67-3963

HARTDEGEN, Francis Joseph, 1934-
CHEMICAL MODIFICATION OF LYSOZYME.

University of Arizona, Ph.D., 1967
Chemistry, biological

University Microfilms, Inc., Ann Arbor, Michigan

CHEMICAL MODIFICATION OF LYSOZYME

by

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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

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THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Francis Joseph Hartdegen

entitled Chemical Modification of Lysozyme

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

Dissertation Director — Date 8/20/66

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ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. John A. Rupley for his direction and interest during the course of the research and the writing of this dissertation.

The financial assistance of the National Science Foundation is gratefully acknowledged.

The author is especially indebted to his wife, Susan, for her understanding, encouragement and, above all, patience.

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ABSTRACT

Lysozyme was reacted with a 2.6 X 10^2 molar excess of iodoacetic acid at pH 5.5 at 40° for 24 hours. The reaction products were separated on Bio-Rex 70, and a protein species was isolated which was shown by amino acid analysis to be unchanged from the parent protein except for the conversion of histidine to 3-carboxymethyl-histidine. Since the modified protein retained 40% of the original activity, histidine is not involved in the bond-breaking steps of the reaction catalyzed by lysozyme, a conclusion confirmed by the failure of inhibitors which are substrate analogs (N-acetylglucosamine and its trimer) to protect against histidine carboxymethylation, and by a lack of a maximum in the rate of reaction at the pH of maximum enzymic activity.

The reaction of lysozyme with iodine in a molar ratio of 1.0 to 0.5 showed that with increasing pH the reaction rate also increased, but the extent of inactivation decreased. The products of reaction with ^{131}I at pH 5.5 were separated on Bio-Rex 70, and a species was isolated which was enzymically inactive and did not contain ^{131}I. In it, one of the six tryptophans had been oxidized to oxindole-tryptophan, determined from its spectrum and by complete enzymic hydrolysis using,

sequentially, pronase, carboxypeptidase A and B, and leucine aminopeptidase. Peptides from a tryptic hydrolysis of the inactive species were separated on Dowex-1 and purified on Sephadex G-25. The one containing an oxindole spectrum was isolated, and amino acid analysis after acid hydrolysis identified tryptophan-108 as the residue modified. Calculation of the peptide yield showed only this tryptophan had been oxidized by iodine. Oxidation of tryptophan-108 was prevented by the presence of inhibitors; no decrease in optical rotation accompanied the oxidation, showing inactivation was not due to gross denaturation. Therefore, it was concluded that tryptophan-108 is an element of the active site of lysozyme.

An unexpected cleavage of the oxidized-tryptophan-108 peptide bond occurred during tryptic hydrolysis or peptide separation, and a mechanism for this is proposed.

It had been reported (K. Hayashi, T. Imoto, G. Funatsu, and M. Funatsu, J. Biochem. Japan, 58, 227 (1965)) that N-bromosuccinimide oxidized tryptophan-62 of lysozyme; this was confirmed by isolation and amino acid analysis of the oxindole-containing peptide. Comparison on Sephadex G-25 of the tryptic peptides from both iodine and N-bromosuccinimide oxidized lysozyme showed they were indeed different.

The rate of oxidation of tryptophan-108 was shown to be half, and that of tryptophan-62 equal to that of the

oxidation of N-acetyltryptophan ethyl ester. The difference in lysozyme reactivity with the two oxidizing agents was explained by postulating that tryptophan-108, while partially buried and therefore not available to the N-bromosuccinimide molecule, is activated in iodine oxidation by neighboring groups, whereas tryptophan-62 is the most exposed and reacts normally with the N-bromosuccinimide.

The function of tryptophan-108 of lysozyme as a binding site is discussed with reference to the known crystal structure.

I. INTRODUCTION

Lysozyme is a low molecular-weight protein which lyses bacterial cell walls and which is widely distributed in nature. These investigations were initiated to elucidate the chemistry of its enzymic action, specifically, to examine the involvement of histidine and tryptophan residues. Hen's-egg-white lysozyme was chosen for this work, since, in addition to its being the most thoroughly characterized lysozyme, it is the protein for which the three-dimensional crystallographic structure has been determined (Blake et al., 1965).

Chemistry of Lysozyme.--Lysozyme is found naturally in plants, animals (both invertebrate and vertebrate), and certain bacteria, occurring in tears, nasal mucosa, saliva, blood serum, milk, and many cells and tissues (Jollès, 1960). It was originally isolated from hen's-egg-white by Alexander Fleming (1922), and was first crystallized by Abraham and Robinson (1937) from nasal mucosa; it is routinely prepared according to the method of Alderton and Fevold (1946), by precipitating the crystals from a 5% sodium chloride suspension of hen's-egg-white at pH 10.5. Lysozyme is an acid and heat stable basic protein of isolectric point 10.5 (Jollès, 1960). In the native state

it is not hydrolyzed by trypsin, chymotrypsin or papain, but is hydrolyzed by pepsin (Jollès, 1960). The natural substrate is the bacterial cell wall and extensive investigations (Shugar, 1952; Salton and Ghuysen, 1960) have been made of its action on the Gram-positive organism Micrococcus lysodeikticus. In this connection, it hydrolyzes β-(1-4)-glycosidic bonds of alternating copolymers of N-acetylglucosamine and N-acetylmuramic acid (from bacterial cells walls) or polymers of N-acetylglucosamine (from chitin) (Salton and Ghuysen, 1960). The primary structure of lysozyme has been determined independently by Jollès et al. (1963) and Canfield (1963b), who found the enzyme to be composed of a single polypeptide chain of 129 amino acids and to have an isoelectric molecular weight of 14,307, calculated from the sequence. There are four internal disulfide bridges of known location (Jollès, Jauregui-Adell, and Jollès, 1964). It is of particular interest that lysozyme has six tryptophans and one histidine residue. Recently, the X-ray crystallographic analysis of lysozyme by Phillips and co-workers (Blake et al., 1965) has produced a Fourier map of the electron distribution at 2 A resolution in which the structure of the molecule can be clearly seen, making lysozyme the first (and at present, the only) enzyme whose three dimensional structure has been established. Crystallographic analysis (D. C. Phillips, unpublished results) at 2 A resolution of the complex formed

from the enzyme and N-acetylglucosamine or its trimer revealed that the binding site of these inhibitors is at a cleft in the surface of the molecule which contains three of the six tryptophans but not the histidine.

Chemical Modification of Enzymes.--Recently, much effort (Putnam, 1953; Fraenkel-Conrat, 1959; Ram et al., 1962) has been devoted to changing the primary structure of enzymes without a concurrent change in secondary or tertiary structure, the purpose being to determine which amino acid side chains are essential to the enzymic activity (both the binding and cleavage processes) as contrasted to those which help maintain the folding of the enzyme protein. If an X-ray crystallographic image of the enzyme is available (as in the case of only lysozyme at present) it then becomes possible to compare chemical information obtained from solution studies with the detailed structural information determined from the crystal. Both types of information are necessary if the mechanism of action of an enzyme is to be understood; crystallographic data can show the active site region of the enzyme molecule and therefore the amino acid side chains which are potentially involved in the enzymic activity; chemical studies alone can show which of these side chains are in fact essential and how they function. It is, of course, necessary to assume the structure in the

the crystal and solution is closely related, and for lysozyme, inhibitor binding studies of both crystalline and solution forms show both conformations are equivalent (L. Butler and J. A. Rupley, unpublished results).

It is of interest that two approaches can be distinguished in chemical modification studies. In the first, extensive modification, all side chains of a certain type are reacted completely and selectively with an appropriate reagent; if the resultant modified protein is still fully active, it is concluded that this amino acid is not necessary for binding or cleavage. For example, Geschwind and Li (1957) fully guanidinated the six lysines of lysozyme with O-methyliso-urea and obtained a fully active enzyme, and concluded the amine side chains are unessential for activity. This contrasts to the observation (Fraenkel-Conrat, 1950) that esterification of the carboxyl groups of lysozyme leads to an inactive enzyme, suggesting that certain of the carboxyl side chains may be essential for activity.

The other approach, minimal modification, is to chemically modify the enzyme to a small extent (preferably changing only one residue) but in such a way as to destroy all of the enzymic activity. This approach was used in these studies for it was felt that more specific information could be so obtained. In a classic example of this procedure, Gundlach, Stein, and Moore (1959) reacted

ribonuclease with iodoacetic acid at pH 5.5 and obtained a fully inactive product in which histidine-119 was carboxymethylated with no other change occurring, clearly implicating this amino acid in the catalytic process. This type of experiment is often successful since chemical agents can react more easily (as does the substrate) with exposed binding sites or abnormally reactive catalytic groups than with less accessible areas of the enzyme.

Chemical Modification of Lysozyme.--Much of the early work on chemical modification of lysozyme implicated histidine and tryptophan. The importance of histidine was inferred by Fraenkel-Conrat (1950), who reacted lysozyme with high concentrations of iodine and reported the reversible loss of activity with the concurrent formation of N-iodohistidine. Additional support for histidine's participation in the catalysis was provided by the photo-oxidation studies of Weil, Buchert, and Maher (1952), who showed a parallel between the decrease of activity and the oxidation of histidine. Ultra-violet inactivation, studied by Shugar (1952), and N-bromosuccinimide induced oxidation, studied by Ramachandran and Rao (1962), also implicated the single histidine as a catalytically important residue. The first definitive experiment was performed by Kravchenko, Kleopina, and Kaverzneva (1962), who reacted lysozyme with iodoacetic acid at pH 5.5 and

separated the resultant products, one of which was fully active although its histidine side chain was carboxymethylated. This should have clearly established that histidine was not involved in the enzymic activity of lysozyme. However, some subsequent work has supported the involvement of histidine. Ferrini (1964) photo-oxidized lysozyme and reported the activity loss was due to histidine oxidation alone; Jauregui-Adell and Jollès (1964) found no histidine reacting in carboxymethylation experiments. In contrast, the recent X-ray analysis (D. C. Phillips, unpublished results) has established that histidine is far removed from the catalytic site of the enzyme molecule, clearly confirming the work of Kravchenko et al. (1962).

Tryptophan has been implicated as a vital side chain through several experiments. Weil et al. (1952) photo-oxidized crystalline lysozyme in the presence of methylene blue and followed the loss in activity as a function of the loss of the various amino acids; at complete enzymic inactivation, all the histidine and tyrosines and four of the tryptophans were oxidized. Ramachandran and Rao (1962) reacted lysozyme with N-bromosuccinimide, studying activity loss as a function of the oxidation of the tryptophan indole to an oxindole, a change that is readily observable in the ultraviolet spectrum. The oxidation of one tryptophan and the histidine led to a 70%

activity loss; oxidization of over four of the six tryptophans was required to achieve full activity loss. It was concluded that histidine and tryptophan were both necessary for activity and that they had an independent role in the mechanism.

It was mentioned that one primary goal of chemical modification is to obtain singly-modified inactive species; the above work on the tryptophans is difficult to interpret because of the unspecific reactions and the presumably complex mixtures of products formed. A pure singly modified enzyme was prepared by Hayashi et al. (1965), who reacted lysozyme at pH 4.5 with N-bromosuccinimide and obtained a product which was completely inactive and had tryptophan-62 oxidized to an oxindole; since no other change had occurred, this clearly established the functional importance of tryptophan-62.

The possible involvement of a second tryptophan can be inferred from the calculation of the ultraviolet absorbance changes which follow the formation of complexes between lysozyme and a substrate, poly-N-acetylglucosamine, a change first observed by Hayashi, Iomoto, and Funatsu (1963). The maximum effect is that expected for the transfer of one to one-and-a-half fully exposed indole groups from a water to an alcohol environment, which is consistent with the X-ray data (D. C. Phillips, unpublished results) that shows three tryptophans in the binding site.

In summary, at least one tryptophan but not the histidine is intimately involved in the catalytic reactivity of lysozyme. The work which follows bears upon the function of the single histidine (done concurrently with the work of Kravchenko *et al*. (1962)) and of tryptophans-62 and 108.

II. EXPERIMENTAL

Materials.--The following crystalline enzymes were purchased from Worthington Biochemical Corporation: Lysozyme (Lot LY 626), Trypsin (Lot TRL 6256), Carboxypeptidase A (Lot COA 693), Carboxypeptidase B (Lot COB 6069), and Leucine Aminopeptidase (Lot LAP 5971). Pronase (Streptomyces griseus protease) was purchased from California Corporation for Biochemical Research (Lot 34045). All enzymes were used without further purification, except trypsin which was digested in 0.063 M HCl (100 mg in 10.0 ml) for 18 hours at 40°, the method of Jollès et al. (1963) for inactivating chymotryptic impurities. Lysozyme was chromatographed by the method of Alderton and Fevold (1946), and was found to contain less than 3% impurities.

Sephadex G-25 Beads (particle size 40- 120 μ) was purchased from Pharmacia. Two analytical grade cationic exchange resins of 200-400 mesh (chloride form), Bio-Rex 70 and Dowex-1 X-2, were purchased from Bio-Rad Laboratories.

A dried cell-wall powder of Micrococcus lysodeikticus was purchased from Worthington Biochemical Corporation.

All other chemicals were of the highest purity commercially available, and deionized water was routinely used.

Protein Concentration.--Protein concentration was determined by optical density measurements made with a Zeiss PMQII spectrophotometer. The extinction coefficient of lysozyme was determined as follows: lysozyme was exhaustively dialyzed against deionized water and the spectrum of an aliquot of the resultant solution was determined in a pH 7.0 buffer. Two 1.0 ml samples (containing 6.4 mg) were dried at 104° to constant weight (obtained in 6 hours) to give an $E^{1\%}_{280\ m\mu}$ of 25.5 $\pm$ 0.1; other reported values are: 21.6 (Shugar, 1952), 25.3 (Bruzzesi, Chiancone, and Antonini, 1965), 26.4 (Sophianopoulos et al., 1962), 26.5 (Canfield, 1963a), 27.1 (Hayashi et al., 1965), 27.2 (Ehrenpreis and Warner, 1956), and 27.3 (Glazer, 1959).

The extinction coefficient of inactive, iodine oxidized lysozyme was similarly determined (using 5.2 mg samples) and was found to be: $E^{1\%}_{280\ m\mu} = 22.7 \pm 0.1$.

Enzymic Activity.--Enzymic activity was measured by the rate of Micrococcus lysodeikticus cell-wall lysis using the method of Shugar (1952). The decrease in turbidity at a wave-length of 450 mμ of a cell-wall suspension of optical density 1.0 was measured as a function of time with a Varicord Model 43 Linear-Log recorder coupled to the Zeiss spectrophotometer. Activity was defined as the initial rate of change of optical density (linear for at

least 30% of the reaction), and was expressed as the percentage of the rate obtained with an equal weight concentration of native lysozyme.

Quantitative Amino Acid Analyses.-- Quantitative amino acid analyses were performed on a Technicon Auto-Analyzer using the modification of Hamilton (1963) of the procedure of Piez and Morris (1960). For samples containing tryptophan, the column temperature was raised from 60° to 62° to achieve separation from arginine. Protein samples were hydrolyzed enzymically or in 6 M HCl at 105° for 18 hours under nitrogen. Since the analytical procedure does not separate cystine from 3-carboxymethylhistidine (1-carboxymethylhistidine is separable from all other amino acids), samples containing this amino acid were first analyzed normally and then were subjected to performic acid oxidation to quantitatively convert cystine to cysteic acid, so isolating the 3-carboxymethylhistidine peak. The positions of the carboxymethyl-histidine isomers in the Piez and Morris (1960) procedure were located by comparing the peaks obtained with a sample of N-acetylhistidine which had been carboxymethylated and acid hydrolyzed by the method of Gundlach et al. (1959) to the positions of these peaks located by Crestfield, Stein, and Moore (1963) in their procedure. The oxidation was performed as follows, according to the method of Hirs (1956): A 10 mg protein

sample was dissolved in 0.25 ml of acetic acid and 0.05 ml of dry methanol; 0.05 ml of performic acid was added before reacting 2-1/2 hours at -10°. The product was diluted in water and lyophilized to dryness *in vacuo* under phosphorous pentoxide.

Preparation of Carboxymethyl-Lysozyme.--Lysozyme (500 mg) was reacted with iodoacetic acid (1.73 gm) in 20 ml of 0.1 M phosphate buffer of pH 5.0 at 40° for 24 hours. The product was desalted by passing it through a 2.4 X 30 cm column of Sephadex G-25 and eluting with a 0.1 M $NH_4C_2H_3O_2$ buffer of pH 8.0; the desalted protein was lyophilized to dryness. The products were separated on a 2.4 X 30 cm column of Bio-Rex 70 which had been equilibrated with a 0.05 M phosphate buffer of pH 7.18. Elution was accomplished by a linear gradient using 4 liters of the washing buffer and 4 liters of a 0.2 M phosphate buffer of the same pH. Fractions of 25 ml were collected and analyzed for optical density and enzymic activity. The reaction was also carried out in the presence of either 0.1 M N-acetylglucosamine or 0.1 M di-N-acetylglucosamine.

Carboxymethylation at Various pH Values.--Lysozyme was reacted with iodoacetic acid at 40° as follows: pH 3.0 (6.2 mg/ml lysozyme, 6.0 mg/ml iodoacetic acid, adjusted to pH 3.0, unbuffered), pH 5.0, 6.0, 7.0, 8.0, and 9.0 (10.5 mg/ml lysozyme, 12.0 mg/ml iodoacetic acid in 0.05 M

citrate or tris-(hydroxymethyl)-amino-mathane buffer), and pH 10.0 (7.5 mg/ml lysozyme, 6.0 mg/ml iodoacetic acid in 0.1 M borate buffer). The half-times of all reactions were determined from a plot of activity against time. At pH 3.0 there was no activity loss in 24 hours; the pH 10.0 half-time was adjusted for concentration to make it equivalent to the pH 5.0 to 9.0 reaction concentrations.

