239, 2516.

Spande, T. F., Witkop, B., Degani, Y., and Patchornik, A. (1970), Adv. Prot. Chem. 24, 98.

Stark, G. R. (1970), Adv. Prot. Chem. 24, 261.

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge. Clarendon Press, Oxford.

Tanford, C., and Wagner, M. C. (1954), J. Amer. Chem. Soc. 76, 3331.

Technicon Technical Bulletin #AAA-1 (1967), Technicon Corporation, Ardsley, New York.

Technicon Technical Bulletin #T-67-101 (1967), Technicon Corporation, Ardsley, New York.

Timasheff, S., Susi, H., Townsend, R., Stevens, L., Gorbunoff, M. J., and Kumosinski, T. F. (1968), p. 173 in Conformation of Biopolymers, Vol. 1, edited by G. N. Ramachandan, Academic Press, New York.

Tojo, T., Hamaguchi, K., Imanishi, M., and Amano, T. (1966), J. Biochem. Tokyo 60, 538.

Viscidi, E., Consiglio, E., and Roche, J. (1966), Biochim. Biophys. Acta 121, 424.

Vogel, A. I. (1959), A Textbook of Practical Organic Chemistry, p. 168. Longmans, Green, and Company, New York.

Whitaker, J. R. (1963), Anal. Chem. 35, 1950.

Wilchek, M., Frensdorff, A., and Sela, M. (1967), Biochem. 6, 247.

Wilcox, P. E. (1967), Methods Enzymol. 11, 605.

Wilcox, P. E., Cohen, E., and Tan, W. (1957), J. Biol. Chem. 228, 999.

Woody, R. W., Friedman, M. E., and Scheraga, H. A. (1966), Biochem. 5, 2034.

Wyckoff, H. W., Tsernoglov, D., Hanson, A. W., Knox, J. R., Lee, B., and Richards, F. M. (1970), J. Biol. Chem. 245, 305.