Reaction of Lysozyme with Iodine at Various pH Values.--Lysozyme was reacted with iodine, using a molar ratio of 1.0 to 0.5, at pH 5.5, 6.5, 8.0, and 10.6. To 100 mg of lysozyme in 10.0 ml of water was added 0.1 ml of the standard iodine solution (0.04 M I_2 in 0.48 M KI). The pH was maintained by the addition of 0.25 M NaOH. The enzymic activity of an aliquot sample was measured before iodine addition and after complete reaction, determined by complete loss of iodine color and cessation of base uptake.

Isolation of an Inactive Lysozyme.--Lysozyme was reacted with a half-molar amount of iodine at pH 5.5 as previously described, except using ^{131}I (0.09 ml of the standard iodine solution plus 0.01 ml of 0.04 M ^{131}I as KI containing 0.73 μC). After complete reaction, the mixture was lyophilized to dryness and 20 mg of the product was separated on a 1.0 X 45 cm column of Bio-Rex 70 which had been equilibrated with a 0.05 M borate buffer of pH 10.0. Elution was accomplished using a linear gradient over 288 ml

from 0 to 0.16 M NaCl in the borate buffer. Fractions of 2.0 ml were collected at the rate of 1.2 ml/minute and diluted with an equal volumn of water. Each fraction was analyzed for its optical density at 280 mμ and for its enzymic activity. Aliquots of 1.0 ml of each fraction were counted to determine ^{131}I, using a Nuclear-Chicago Model 192A Ultrascalor attached to a Model 1810 Radiation Analyzer.

For the routine preparative separation of the products, a sample of the iodine reaction mixture containing 500 mg protein was chromatographed on a 2.4 X 45 cm column of Bio-Rex 70 using a 7 liter linear gradient at a rate of 2.5 ml/minute. Fractions of 25 ml were collected.

Complete Enzymic Hydrolysis. Hydrolysis by pronase, leucine aminopeptidase and carboxypeptidase A and B was carried out in three steps to prevent hydrolysis of one protease by the others. Protein (25 mg) and pronase (4.0 mg) were dissolved in 1.0 ml of 0.1 M tris-(hydroxymethyl)-amino-methane buffer of pH 7.25 and were reacted for 98 hours at 40° (0.025 ml of toluene was present to minimize bacterial growth). The sample was then boiled for 5 minutes, 1.19 mg carboxypeptidase A and 1.0 mg carboxypeptidase B were added, and the volume was brought to 1.0 ml with water. After 42 hours at 40°, the sample was again boiled for 5 minutes, leucine aminopeptidase (0.75 mg) was

added and the solution was made 0.058 M in $MnCl_2$ and brought to 1.0 ml in volume. After reacting for 42 hours at 40°, the sample was boiled for 5 minutes and lyophilized to dryness. A blank without the initial protein sample was run under identical conditions.

Difference Spectrum.--Solutions of native and iodine-inactivated lysozyme of the same molarity were prepared by adjusting their optical densities at a wave-length of 280 mμ to 0.761 and 0.678 respectively, in accordance with the extinction coefficients. The optical density of native lysozyme versus iodine-inactivated lysozyme was read at intervals of one millimicron between 230 and 350 mμ.

Specific Rotations.--Specific rotations of 400 μM samples of native and iodine inactivated lysozyme were determined at a wave-length of 589 mμ using a Bendix Automatic Polarimeter Model 143A standardized with dextrose.

Reaction with Iodine in the Presence of Inhibitors.--Lysozyme was reacted with iodine at pH 5.5 as described above, but in the presence of either 0.1 M N-acetylglucosamine or 0.1 M di-N-acetylglucosamine. Each solution was lyophilized to dryness after complete reaction and a 10.0 mg sample was separated on a 1.0 X 45 cm column of Bio-Rex 70. The effluent was analyzed automatically using a

Technicon AutoAnalyzer to determine ninhydrin-positive material.

Reduction and Carboxymethylation.--The method of Jollès et al. (1963) was used in preference to that of Canfield and Anfinsen (1963) since it was experimentally more suitable for small samples. In both methods, acid hydrolysis of the product showed cystine had been quantitatively convereed to S-carboxymethylcysteine and that methionine was almost completely lost (probably by conversion to homoserine), with no other change occurring. Protein samples (100 mg) were dissolved in 20 ml of 8 M urea, and 0.2 ml of mercaptoacetic was then added. The pH was adjusted to 8.5 with 50% NaOH before reacting for 6 hours under nitrogen at room temperature. The pH did not change during the reaction. Iodoacetic acid (920 mg) was added to the solution of reduced protein and the pH was maintained at 8.5 by the addition of 1.0 M NaOH. After 2 hours, the protein was exhaustively dialyzed against water and lyophylized to dryness. Amino acid analysis after acid hydrolysis was done after every preparation, and the sample was discarded if it revealed more than 3% of one residue of cysteine. No peptide analysis ever revealed a measurable amount of cysteine.

Tryptic Hydrolysis.--A 100 mg sample of reduced carboxymethyl-protein in 10.0 ml of water was reacted with

4.0 mg of chymotrypsin-free trypsin for two hours at 40° while maintaining the pH at 8.0 by the addition of 0.1 M NaOH. The tryptic peptides were lyophylized to dryness. In a typical experiment, 8.5 moles of base were used per mole of protein.

The initially insoluble sample usually dissolved completely within 20 minutes; if it did not, the pH was raised to 10.0 with concentrated NH_4OH and the temperature was raised to 60° to solubilize the sample, the method initially used by Canfield (1963a). The temperature and pH were then lowered causing the protein to be precipitated in a form well enough dispersed to be easily hydrolyzed by another 4.0 mg addition of trypsin.

Peptide Separation on Dowex-1.--Peptide samples (10 to 25 mg) were separated on a 1.0 X 30 cm column of Dowex-1 at room temperature. The resin was prepared by washing alternatively with 1 M NH_4OH, water, 1 M HCl, water, 1 M NH_4OH and was then equilibrated with 0.05 NH_4OAc buffer of pH 8.0 before packing the column. The sample was eluted by pumping at 0.6 ml/minute a linear gradient obtained by using two 100 ml chambers, the first containing the equilibrating buffer, the second 2.0 M acetic acid. Fractions of 3.0 ml were collected and analyzed for optical density at 250, 266, and 280 mμ.

Normally, Dowex-1 separations were performed directly on the product of a tryptic hydrolysis, leading to good yields (see Chapter III for percentages). Purification of the product of a Sephadex G-25 or Dowex-50 separation on Dowex-1 led to very poor yields (0 to 25%) of tryptophan containing peptides; this sequence was therefore seldom used.

A longer column (150 cm) of Dowex-1 gave a recovery of tryptophan peptides only half as great as the short column, while the separation was not significantly improved. Dowex-1 separations were also carried out at 40° and 60° but produced no better resolution, perhaps owing to extensive gas release in the column.

Published methods for separation of lysozyme tryptic peptides, for example Jollès et al. (1963) and Canfield (1963a), were not used since they employed organic bases (for example, pyridine and collidine) which imparted a high optical density to the effluent solution, requiring ninhydrin analysis of the fractions.

Short Dowex-50 columns (10 cm) were tried but resulted in low yields of tryptophan containing peptides, and in peaks which could not be purified by Sephadex G-25.

Peptide Separation on Sephadex G-25.--Sephadex G-25 was sized before packing by stirring 100 gm of resin in 500 ml of water and after 10 minutes removing the unsettled

material by suction; the procedure was repeated three times. Peptide samples (10 to 25 mg) were separated on a 1.0 x 75 cm column of Sephadex G-25 by eluting with a 0.05 M $NH_4C_2H_3O_2$ buffer of pH 8.0 at a pumping rate of 0.3 ml/minute. Fractions of 3.0 ml were collected and analyzed for optical density. Under these conditions, it was found that salt (NaCl) eluted at Fraction 16, and the void volume corresponded to approximately Fraction 3.

Sephadex G-25 separations of the products of a tryptic hydrolysis gave the largest yields (85 to 95%) of any column packing used. The yields obtained with it were reduced (to between 55 and 75%) when purifying peptides separated on Dowex-1, and tryptophan peptides separated on Dowex-50 were completely lost when placed on Sephadex G-25.

Separation of tryptic peptides of lysozyme on either Sephadex G-10 or G-50 resulted in poorly defined peaks.

Peptide separation on Bio-Gel P-10 gave, on occasion, excellent results. However, the sample was usually completely lost on the column, and this material was consequently not used.

Blocking of Arginine Cleavage with Benzil.--Reduced-carboxymethyl-lysozyme was reacted with benzil, according to the method of Itano and Gottlieb (1963), in order to modify the arginine residues and produce a smaller

number of peptides after tryptic hydrolysis. This method was reportedly successful for modifying oxidized insulin which contains no tryptophan; it was found in these studies with lysozyme that the method appears to work when peptides are detected with a ninhydrin reagent; however, tryptophan was extensively destroyed during the reaction with benzil, as evidenced by a loss of its ultra-violet absorption spectrum.

Preparation of Peptides of Heat Denatured Lysozyme.--Lysozyme was reacted with a half-molar amount of iodine at pH 5.5 as described above, and 1.0 gm of the product in 100 ml of water was heated at 100° for four hours. The insoluble denatured protein was hydrolyzed with 20 mg of trypsin at 40° for 18 hours. The pH was maintained at 8.0 by the addition of 0.2 M NaOH; the total base uptake was 5.3 moles per mole of protein, indicating that hydrolysis was 60% complete, relative to the reaction of the reduced-carboxymethyl-lysozyme. Insoluble protein was removed by centrifugation, and the supernate was lyophilized to dryness. The entire sample was separated on a column of Dowex-1 as before.

Sedimentation Coefficients.--Sedimentation coefficients of 7.5 mg/ml samples of reduced-carboxymethyl-native and iodine-inactivated-lysozyme were determined in 0.1 M tris-(hydroxymethyl)-amino methane buffer of pH 7.0,

containing 8.0 M in urea to solubilize the sample. Determinations were made with a Spinco Model E Ultracentrifuge at 22.3° (native) and 22.1° (iodine-inactivated).

Oxidation of Lysozyme by N-Bromosuccinimide.--Tryptophan-62 of lysozyme was oxidized by the method of Hayashi et al. (1965). Lysozyme (2.0 gm) was dissolved in 50 ml of 0.1 M $NaC_2H_3O_2$ buffer of pH 4.5. A solution of 26.7 mg of N-bromosuccinimide in 3.0 ml of water was added dropwise with stirring at 0°. After reacting 30 minutes, the solution was dialyzed exhaustively and lypophilized to dryness.

Rate of Iodine Oxidation of Lysozyme.--The rates of iodine oxidation of lysozyme and a tryptophan ester were determined by measuring the rates of disappearance of the iodine color. The standard iodine solution (0.5 ml) was added to the protein or amino acid in 50.0 ml of 0.1 M $NaC_2H_3O_2$ buffer of pH 5.5 and the decrease of optical density at a wave-length of 410 mμ was measured as a function of time. This wave-length, although not the maximum for iodine absorption, was chosen because undiluted measurements could be taken, and no other species absorbed in this region. The reaction mixtures contained either 500 mg (34 μ moles) of lysozyme, or 6.9 mg (34 μ moles) of tryptophan, or 9.3 mg (34 μ moles), 18.6 mg (68 μ moles) or

27.9 mg (102 μ moles) of N-acetyltryptophan-ethyl-ester. The spectra of the products of the reactions were determined, showing oxindole formation in all cases except with free tryptophan. The pH of the final solution in all cases remained unchanged from the initial.

Rate of N-Bromosuccinimide Oxidation of Lysozyme.--One gram of lysozyme (67 μ moles) and 18.6 mg (67 μ moles) of N-acetyltryptophan-ethyl-ester were dissolved together in 50.0 ml of 0.1 M $NaC_2H_3O_2$ buffer of pH 4.5, and 18.6 mg (67 μ moles) increments of N-bromosuccinimide in 1.0 ml of water were added; samples (0.5 ml) were taken after 0, 1, 2, 3, 4, and 12 increments. Samples were separated on Sephadex G-25 as previously described, and analyzed for optical density at wave-lengths of 250 and 280 mμ.

III. RESULTS

Preparation of Carboxymethyl-Lysozyme.--Although histidine has been implicated as an important residue of lysozyme (Fraenkel-Conrat, 1950; Weil et al., 1952; Ramachandran and Rao, 1962; Ferrini, 1964), in all cases, analysis and activity measurements were made on unseparated reaction products. To better evaluate the role of this amino acid, lysozyme was carboxymethylated under conditions known to selectively modify histidine, and a pure mono-carboxymethyl-enzyme was isolated.

Lysozyme was reacted with a 2.6×10^2 molar excess of iodoacetic acid at pH 5.0 and 40° for 24 hours. The reaction products were desalted on Sephadex G-25 and were separated on Bio-Rex 70 to give two principal fractions (A and B), shown in Fig. 1. The recovery of optical density after desalting and separation was 65%; of the recovered protein, component A represented 84% and component B represented 16%. Two 10 mg samples of protein from both components were acid hydrolyzed and analyzed for amino acid content, giving the composition shown in Table 1. Component A lacked histidine with no other significant change in amino acid composition from native lysozyme; it exhibited 40% of the enzymic activity of the parent protein. Amino acid analysis after performic acid oxidation and acid

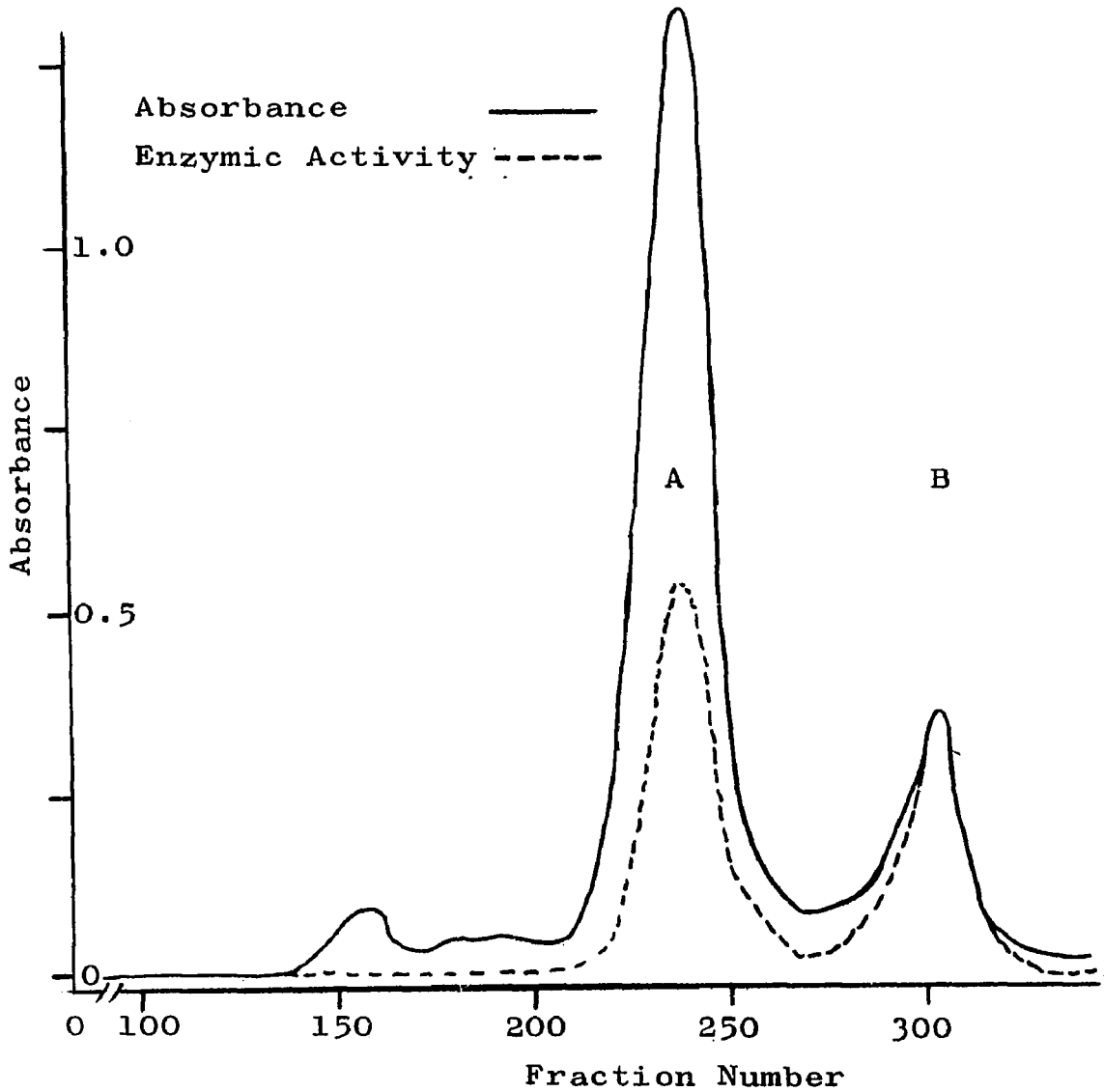

Fig. 1. Bio-Rex 70 Separation of the Products of the Carboxymethylation of Lysozyme at pH 5.5.

Enzymic activity values are relative, adjusted by a ratio so that the value for the peak fraction of component B (fraction 304) equals the absorbance value.

Table 1. Amino Acid Analyses of Components A and B of the Carboxymethylation of Lysozyme.

Amino Acid	Theory[b]	A[a]	B[a]
aspartic acid	21	20.3	20.4
Threonine	7	7.2	7.4
serine	10	10.1	9.9
glutaminc acid	5	4.4	4.6
proline	2	1.8	2.4
1-CM-histidine	0	0	0.13
glycine	12	11.2	11.3
alanine	12	12.6	12.3
1/2-cystine and 3-CM-histidine	8	7.3	5.8
valine	6	6.1	6.2
methionine	2	2.0	1.9
isoleucine	6	5.8	5.7
leucine	8	7.7	7.9
tyrosine	3	3.2	3.3
phenylalanine	3	3.1	3.2
lysine	6	6.1	6.6
histidine	1	0.01	0.77
arginine	11	10.8	12.9

a. Determinations were performed in duplicate; no corrections were made for losses in hydrolysis. All values were standardized on aspartic acid and alanine.

b. From Canfield (1963b) and Jolles et al. (1963).

hydrolysis showed that histidine had been converted to 3-carboxymethylhistidine (no 1-carboxymethylhistidine was detected). Component B, with the mobility in this chromatographic system equal to native lysozyme as determined by an independent run of a pure native sample, was not homogeneous, demonstrated both by its slightly less than theoretical histidine content and by the changing ratio of optical density to enzymic activity in the fractions corresponding to it. It is of interest that 1-carboxymethylhistidine was present in component B, suggesting that it contained the other mono-carboxymethyl-lysozyme.

The measured activity of component A indicates that histidine is not in the active site of lysozyme, and as further evidence, 0.1 M N-acetylglucosamine or di-N-acetyl-glucosamine added, respectively, at 2 and 100 times greater concentrations than required for 50% inhibition gave patterns identical to Fig. 1, showing no protection of the histidine by bound inhibitors.

Carboxymethylation at Various pH's.--The rate of enzymic inactivation of lysozyme by iodoacetic acid as a function of pH is shown in Fig. 2. The rate increased monotonically from pH 3.0 to 10.0, showing no maximal rate of inactivation in the pH range of maximal enzymic activity (pH 5.3), as was obtained for the critical histidine of

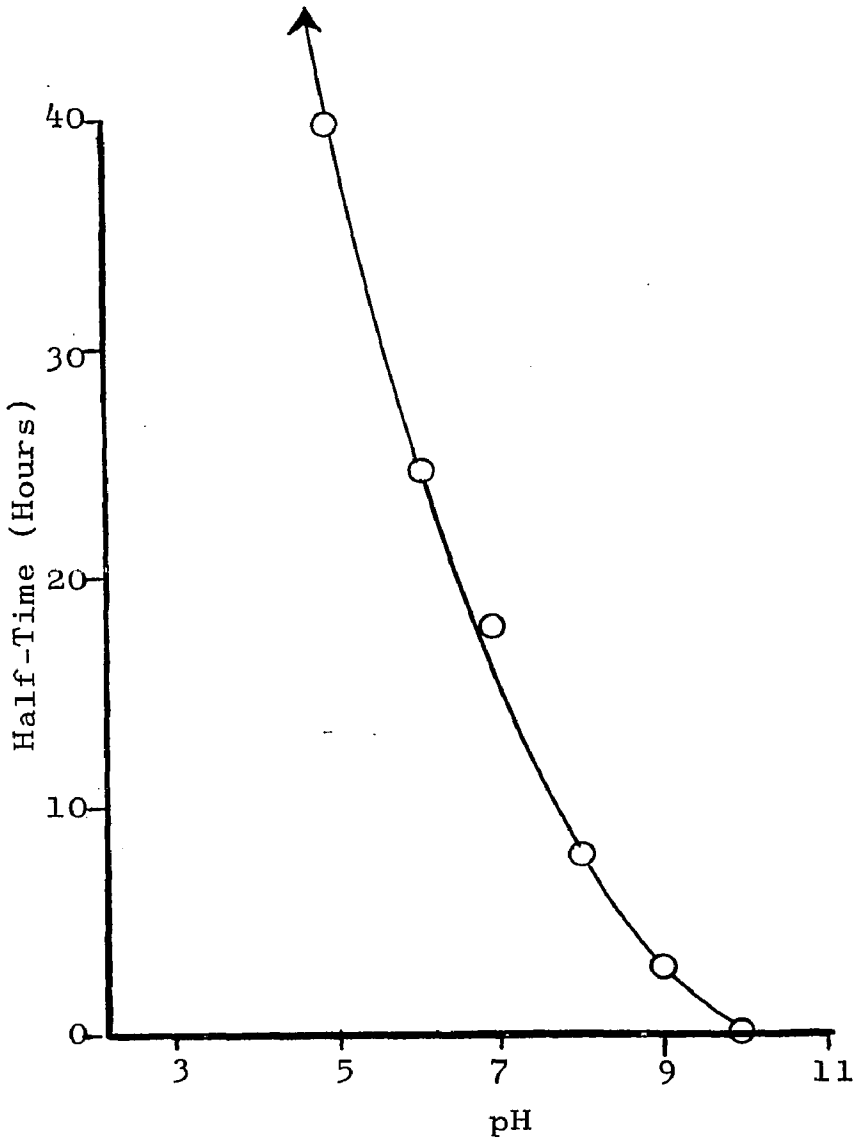

Fig. 2. The pH Rate Profile for the Inactivation of Lysozyme by Carboxymethylation.

Half-times were determined from the rate of loss of activity at each pH.

ribonuclease (Bernard and Stein, 1959; Gundlach et al., 1959), and is further evidence against the participation of the single histidine of lysozyme. Also, no fully inactive derivatives were found upon chromatographic separation of the products obtained at these pH values.

Reaction of Lysozyme with Iodine.--Fraenkel-Conrat (1950) has shown that the reaction of lysozyme with a 35 fold molar excess of iodine at pH 7.6 leads to partial enzymic inactivation; the possible side chains involved are shown in Table 2. Reaction under these conditions leads to extensive modification and precipitation of the protein and it is difficult to interpret the results with respect to the specific contributions of the individual amino acids. Therefore, low iodine concentrations were used which did not cause protein precipitation, and an inactive singly-modified enzyme was produced.

When lysozyme was reacted with iodine in a molar ratio of 1.0 to 0.5, there was a ten-fold increase in rate between pH 5.5 and 10.5; however, the inactivation after complete reaction is greater at the lower pH, as shown in Table 3.

This observation is easily explained if more than one residue reacted with iodine. The reactions of tryptophan, histidine and tyrosine are all pH dependent; however, this dependency is greatest with tyrosine, since the

Table 2. Amino Acid Side Chains Which React With Iodine.

Amino Acid	Products Possible[a]
Tyrosine	Monoiodotyrosine Diiodotyrosine
Methionine	Methionine periodide Methionine sulfoxide
Cysteine	Sulfenyl iodide Disulfide (Cystine) Sulfenic Acid
Histidine	Mono-C-iodohistidine Di-C-iodohistidine
Tryptophan	Oxindole tryptophan

a. Ramachandran (1956).

Table 3. Rate and Enzymic Activity Remaining After Reaction of Lysozyme with a Half-Molar Amount of Iodine at Various pH Values.

pH	Half-Time (min.)	% Activity Remaining
10.6	2	93
8.0	5	82
6.5	10	76
5.5	20	70

phenolate ion is iodinated significantly faster than the phenol. If inactivation were caused by a tryptophan reaction and not by tyrosine iodination, an increase of pH leading to a faster rate would increase the amount of tyrosine iodinated over the amount of tryptophan reacted since there was a small fixed amount of iodine present. Therefore, the total inactivation would decrease at higher pH values.

Separation on Bio-Rex 70 of the products of the reaction at a pH other than 5.5 gave at least four species; all were partially active. Since an inactive product was desired, these were not studied further.

Isolation of an Inactive Lysozyme. Lysozyme was reacted at pH 5.5 with a half-molar amount of iodine labeled with ^{131}I. Bio-Rex chromatography of the reaction mixture, shown in Fig. 3, separated three principal components, with a recovery of the applied optical density of 76%. The relative amount of protein, the enzymic activity, and the iodine content of the three components are given in Table 4.

Component B eluted at the position of the unmodified protein, determined by chromatography of native lysozyme under identical conditions; the presence of ^{131}I in this peak shows it is heterogeneous, a conclusion also supported by its changing ratio of optical density to enzymic

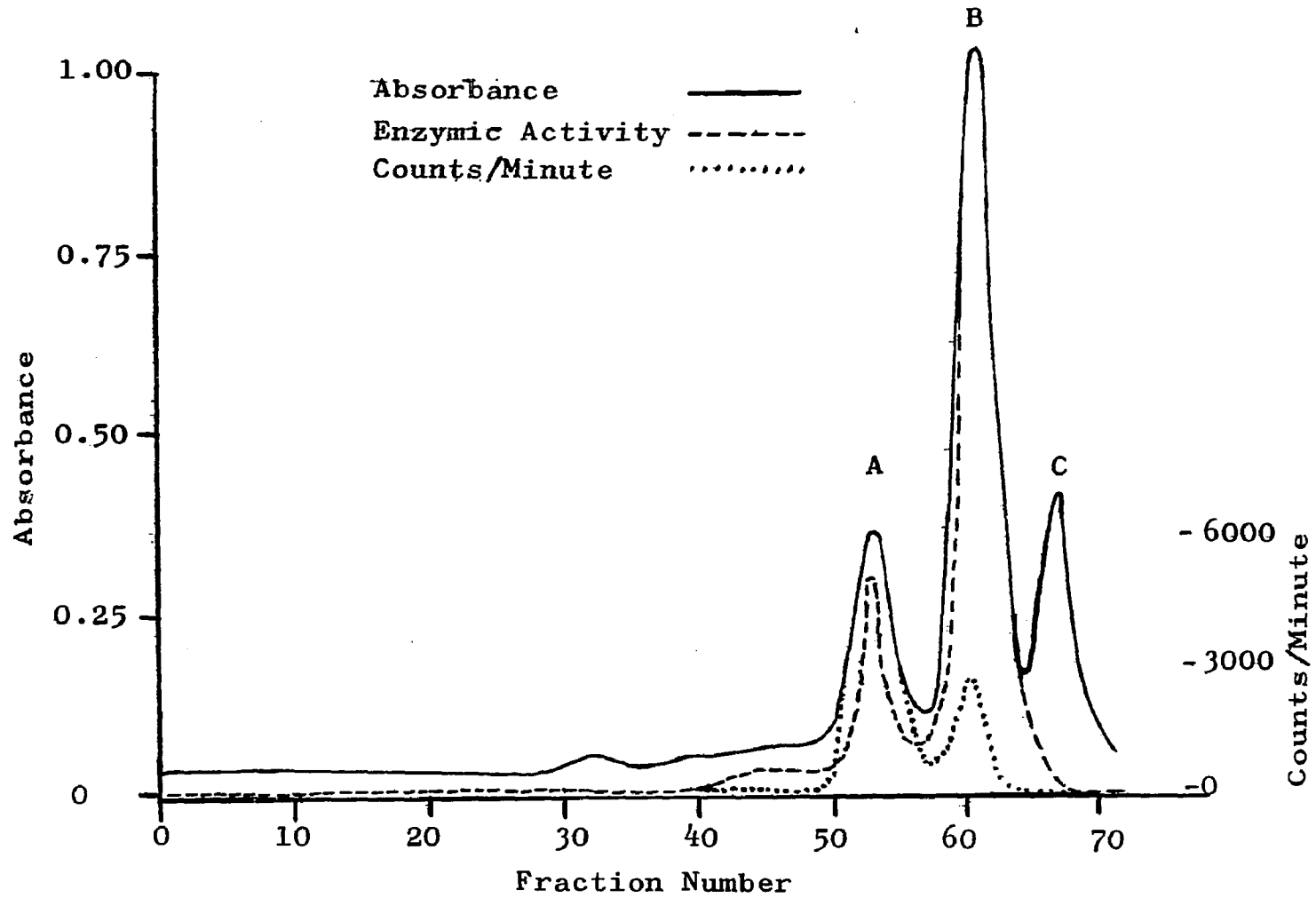

Fig. 3. Bio-Rex 70 Separation of the Products of the Reaction of Lysozyme with Iodine at pH 5.5.

Enzymic activity values were adjusted to equal the absorbance value for fraction 61. Counts/minute scale was adjusted to equal the absorbance value for fraction 53, which contains 1.08 moles of (I) per mole of protein.

Table 4. Products of the Reaction of Iodine and Lysozyme at pH 5.5.

Component	% of Total Sample	% of Native Activity	Moles (I) per Mole Protein
A	20	75	1.08
B	55	87	0.22
C	25	0	0

activity. This component is presumably mainly native lysozyme with perhaps a small amount of a species both iodinated and oxidized.

The fastest moving component A is a partially active, iodine-containing species which retains 75% of the enzymic activity of native lysozyme. From Table 2 it can be seen that the iodinated residue in this component could be tyrosine, methionine, or histidine.

Table 4 shows that half of the lysozyme reacted with iodine, and half of the protein which reacted was mono-iodinated (component A). Since only a half-mole of iodine was added per mole of protein, the reaction to produce component C was with an equimolar amount of iodine and lysozyme. This species, which retains less than 1% of the original activity, is presumably, then, singly modified, since all possible oxidative reactions (with methionine or tryptophan) require at least one mole of I^+ ion.

Only those fractions corresponding to component C which showed no enzymic activity were pooled and desalted on Sephadex G-25. Chromatography of this material on Bio-Rex 70 gave a single peak, showing components A and B had been completely removed, and this product was used in the following experiments.

Determination of the Side Chain Oxidized by Iodine.--The possible oxidative conversions in lysozyme (Table 2) are tryptophan to an oxindole and methionine to a sulfoxide; there is no cysteine present. The determination of amino acid composition after acid hydrolysis cannot measure these reactions, owing to the destruction of tryptophan and the conversion of methionine sulfoxide to methionine during the hydrolysis (Floyd, Camnaroti, and Lavine, 1963). Therefore, the inactive derivative C and the native protein were analyzed for amino acid composition after both acid and total enzymic hydrolysis (using in this order, pronase, carboxypeptidase A and B and leucine amino peptidase). The results are shown in Table 5. The only significant difference was the loss of approximately one residue of tryptophan in the inactive enzyme. The absence of changes in histidine or tyrosine is in accord with the lack of ^{131}I in the inactive protein. The small difference in methionine content after enzymic hydrolysis was shown to be unreal by amino acid analysis after carboxymethylation, performic

Table 5. Amino Acid Analyses of Native and Iodine-Inactivated-Lysozyme.

Acid	Theory[a]	Acid Hydrolysis		Enzymic Hydrolysis		Carboxymethyl-Oxid. Acid Hyd.	
		Nat.	Mod.	Nat.	Mod.	Nat.	Mod.
Try	6	--	--	6.0	4.8	--	--
Met	2	2.2	2.3	2.0	1.5	.5	.4
Met Sulf.	--	--	--	--	--	.2	.1
Asp	21 (8)	20.8	20.7	7.8	7.6	21.0	21.0
Thr	7	6.8	7.1	--	--	7.5	7.2
Ser	10	8.5	9.3	10.3	10.5	9.8	9.6
Glu	5 (2)	4.9	5.1	2.4	2.4	5.3	5.2
Pro	2	2.2	2.6	1.5	1.4	2.1	2.1
Gly	12	12.2	11.8	10.3	10.0	12.0	12.0
Ala	12	12.3	12.2	12.3	12.5	12.0	12.0
1/2 Cys	8	5.3	6.2	6.7	6.2	.6	.5
Val	6	5.7	5.7	6.3	6.4	6.4	5.4
Ileu	6	5.5	5.5	6.1	6.2	5.2	4.7
Leu	8	7.7	7.5	7.8	8.0	7.8	7.8
Tyr	3	3.1	3.0	3.4	3.4	.8	.7
Phe	3	3.1	3.0	3.1	3.2	2.8	2.8
Lys	6	5.9	6.1	6.3	6.3	--	--

Table 5--Continued

His	1	1.0	1.0	1.1	1.0	--	--
Arg	11	12.1	12.1	12.3	12.3	11.0	10.0

Figures in parentheses give the number of residues not present as amides.

All determinations were performed in duplicate.

All values were standardized on aspartic acid and alanine.

Acid hydrolysis: no corrections made for losses.

Enzymic hydrolysis: glutamine, asparagine and threonine were not separated by the analytical procedure. Corrections (0.1 to 1.8 residue equivalent determined by control analyses) were applied for the amino acids released by self-digestion of the hydrolyzing enzymes.

a. Canfield (1963b).

acid oxidation and acid hydrolysis. This series of reactions, suggested by Ray and Koshland (1962) converts methionine sulfoxide, but not methionine, to the sulfone; none of this was found (Table 5).

The above results show the inactive derivative to be identical to native lysozyme except for the oxidation of one of the six tryptophans. If this assumption is correct it should be verifiable from absorbance measurements, since Patchornik et al. (1960) have shown that in the oxidation of the tryptophan indole to an oxindole the absorption maximum shifts from 280 to 250 mμ, the original trough. Extinction coefficients of native and iodine-inactivated-lysozyme were measured, permitting the difference spectrum for the two species to be determined. As shown in Fig. 4, it is nearly identical in shape and magnitude to the published (Patchornik et al., 1960) difference spectrum for tryptophan and oxidized tryptophan. Comparison of the magnitude of the difference spectra at 280 mμ showed that 0.96 moles of tryptophan had been oxidized per mole of lysozyme.

The foregoing results show that oxidation of one of the six tryptophans, no other change occurring, abolishes the enzymic activity of lysozyme. The modification of only one residue is consistent with the stoichiometry considered in the previous section.

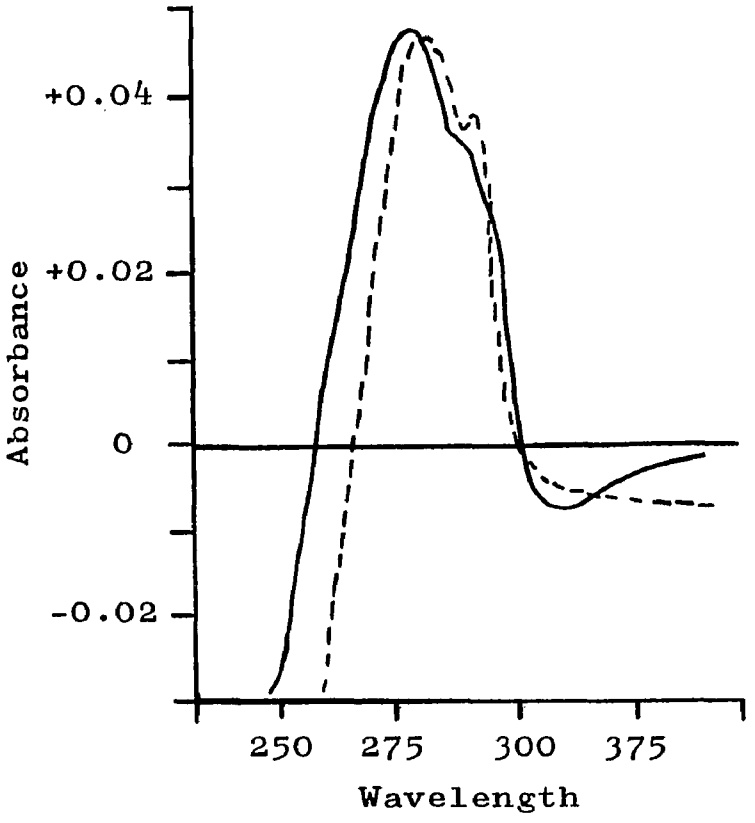

Fig. 4. Difference Spectrum of Native vs. Iodine-Inactivated-Lysozyme.

Solid line: Difference spectrum of native vs. iodine-inactivated-lysozyme, adjusted to 0.10 m_M_.

Broken line: Difference spectrum of tryptophan vs. oxidized-tryptophan at concentrations of 0.10 m_M_. From Patchornik _et al_. (1960).

Specific Rotation of Oxidized Lysozyme.--The oxidative modification of lysozyme by iodine leads to a slight positive shift in the specific rotation. The specific rotations of native and iodine-inactivated-lysozyme were determined using 400 μM solutions and were: $[\alpha]_{D,\ native} = -50.4$ and $[\alpha]_{D,\ oxidized} = -42.6$. The small and positive change in rotation is of interest, in that it rules out extensive unfolding of the molecule following oxidation. Consequently, reaction with iodine likely destroys the enzymic properties through modification of a residue at the active site. This will be seen more clearly from the data in the following section.

Inactivation of Lysozyme in the Presence of Inhibitors.--If tryptophan were indeed in the active center of lysozyme, the production of the inactive species should be retarded if the enzyme is complexed to an inhibitor which is a substrate analog. Therefore, lysozyme was reacted at pH 5.5 with half-molar amounts of iodine in the presence of 0.1 M N-acetylglucosamine or the corresponding disaccharide, the same concentrations used in the carboxymethylation experiments discussed in preceding sections. The products of the reactions were separated on Bio-Rex 70 to give the patterns shown in Fig. 5. With added N-acetylglucosamine (NAGA) or disaccharide (DAGA), the quantity of inactive modification obtained was 75% and 100%

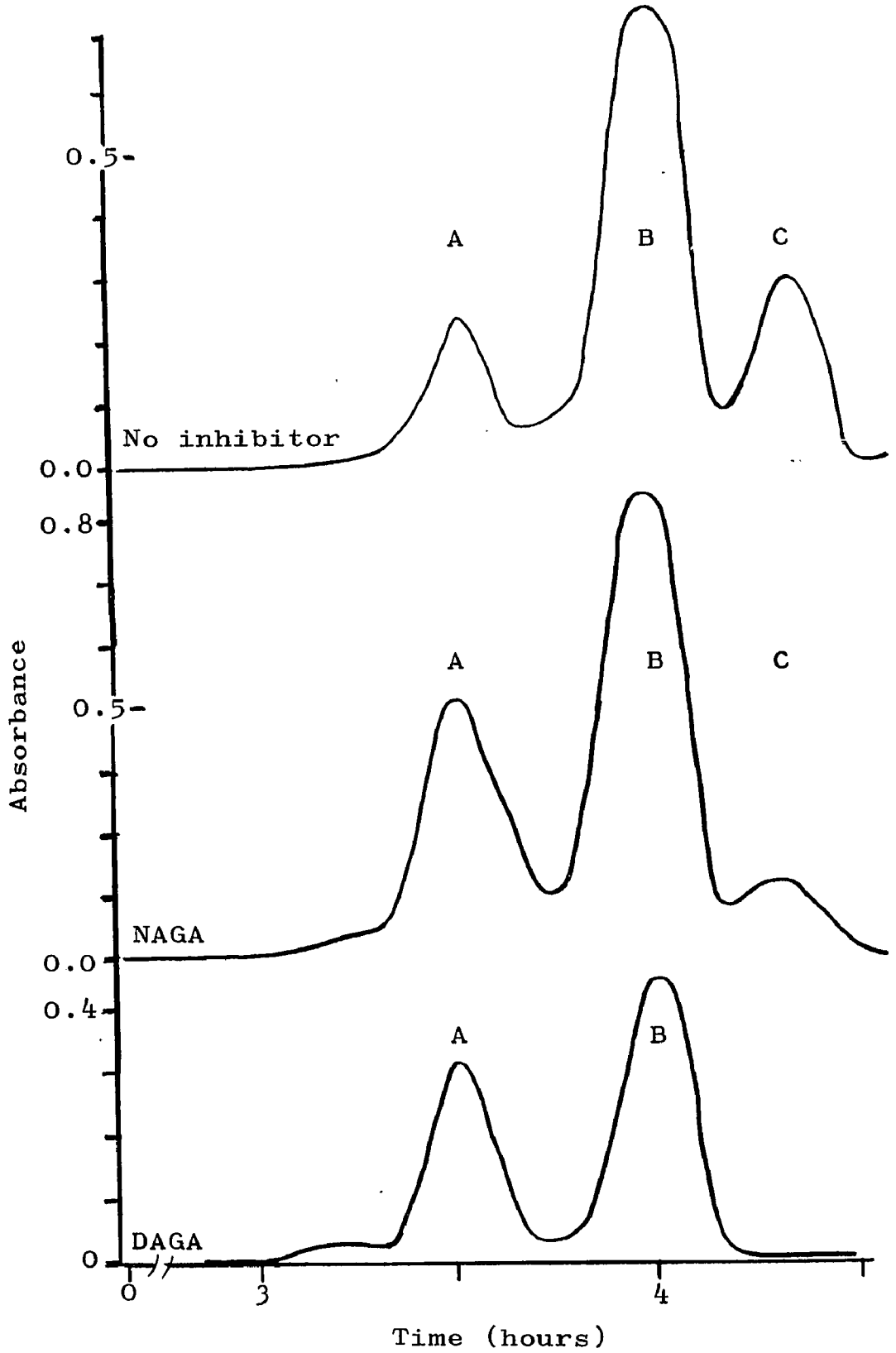

Fig. 5. Bio-Rex 70 Separation of the Products of the Reaction of Lysozyme with Iodine in the Presence of Inhibitors.

less, respectively, than in the absence of an inhibitor. The identification of each species was based upon the position of the peak; independent chromatographic analysis of native lysozyme in the presence of these inhibotors showed there was no effect of the saccharide on the elution behavior. Table 6 gives the relative amounts of the three reaction products. It can be seen that in the presence of inhibitors, as the third component (inactive lysozyme) decreases in size, the first component (which probably represents tyrosine iodination) increases. The relative amount of the second component (mainly native lysozyme) remains reasonably constant since the small fixed amount of iodine originally added was completely consumed in all cases. This behavior is consistent with tryptophan being part of or near the catalytic site of lysozyme, and furthermore suggests that a unique tryptophan is oxidized.

Table 6. Relative Amounts of Products After Iodine Reaction in the Presence of Inhibitors.

	% of Total Product		
Inhibitor	Component A	Component B	Component C
None	20	55	25
0.1 M NAGA	32	61	6
0.1 M DAGA	40	60	0

Location in the Sequence of the Oxidized Tryptophan, General Comments.--There are six tryptophan side chains in the lysozyme molecule; their locations in the sequence (Jollès et al., 1963; Canfield, 1963b) and their positions in the three-dimensional structure (Blake et al., 1965) are known. The identification of that tryptophan whose oxidation by iodine leads to enzymic inactivation is important in order to specify an element of the catalytic region of the enzyme as a preliminary step in understanding the properties of this particular residue.

Native and iodine-inactivated lysozyme were reacted with mercaptoacetic acid (to unfold), the newly generated sulfhydryl groups were carboxymethylated with iodoacetic acid (to prevent air reoxidation), and the product was hydrolyzed by trypsin, a specific proteolytic enzyme which cleaves at the C-terminal end of lysine and arginine residues. The tryptic peptides were separated on Dowex-1 and purified on Sephadex G-25. The peptide containing oxidized tryptophan was identified by its spectrum, and its amino acid composition after acid hydrolysis permitted the location of its position in the sequence. The details of these steps is described in subsequent sections.

The amino acid sequence of native lysozyme is shown in Fig. 6. The positions of the six tryptophans are: 28, 62, 63, 108, 111, and 123. Hydrolysis by trypsin results in eighteen peptides, each having a unique amino acid

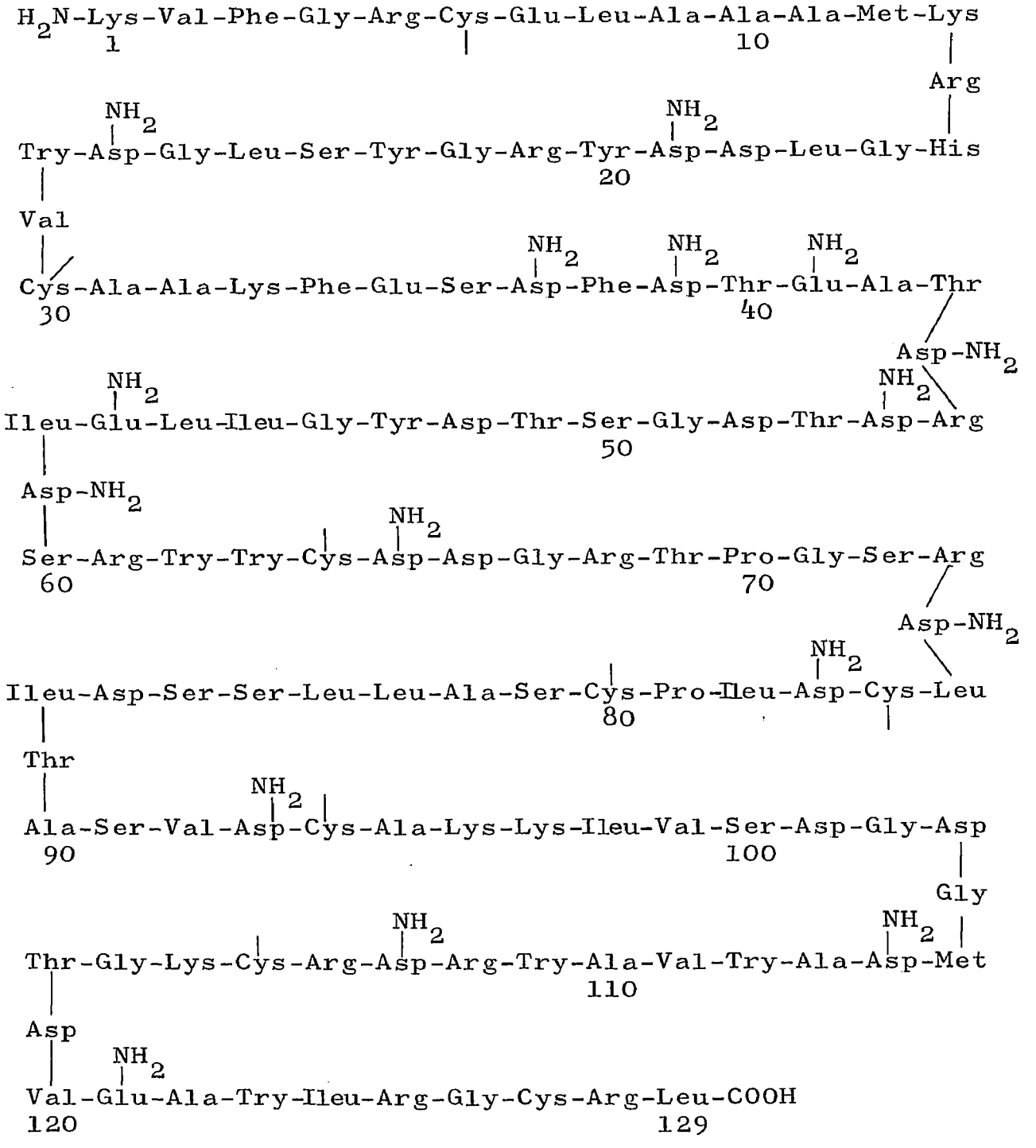

Fig. 6. Amino Acid Sequence of Lysozyme (Canfield, 1963a).

composition. Since tryptophan and tyrosine are the only amino acids absorbing in the ultraviolet region of the spectrum (phenylalanine, histidine and cystine having a negligible absorption at the concentrations used in these experiments), it was experimentally advantageous to analyze the effluent fractions of peptide separation columns using ultraviolet absorption, rather than ninhydrin analysis of alkaline digests. This does not affect the estimates of peptide purity, which were based only upon amino acid analysis and not upon apparent chromatographic homogeneity. The amino acid compositions of all peptides containing tryptophan or tyrosine are summarized in Table 7, in which the peptides are designated as T-1 to T-6. The nomenclature of other authors is also given in this table. Peptides T-4 and T-5 contain two tryptophans each, Peptides T-2 and T-6 contain one each. In all chromatographic separations, each fraction was measured for optical density at wave-lengths of both 250 mμ (the peak of oxindole-tryptophan and the trough of tryptophan) and at 280 mμ (the peak of tryptophan and a region of low oxindole absorption).

Separation of the Tryptic Peptides of Lysozyme.--The tryptic peptides of both native (18.8 mg) and iodine-inactivated-lysozyme (16.8 mg) were separated on Dowex-1; the optical density at 250 and 280 mμ of each fraction is

Table 7. Amino Acid Composition of Tryptophan and Tyrosine Containing Peptides of Lysozyme.

Acid	T-1	T-2	T-3	T-4	T-5	T-6	T-5 (108)	T-5 (111)
Try		1		2	2	1	1	1
Tyr	1	1	1					
Asp	2	1	4	2	3	1	3	
Thr			2			1		
Ser		1	2		1		1	
Glu			1			1		
Gly	1	2	2	1	2	1	2	
Ala		2			2	1	1	1
Val		1			2	1	1	1
1/2 Cys		1		1				
Met					1		1	
Ileu			2		1	1	1	
Leu	1	1	1					
His	1							
Arg	1		1	1	1	1		1
Lys		1						
TOTAL	7	12	16	7	15	9	11	4
A.	15-21	22-33	46-61	62-68	98-112	117-125	98-108	109-112

Table 7--Continued

B.	T-5	T-6	T-8	T-9	T-13	T-16
C.	T-11	T-14	T-13	T-17	T-16	T-10

A. Sequence positions.
B. Peptide nomenclature: Canfield (1963a).
C. Peptide nomenclature: Jollès *et al*. (1963).

Asparagine and glutamine are included with the respective free amino acids.

shown in Figs. 7 and 8. The minimal number of tryptophan and tyrosine peptides present were four in the native and seven in the iodine-inactivated.

It can be seen from Fig. 8 that all peptides of native lysozyme have an optical density higher at 280 mμ than at 260 mμ which is expected for either unmodified tryptophan or tyrosine. Peptides M-E and M-G from oxidized lysozyme, however, have absorptions indicating the presence of oxindole.

Fractions corresponding to each peak were dried in vacuo and purified on Sephadex G-25. The results of these separations are shown in Figs. 9 and 10. Fractions M-E and N-E each contain at least two peptides, designated M-Ea and M-Eb, and N-Ea and N-Eb, respectively.

Recovery of Peptides.--The total recovery of the applied optical density after Dowex-1 separation was 83% and 85% for the peptides of the modified and native protein, respectively. Although the missing 15% could represent a complete loss of one tryptophan peptide and complete recovery of all the others, this is unlikely since losses of this magnitude are expected during Dowex-1 separations (Jollès, 1963).

The per cent of the recovered optical density represented by each peak of the Dowex-1 separations is shown in Table 8. Calculations were made on the basis of

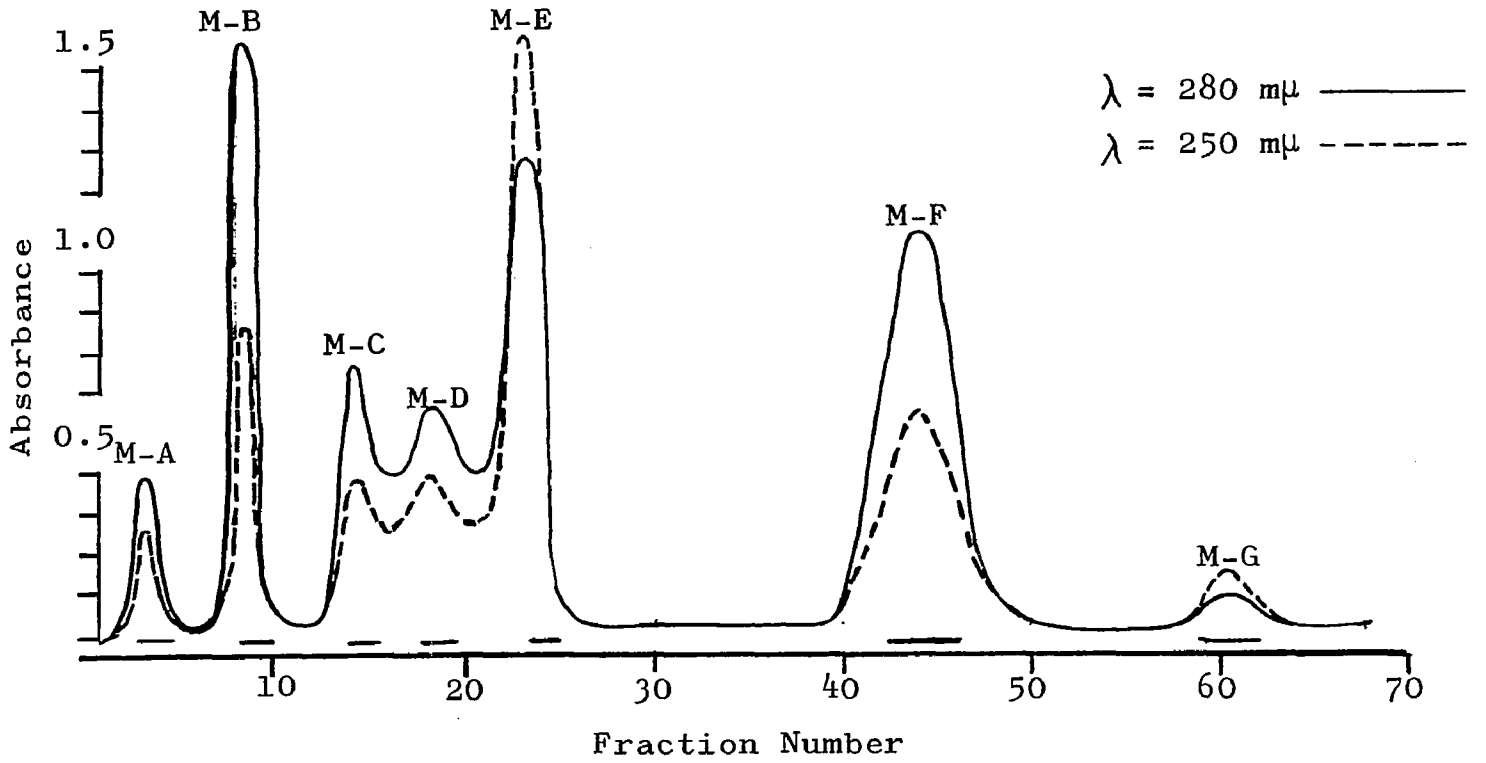

Fig. 7. Dowex-1 Separation of the Tryptic Peptides of Reduced-Carboxymethyl-Iodine-Inactivated-Lysozyme.

Fractions over solid line were pooled and purified by Sephadex G-25.

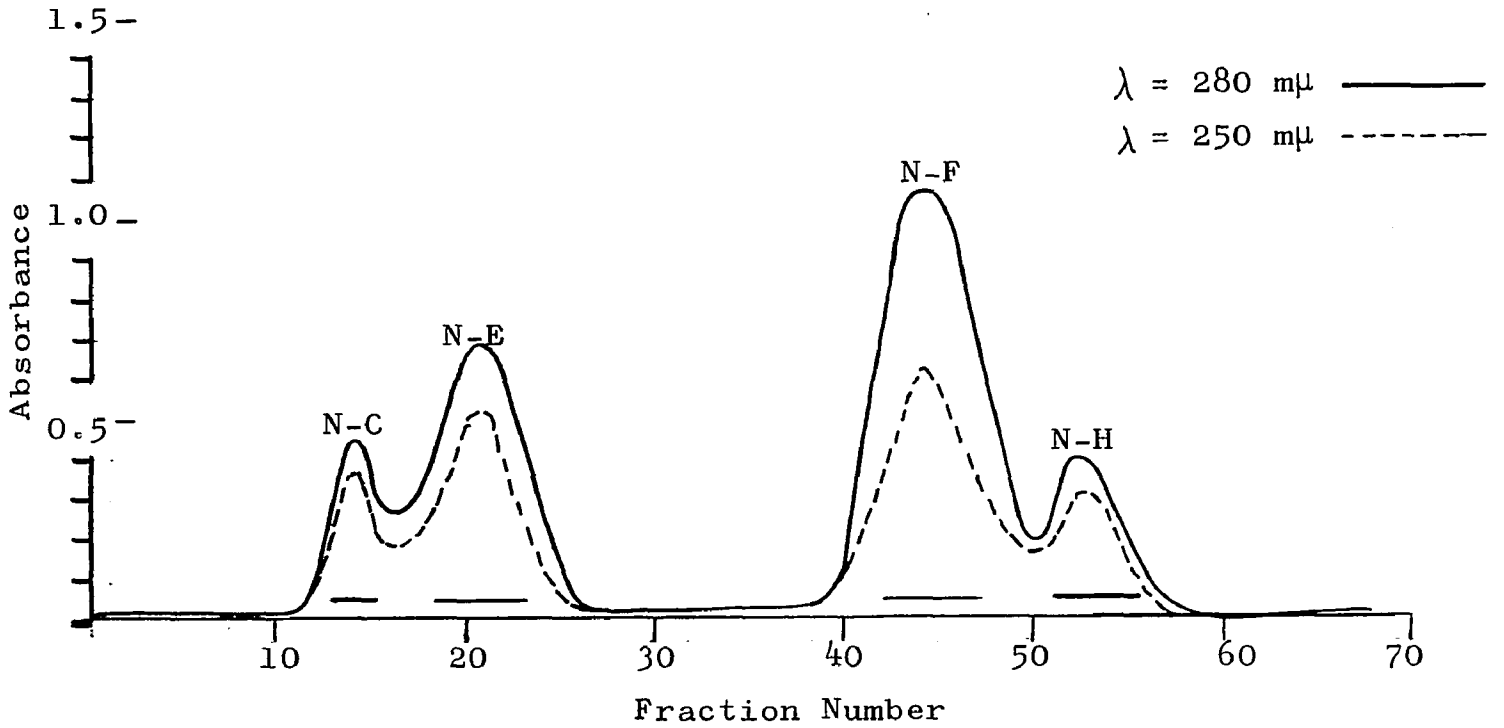

Fig. 8. Dowex-1 Separation of the Tryptic Peptides of Reduced-Carboxymethyl-Native-Lysozyme.

Fractions over solid line were pooled and purified by Sephadex G-25.

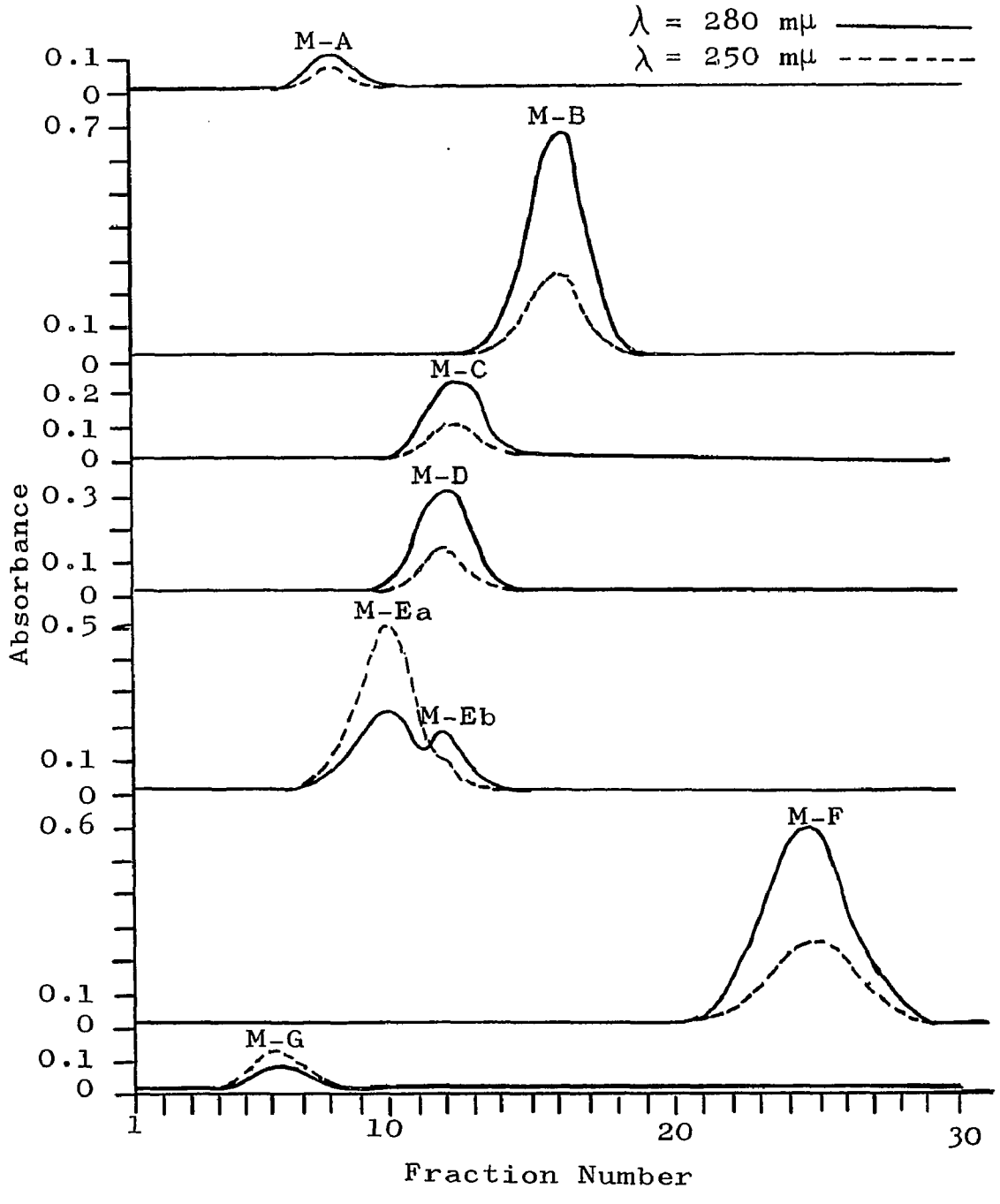

Fig. 9. Sephadex G-25 Purification of Peptides from Iodine-Inactivated-Lysozyme.

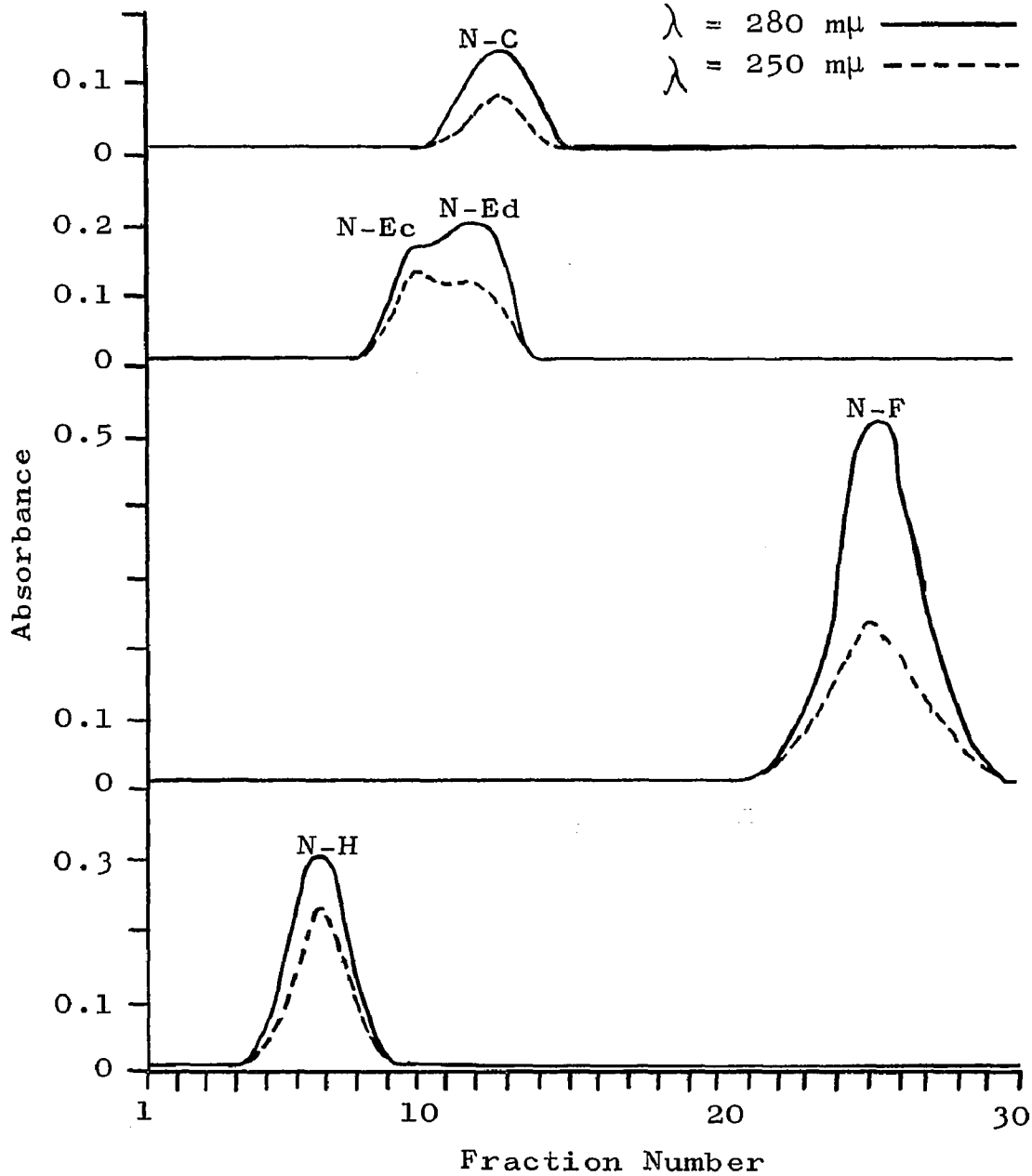

Fig. 10. Sephadex G-25 Purification of Peptides from Native-Lysozyme.

Table 8. Recoveries, Positions, and Identifications of Peptides of Reduced-Carboxymethyl-Native and Iodine-Inactivated-Lysozyme.

Peak	% Total	Position		Identification
		Dowex	Sephadex	
	Peptides of Iodine-Inactivated-Lysozyme			
M-A	3.9	3	6	Incomplete Tryptic Hydrolysis
M-B	14.4	8	16	T-5 (111)
M-C	10.1	14	13	T-1
M-D	14.4	18	12	T-2 or 3
M-Ea	17.0	23	10	T-5 (108)
M-Eb	6.6	23	12	T-2 or 3
M-F	32.2	44	25	T-4
M-G	1.4	61	6	T-5 (108)
	Peptides of Native-Lysozyme			
N-C	8.7	14	13	T-1
N-Ec	12.6	21	10	T-2, 3 or 5
N-Ed	15.7	21	12	T-2, 3 or 5
N-F	50.6	44	25	T-4
N-H	12.4	52	7	Mixture

absorption at 266 mμ, the isosbestic point of the indole-oxindole system. The per cent total recovery of peptides M-E and N-E was separated into the two components which they each contain using the ratio of these obtained in the Sephadex G-25 separations. Assuming that the tyrosines and tryptophans in these peptides absorb with a molar extinction coefficient equal to the free amino acids, a single tryptophan would contribute 15.1% and a single tyrosine 3.4% of the total absorption, not corrected for column losses.

Spectra of Tryptic Peptides.--The identification of the peptide containing the oxidized tryptophan is based upon its spectrum. Fig. 11 shows the spectra of the free amino acids tryptophan, tyrosine, oxidized-tryptophan and an equimolar mixture of normal and oxidized-tryptophan; each spectrum is readily distinguishable from the others.

The absorption spectra of the Sephadex G-25 purified tryptic peptides are shown in Figs. 12 and 13; they are characteristic of tryptophan, except those for peptides M-C and N-C, which have tyrosine spectra, and for peptides M-Ea and M-G, which both have spectra identifying them as containing oxidized-tryptophan, but not with an equimolar amount of normal tryptophan.

Amino Acid Analyses of Tryptic Peptides.--Fractions corresponding to peaks in the Sephadex G-25 patterns were analyzed for amino acid composition. The results for

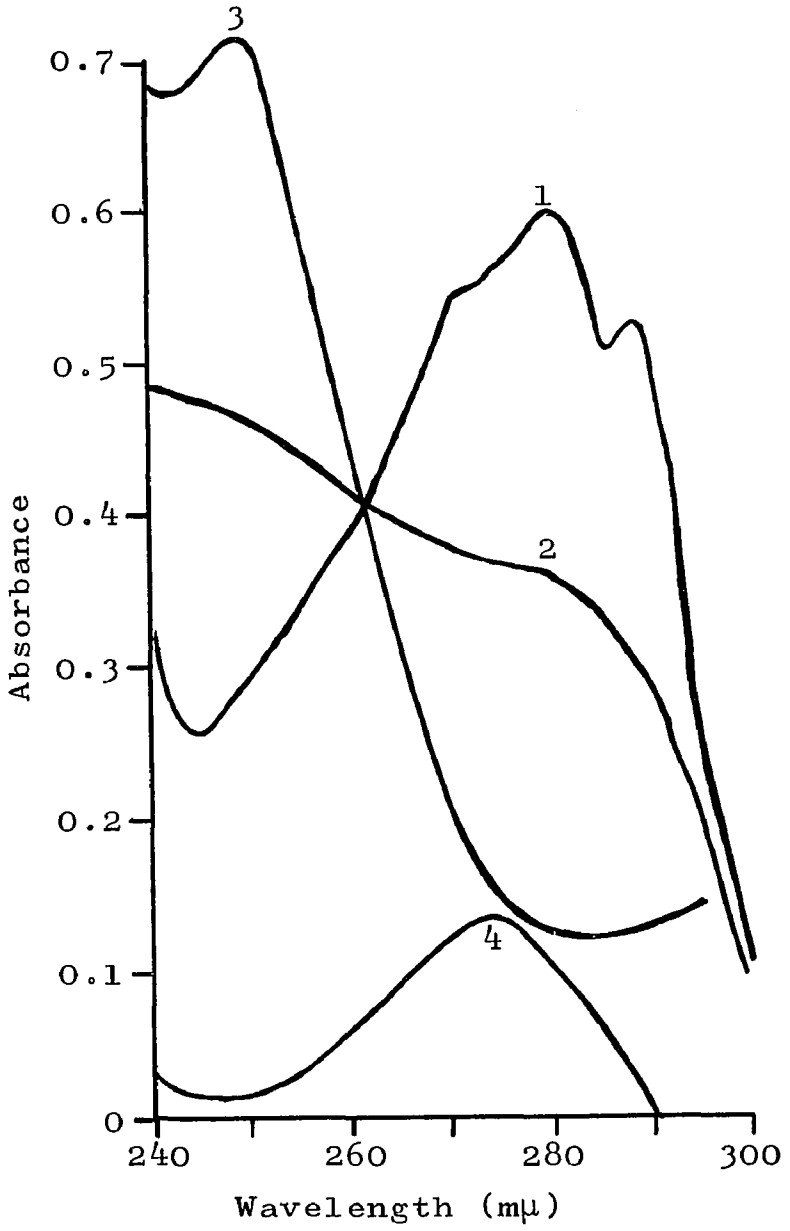

Fig. 11. Spectra of Amino Acids which Absorb in the Ultraviolet Region

1. Tryptophan (0.10 mM, pH 4.0).
2. Tryptophan (0.05 mM) and N-bromosuccinimide-oxidized-tryptophan (0.05 mM). Calculated from spectra 1 and 3.
3. N-bromosuccinimide-oxidized-tryptophan (0.10 mM, pH 4.0).
4. Tyrosine (0.10 mM, pH 4.0).

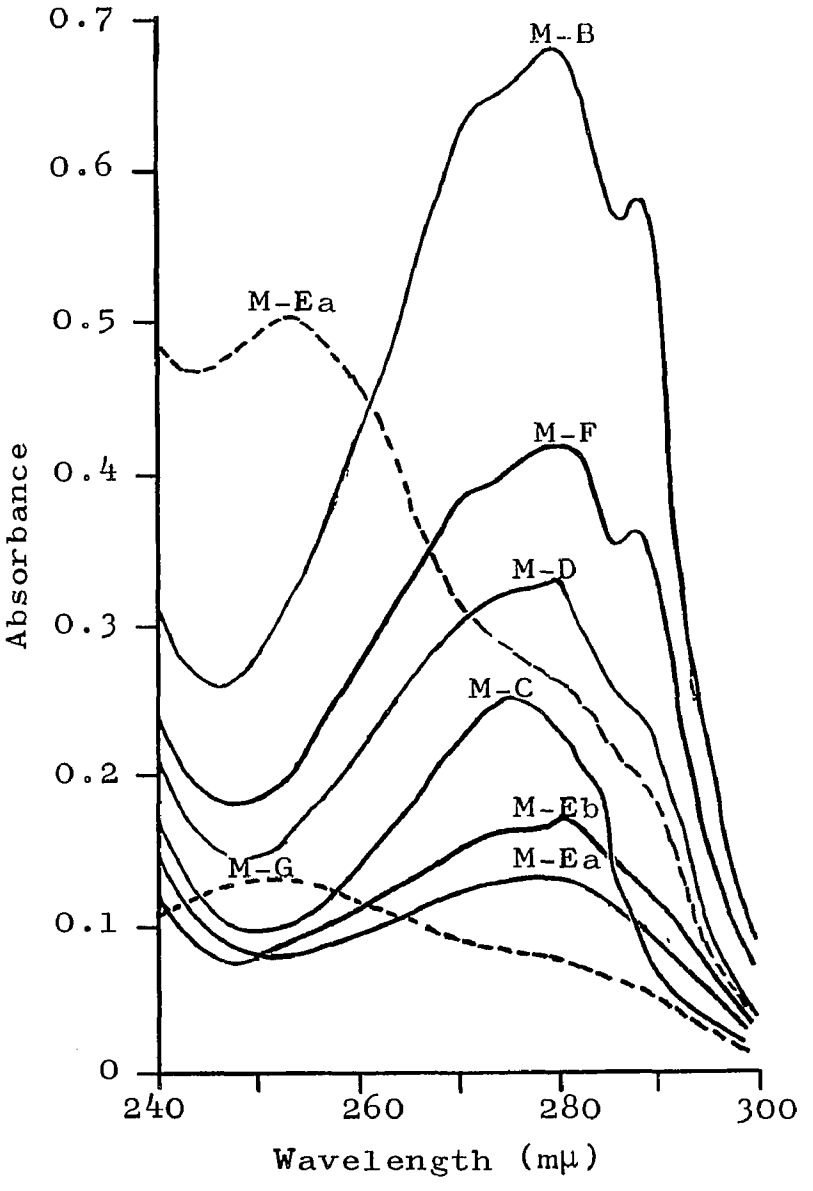

Fig. 12. Spectra of Peptides from Iodine-Inactivated-Lysozyme.

Spectra of the peak fractions of the Sephadex G-25 purifications (Fig. 9); all spectra were read in the eluting buffer (0.05 M NH_4OAc, pH 8.0).

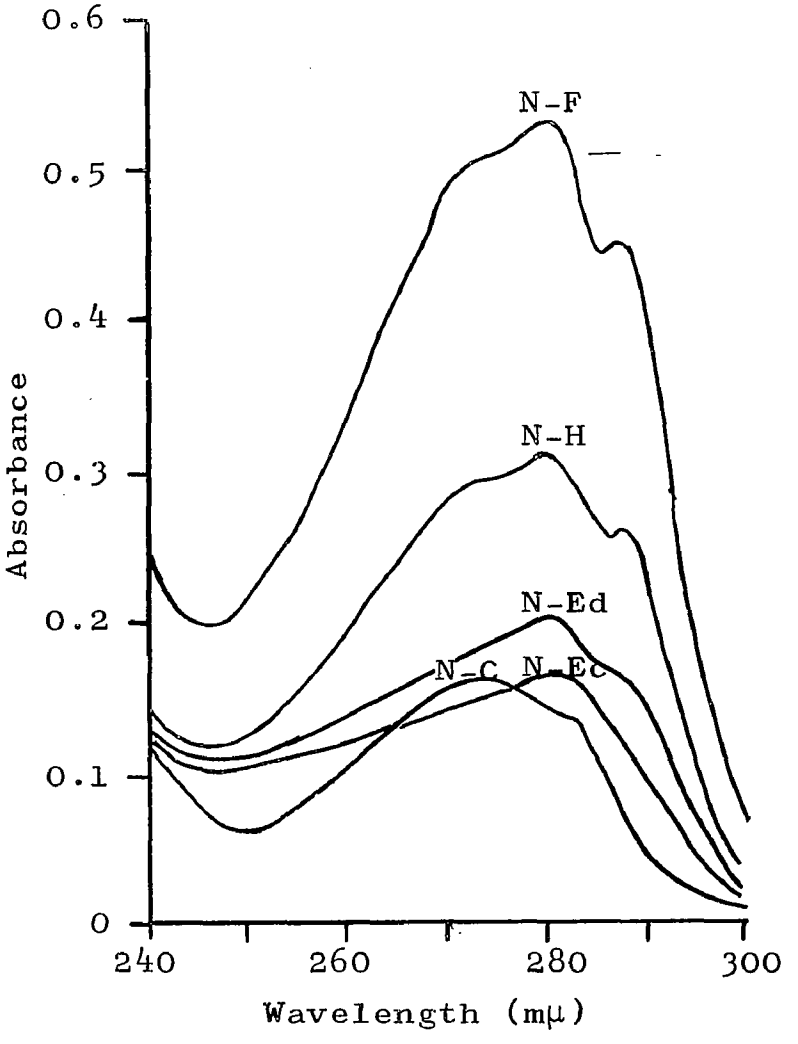

Fig. 13. Spectra of Peptides from Native-Lysozyme.

Spectra of the peak fractions of the Sephadex G-25 purifications (Fig. 10); all spectra were read in the eluting buffer (0.05 M NH_4OAc, pH 8.0).

peptides M-B, M-Ea, M-F, N-C, and N-F after acid hydrolysis are shown in Tables 9 and 10. The values given are ratios based on the quantity of tryptophan determined by optical density measurements and assumed to be either 1.00 or 2.00. Since tryptophan is destroyed in acid hydrolysis, it was replaced by a known amount of norleucine added as an internal standard to the peptide before hydrolysis and analysis. The absolute recoveries (moles of norleucine recovered in analysis per mole of norleucine added to the peptide) ranged from 75 to 80%.

The amino acid analysis of peptides M-F and N-F, both of which elute in fraction 44 on Dowex-1 and fraction 25 on Sephadex G-25, show they are both identical and are peptide T-4. Therefore the residue oxidized by iodine is not tryptophan-62 or 63.

If peptide T-5 were cleaved C-terminal to tryptophan-108, two new peptides would result: T-5 (108) and T-5 (111). The analyses show that peptides M-Ea and M-B correspond, respectively, to these. The low molar values of isoleucine and valine of M-Ea occur because the N-terminal valyl-isoleucyl bond is difficult to cleave; Canfield (1963a) reported values of 0.42 (valine) and 0.58 (isoleucine) moles per mole of peptide in his analysis of peptide T-5.

Cleavage after tryptophan-108 was unexpected. It can be seen from Fig. 8 that there is no peak in the region

Table 9. Amino Acid Analyses of Tryptic Peptides of Reduced-Carboxymethyl-Iodine-Inactivated-Lysozyme.

Acid	M-B	T-5 (111)	M-Ea	T-5 (108)	M-F	T-4
Try or Oxid-Try	1.00	1	1.00	1	2.00	2
Tyr	--		--		--	
Asp	.01		3.08	3	2.02	2
Thr	--		--		--	
Ser	.02		.95	1	.03	
Glu	.01		.03		.03	
Gly	.04		2.13	2	.97	1
Ala	1.02	1	.97	1	.01	
Val	1.00	1	.31	1	.04	
1/2 Cys	--		.08		.81	1
Met	--		.28	1	--	
Ileu	--		.27	1	.01	
Leu	.01		--		--	
Phe	--		--		--	
His	--		--		--	
Arg	1.06	1	.02		1.03	1
Lys	.01		.01		--	

Values are the ratios of the amino acids to tryptophan (determined by absorbance) taken as 1.00 or 2.00. 1/2 Cys determined as carboxymethylcysteine.

Table 10. Amino Acid Analyses of Tryptic Peptides of Reduced-Carboxymethyl-Native-Lysozyme.

Acid	N-C	T-1	N-F	T-4
Try	--		2.00	2
Tyr	1.00	1	--	
Asp	2.07	2	2.05	2
Thr	--		--	
Ser	.01		--	
Glu	.02		--	
Gly	1.08	1	1.00	1
Ala	.01		.01	
Val	.08		.03	
1/2 Cys	--		.87	1
Met	--		--	
Ileu	--		--	
Leu	1.00	1	--	
Phe	.07		--	
His	1.00	1	--	
Arg	1.04	1	1.02	1
Lys	.09		.01	

Values are the ratios of the amino acids to tyrosine (taken as 1.00) or tryptophan (taken as 2.00), determined by absorbance. 1/2 Cys determined as carboxymethylcysteine.

of fraction 9 (the position of M-B), therefore this cleavage did not occur in native lysozyme. (This conclusion will be further strengthened in the discussion of Sephadex G-25 separations of peptides.) The spectrum of M-Ea identifies it as the one containing an oxidized tryptophan, and tryptophan-108 is therefore the side chain whose oxidation causes enzymic inactivation. Peptide M-G, obtained in too low a yield for analysis, also has an oxindole spectrum and will be discussed in the section dealing with the tryptic peptides of heat-denatured oxidized lysozyme.

Amino acid analysis of peptide N-C showed it to be T-1 which contains tyrosine but not tryptophan; this identification was verified by the spectra. Since peptide M-C elutes in the same position on Dowex-1 and Sephadex G-25 as does N-C, and since it also has a tyrosine spectrum, these peptides are probably identical.

Peptide M-A represents too small a fraction of the total sample to be a tryptophan peptide and amino acid analysis showed it to be similar, but not identical, to native lysozyme; its early elution on Sephadex G-25 indicates it to be of large molecular-weight. It is therefore likely a large fragment resulting from incomplete tryptic hydrolysis.

Amino acid analysis of peptide N-H showed it to be a complex mixture; the ratio of serine to tryptophan was

one to twenty, therefore this peak could contain no more than 10% of peptide T-5. This peptide therefore must be in fractions N-Ec or N-Ed.

The identification of the peptides of native and iodine-inactivated lysozyme are shown in Table 8.

Amino acid analyses were also performed after exhaustive leucine aminopeptidase hydrolysis. The results in no case conflicted with those found by acid hydrolysis. However, reproducible results were not obtained, particularly in the cleavage of the tryptophanyl-carboxymethyl-cysteine bond in peptide T-5, whose cleavage, under identical conditions, varied from 100% to 0%.

Sedimentation Coefficients.--In order to determine whether cleavage after tryptophan-108 occurred during reaction with iodine, samples of native and iodine-inactivated-reduced-carboxymethyl-lysozyme were analyzed using the ultracentrifuge; in each case only one peak was obtained. The sedimentation coefficients ($S_{20,\,w}$) were 1.10 for native and 1.37 for iodine-inactivated lysozyme. The protein concentration in both cases was 7.5 mg/ml, determined by optical density measurements. Measurements were performed in 0.1 M tris-(hydroxymethyl)-amino-methane buffer of pH 7.0, containing 8.0 M urea; for this system, the solvent density was 0.9978 gm/ml and the viscosity was 0.9469, determined in this laboratory. The $S_{20,\,w}$ was calculated using the equation of Schachman (1957).

Peptides of Heat Denatured Modified Lysozyme.--

Fig. 12 shows that there are two oxindole containing peptides (M-Ea and M-G) in iodine-inactivated-lysozyme, although one of these (M-G) represents only 1.4% of the total optical density, significantly less than the 15.1% contribution expected for a full tryptophan residue. In order to analyze this peptide and to determine if there were any other oxindole peptides occurring in low yield, a chromatographic separation of a large sample was run. For this experiment, the protein was unfolded by heat denaturation prior to tryptic hydrolysis. Although this method does not cleave disulfide bonds, peptide T-5 does not contain cysteine and should therefore appear in the chromatographic pattern as before. There are only two methionine residues in lysozyme, one of which is in peptide T-5. Since heat denaturation has the advantage of not causing methionine destruction as does reduction and carboxymethylation, this amino should now be found in the oxidized peptide.

Dowex-1 separation of a large sample of a tryptic hydrolysate of the heat-denatured reaction product of lysozyme and iodine gave the pattern shown in Fig. 14. There were four peaks with a greater absorbance at 250 than at 280 mμ; they were purified on Sephadex G-25, giving the patterns shown in Fig. 15. The spectra of the peak fractions of these separations is shown in Fig. 16,

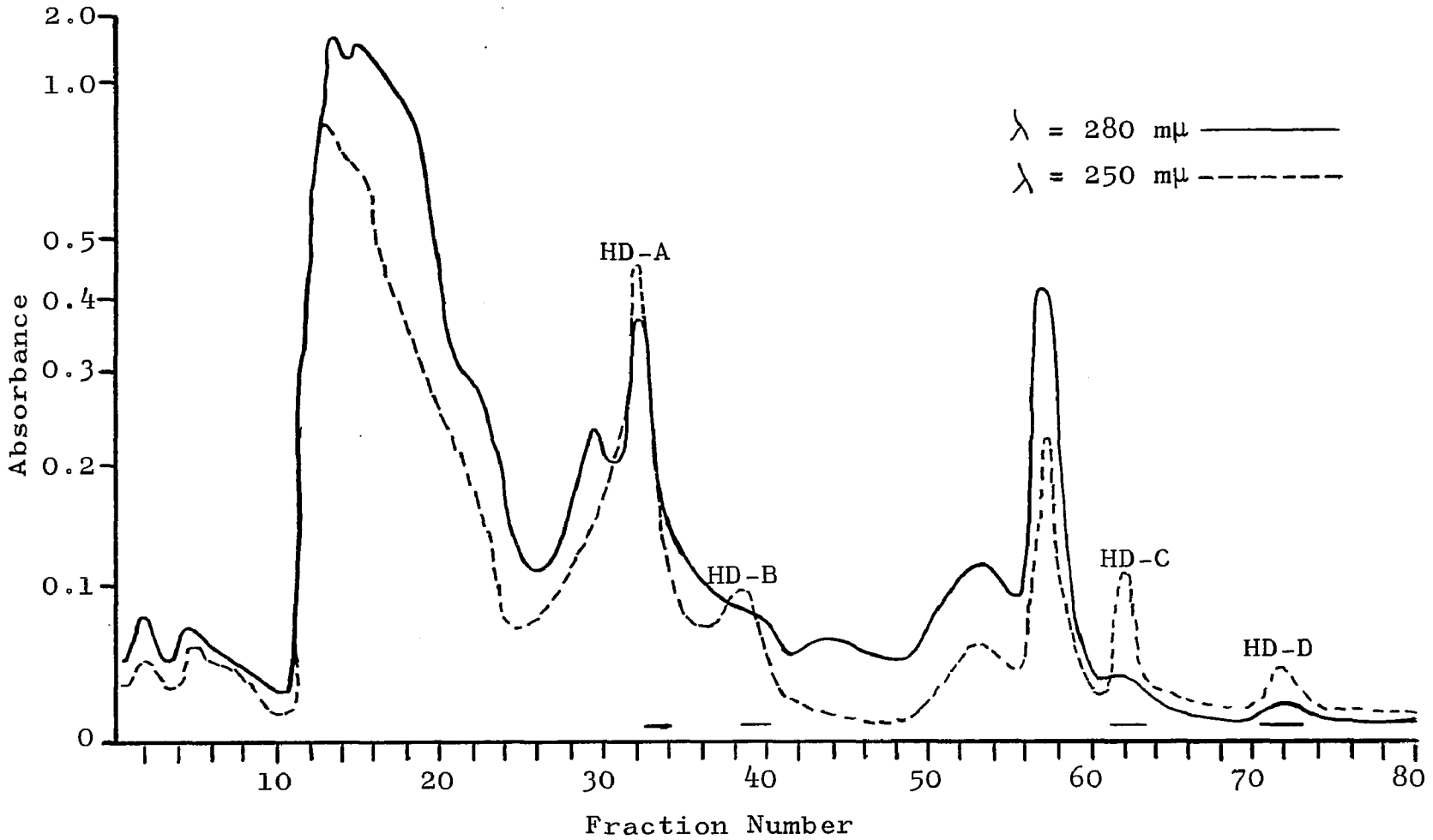

Fig. 14. Dowex-1 Separation of the Heat-Denatured and Tryptic Hydrolyzed Products of the Reaction of Lysozyme and Iodine.

Fractions over solid line were pooled and purified by Sephadex G-25.

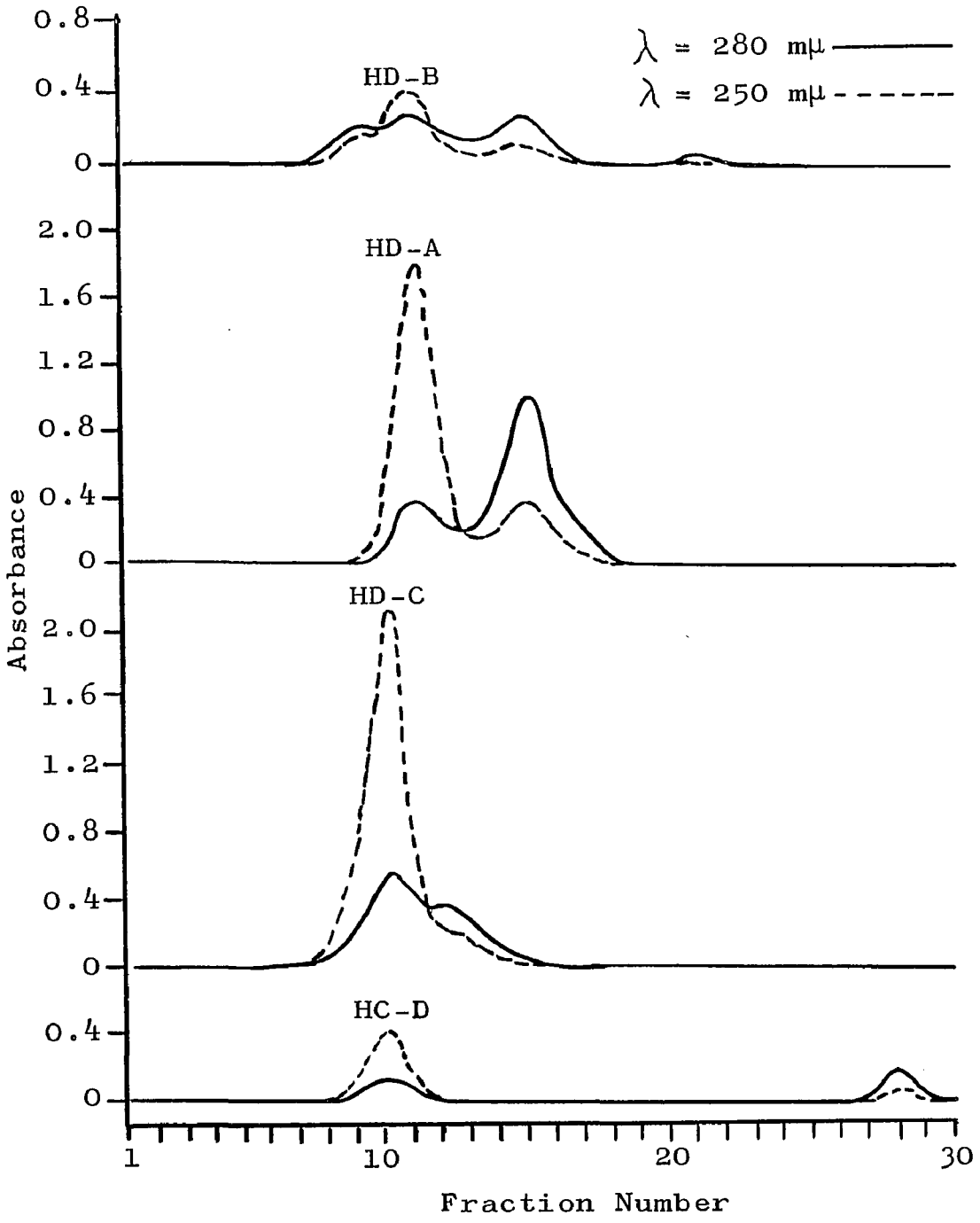

Fig. 15. Sephadex G-25 Purification of Oxidized Peptides from Heat-Denatured Lysozyme.

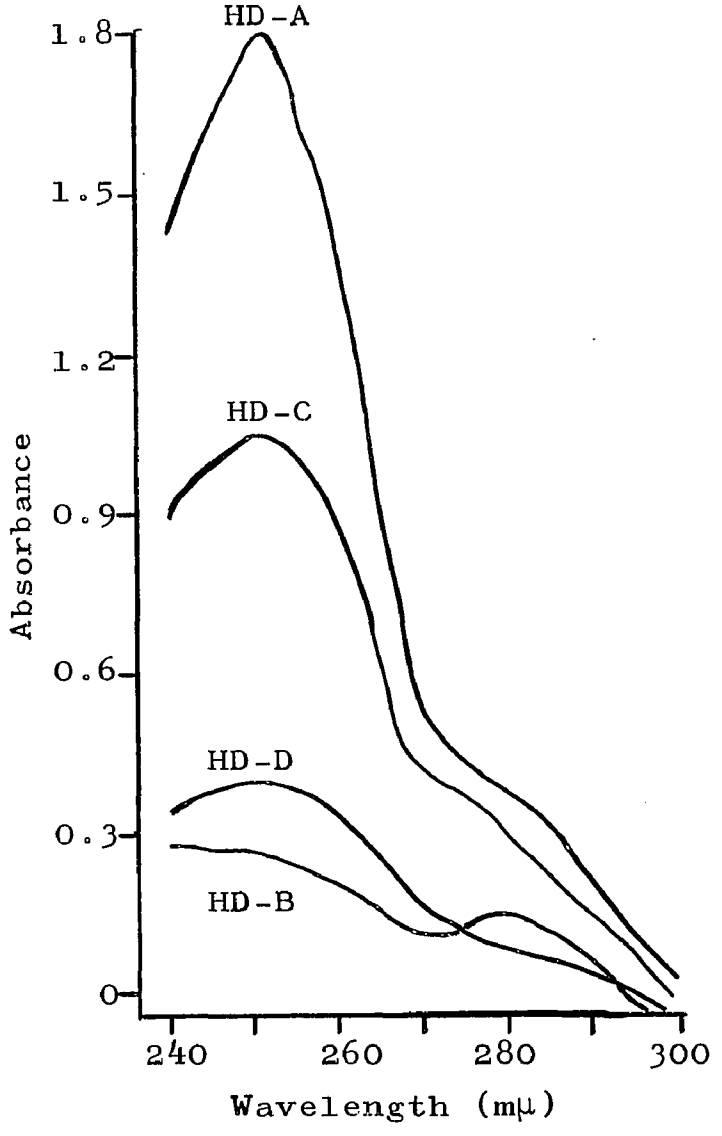

Fig. 16. Spectra of Peptides from Heat-Denatured Iodine-Inactivated-Lysozyme.

Spectra of the peak fractions of the Sephadex G-25 purifications (Fig. 15); all spectra were read in the eluting buffer (0.05 M NH_4 OAc, pH 8.0).

revealing the presence of oxindole in peptides HD-A, HD-C, and HD-D. Peptide HD-B was still impure, as can be seen from its Sephadex G-25 pattern. From their Dowex-1 and Sephadex G-25 elution positions, HD-A is equivalent to M-Ea and HD-C is equivalent to M-G.

The amino acid compositions after acid hydrolyses of peptides HD-A, HD-C, and HD-D are shown in Table 11, together with that of M-Ea (from Table 9) for comparison. Component HD-B had a composition uninterpretable in terms of a lysozyme peptide; it is a complex mixture which could not be purified by the chromatographic systems used. The three other components (HD-A, HD-C, and HD-D) are seen to be identical and correspond to peptide T-5 (108).

Oxidation of Tryptophan-62.--Recently, Hayashi et al. (1965) have reported that N-bromosuccinimide oxidizes tryptophan-62 of lysozyme, and that the modification is enzymically inactive. Unlike iodine oxidation, the reaction is specific in that only one product is formed. Since the over-all reaction is the same as reported here, i.e., oxidation of a single tryptophan to an oxindole, it is unexpected that two oxidizing agents attack different tryptophans. Therefore, the N-bromosuccinimide-oxidized-lysozyme was prepared in order to show that it and the iodine-oxidized were indeed different, and to confirm in this way both this work and that of Hayashi et al. (1965).

Table 11. Amino Acid Analyses of Tryptic Peptides of Iodine-Oxidized Peptides of Heat-Denatured Lysozyme.

Acid	T-5 (108)	HD-A	HD-C	HD-D	M-Ea
Try	1	--	--	--	1.00
Tyr		.06	.13	.02	--
Asp	3	2.78	3.21	2.86	3.08
Thr		--	.30	.03	--
Ser	1	1.00	1.00	1.00	.95
Glu		.02	.13	.03	.03
Pro		--	--	--	--
Gly	2	2.26	2.04	1.91	2.12
Ala	1	.93	.92	.83	.97
Val	1	.21	.19	.18	.31
CM-Cys		--	--	--	.08
Met	1	.88	.87	.90	.28
Ileu	1	.16	.31	.19	.27
Leu		--	.07	.01	--
Phe		--	--	--	--
His		--	--	--	--
Arg		.02	.02	.01	.02
Lys		.02	--	.03	.01

Values were standardized on serine as 1.00 for peptides from heat-denatured lysozyme. Peptide M-Ea was standardized on tryptophan as 1.00.

Lysozyme was reacted with an equimolar amount of N-bromosuccinimide at pH 4.5 by the method of Hayashi et al. (1965). The product and samples of both native and iodine-inactivated-lysozyme were each reduced, carboxymethylated and then hydrolyzed by trypsin, in reactions run in parallel. Samples (10 mg each) of each of these three proteins were separated on Sephadex G-25 giving patterns shown in Fig. 17. The recovery of the applied optical density in the separations of native, N-bromosuccinimide-inactivated and iodine-inactivated was 91%, 80%, and 64% respectively. (The low yield for the iodine-inactivated-protein was due to incomplete insolubility in the Sephadex G-25 buffer of the sample in this particular preparation.) The absorption at 280 mμ is consistently higher than at 250 mμ for the peptides of the native protein, as is expected. In both the native and iodine-inactivated samples there is a peak, representing 20.7% and 21.6% of the total optical density at fraction 25, the position of peptides N-F and M-F, which have been shown to contain tryptophan-62 and 63. The spectra of these peaks were both that of normal tryptophan, showing iodine had not oxidized either tryptophan-62 or 63. In the pattern for N-bromosuccinimide-inactivated lysozyme, the peak in this region (fraction 25) contains only 2.7% of the total optical density (13% the size of the corresponding peak in the native). This peptide could be the result of

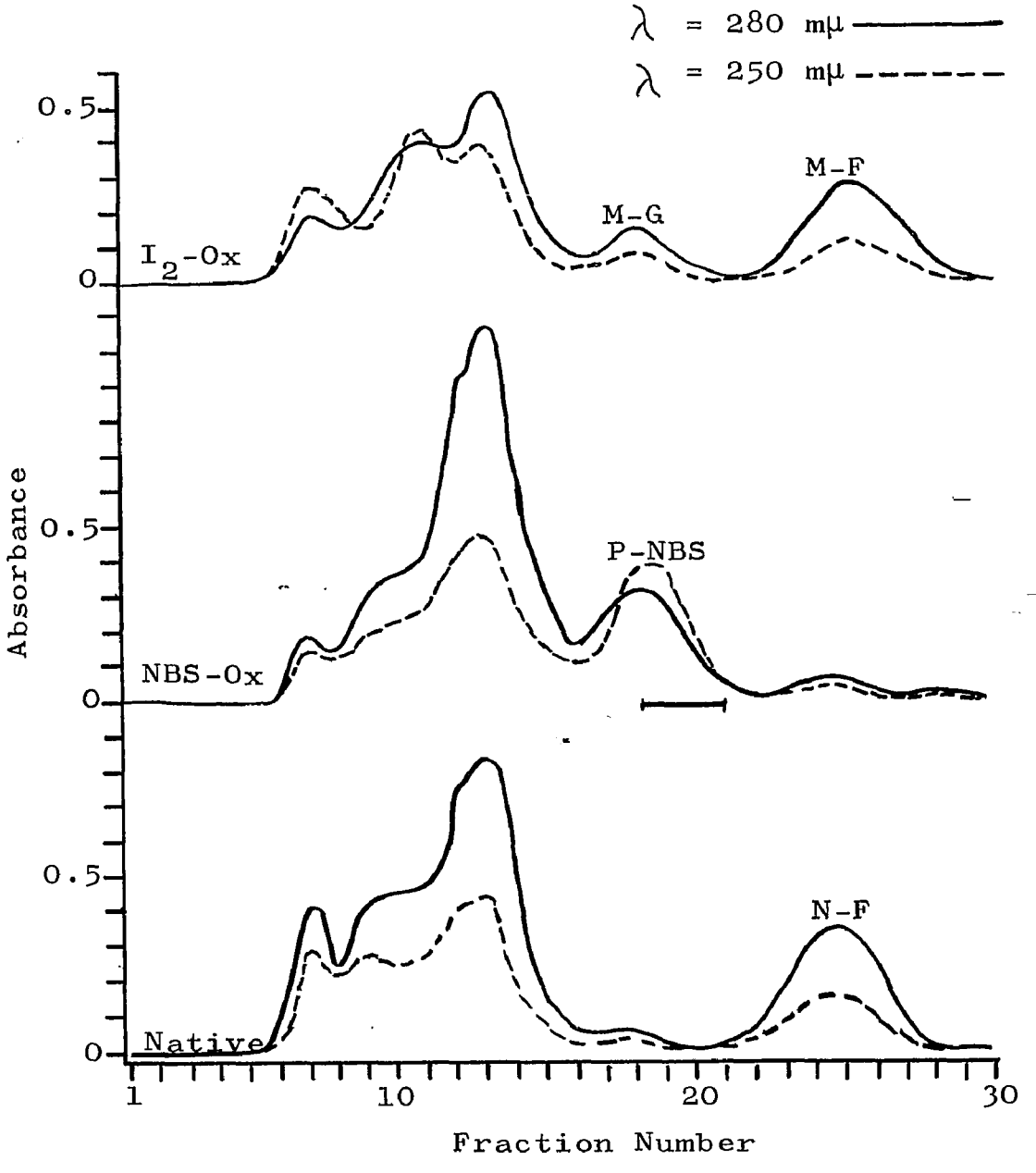

Fig. 17. Sephadex G-25 Separation of Tryptic Peptides from Reduced-Carboxymethyl-Lysozymes. Native, Iodine-Oxidized and N-Bromosuccinimide-Oxidized.

Fractions over solid line (in N-bromosuccinimide-oxidized-lysozyme) were pooled and purified by Dowex-1.

either unreacted native lysozyme in the protein sample, or oxidation by N-bromosuccinimide of a tryptophan other than 62 or 63 to the extent of 13%.

In the N-bromosuccinimide-inactivated sample a peak appears at fraction 19 (P-NBS) which shows a high absorption at 250 mμ and which is absent in the native and appears as a scarcely resolved shoulder at fraction 19 in the iodine-inactivated lysozyme. Fraction 19 (P-NBS) was purified on Dowex-1, giving the pattern shown in Fig. 18. One of the components has a higher absorption at 250 mμ; the peak fraction of this peptide was dried and acid hydrolyzed giving the analysis shown in Table 12. It was identical with peptide T-4, containing tryptophan-62 and 63. The spectrum of this peptide (Fig. 19) shows it contains one normal and one oxidized-tryptophan by comparison with Fig. 11, showing N-bromosuccinimide oxidizes either tryptophan-62 or 63, but not both, and not tryptophan-108, in agreement with the results of Hayashi et al. (1965).

Fig. 17 also shows peptide M-B (which contains tryptophan-111) present at fraction 18 in the iodine-inactivated sample, but absent in the native, demonstrating that the 108-109 bond was not split during the reduction, carboxymethylation or tryptic hydrolysis of the native protein.

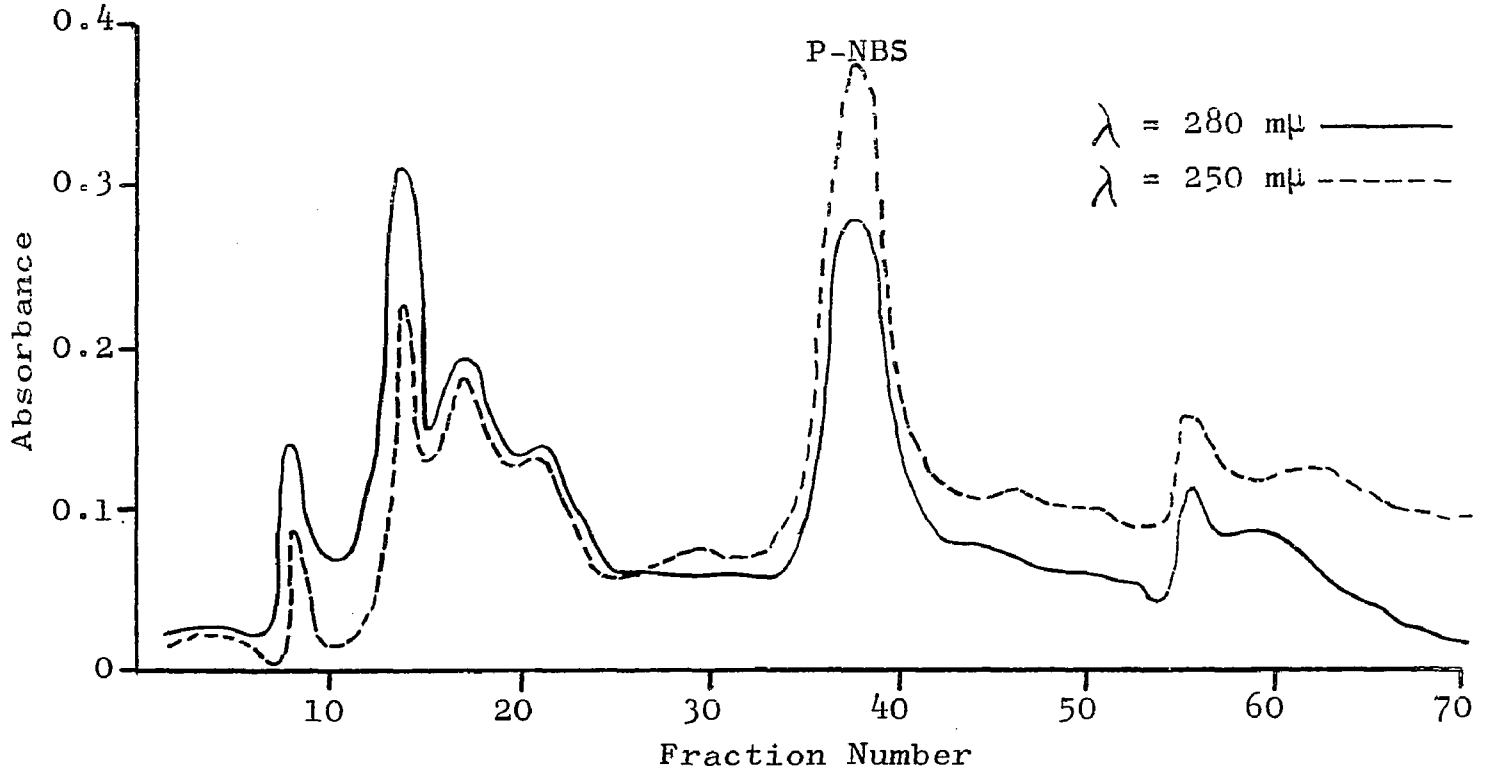

Fig. 18. Dowex-1 Purification of Peptide P-NBS.

Table 12. Amino Acid Analysis of the Peptide Oxidized by N-Bromosuccinimide.

Acid	T-4	P-NBS	CT-11-b
Try	2	--	--
Asp	2	1.98	1.98
Gly	1	1.00	1.00
Arg	1	1.00	.78
Cys	1	.67	.49
Thr		.03	.01
Ser		.05	.08
Glu		.05	.01
Pro		.00	.00
Ala		.06	.07
Val		.09	.01
Met		.01	.00
Ileu		.01	.00
Leu		.03	.00
Tyr		.02	.00
Phe		.03	.00
Lys		.04	.02
His		.00	.00

Results of amino acid analysis after acid hydrolysis. All values are the ratio of the individual amino acids to glycine taken as 1.00. Peptide CT-11-b results are by Hayashi et al. (1965) for the peptide showing an oxindole-indole spectrum.

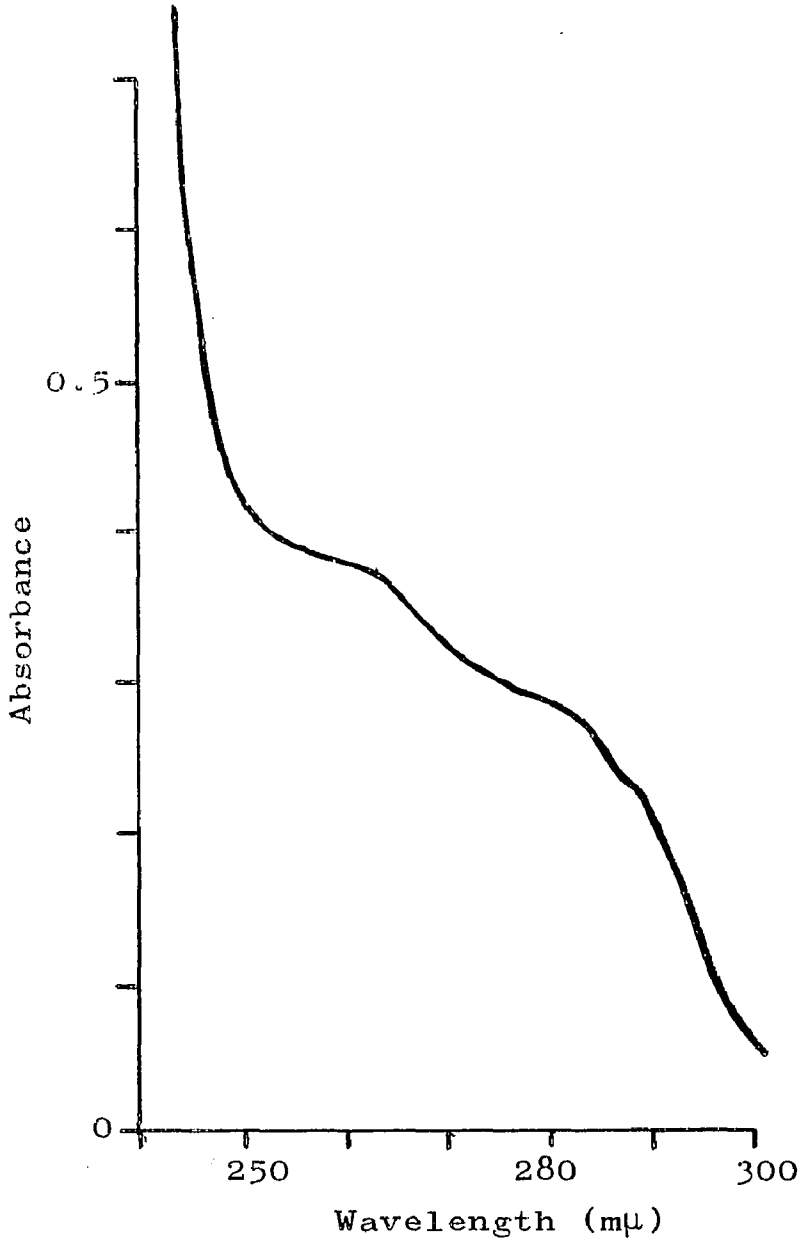

Fig. 19. Spectrum of Peptide P-NBS

Spectrum of fraction 38 of Fig. 18, read in eluting buffer.

Rate of Oxidation of Tryptophan-108 by Iodine.--In an attempt to explain why iodine and N-bromosuccinimide specifically oxidize unique but different tryptophan residues in lysozyme, the rates were measured for these oxidations and for a model tryptophan-containing compound.

One mole equivalent of lysozyme and one, two or three mole equivalents of N-acetyltryptophan ethyl ester (NATEE) were reacted with one-half mole of iodine; the rate of disappearance of the iodine color was measured as a function of time. These results were plotted according to the second-order rate equation, and are shown in Fig. 20. (The extent of reaction in each case was at least 40%.) The rate constants calculated from these curves are shown in Table 13.

Table 13. Rate of Oxidation of Lysozyme and N-Acetyltryptophan Ethyl Ester by Iodine.

Half-Mole Iodine Plus:	K_2 (liters/mole sec.)
One mole lysozyme	1.17
One mole NATEE	1.03
Two moles NATEE	0.93
Three moles NATEE	0.93

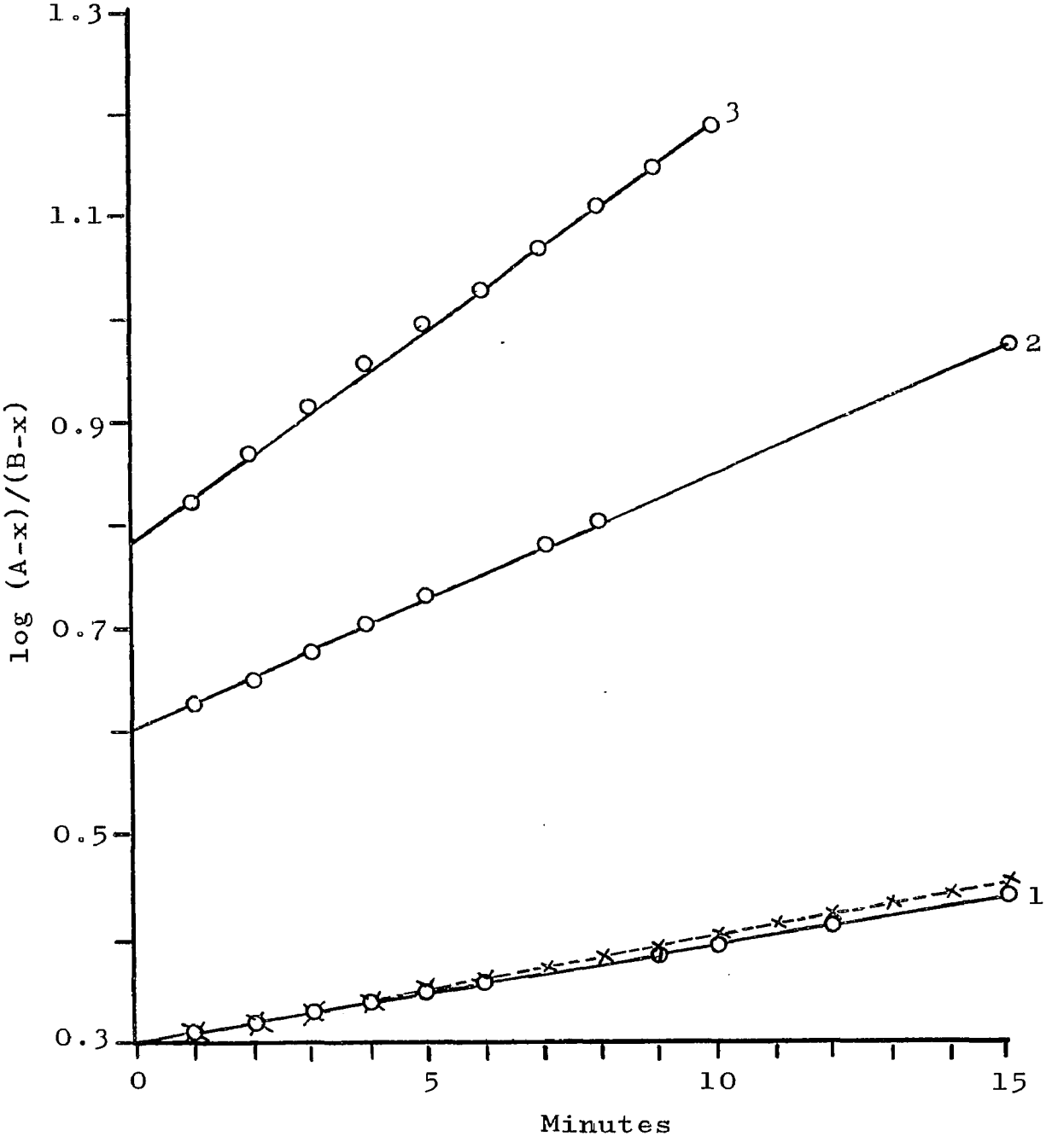

Fig. 20. Rate of Reaction of Lysozyme and N-Acetyltryptophan Ethyl Ester with Iodine

Plot of the second-order rate curve, where A = 340 μM I_2; x was determined by the decrease in optical density of the iodine color at 410 mμ.

B = 680 μM lysozyme (broken curve); 680 μM NATEE (curve 1); 1360 μM NATEE (curve 2) and 2040 μM NATEE (curve 3).

Free tryptophan reacts instantly with iodine to give a red color with no oxindole formation; this reaction was not further studied.

The above reaction of lysozyme was carried out under conditions where tryptophan-108 had been shown to be oxidized. From Table 13 it was concluded that this tryptophan was oxidized at the same rate as the tryptophan ester. However, the lysozyme reaction gives two major products which are obtained in about equal yields (components A and C in Fig. 3) and which result from iodination and oxidation, respectively. Consequently, the rates of these reactions are approximately equal, and half the measured rate of the iodine disappearance. The rate of oxidation of tryptophan-108 is therefore half the rate of N-acetyl-tryptophan ethyl ester oxidation to an oxindole.

Rate of N-Bromosuccinimide Oxidation of Lysozyme.--The rate of oxidation of lysozyme or NATEE by N-bromosuccinimide could not be measured in a manner similar to that by iodine since the reaction is immeasurably fast at a concentration of a thousand-fold less than that used by Hayashi et al. (1965) for the modification. Consequently, a different procedure was used.

Lysozyme and NATEE (67 μmoles each) were oxidized together by the addition of 67 μmole increments of N-bromosuccinimide. Samples were taken after 0, 1, 2, 3, 4,

and 12 additions, and the reaction products were separated on Sephadex G-25. In control experiments using pure samples, it was found that lysozyme and oxidized lysozyme eluted at fraction 9, oxidized NATEE at fraction 22 and unreacted NATEE at fraction 30. The patterns obtained for 0, 1, and 2 additions of N-bromosuccinimide are shown in Fig. 21. Patterns after all higher additions of N-bromosuccinimide were the same as after 2 additions in that there were peaks at fractions 9 and 22 but not at 30; the additional N-bromosuccinimide changed only the spectrum of the protein in fraction 9. All peaks appearing at fraction 22 had the oxindole spectrum, all peaks at fraction 30 had the normal tryptophan spectrum. The spectrum of the protein appearing at fraction 9 after each N-bromosuccinimide addition is shown in Fig. 22.

The Sephadex G-25 separation after addition of one mole of N-bromosuccinimide per mole of protein was the only one which showed both oxidized (fraction 22) and normal (fraction 30) NATEE. The ratio of the areas of these peaks was 55.5% and 45.5% respectively, using experimentally determined extinction coefficients of: $\epsilon m\underline{M}$ = 6.02 (280 mμ), 2.75 (250 mμ) for NATEE; $\epsilon m\underline{M}$ = 1.25 (280 mμ), 6.25 (250 mμ) for oxidized-NATEE.

To determine the extent of lysozyme oxidation in each case, the 280/250 mμ absorption ratio was calculated for lysozyme with one, two, and three tryptophans oxidized,

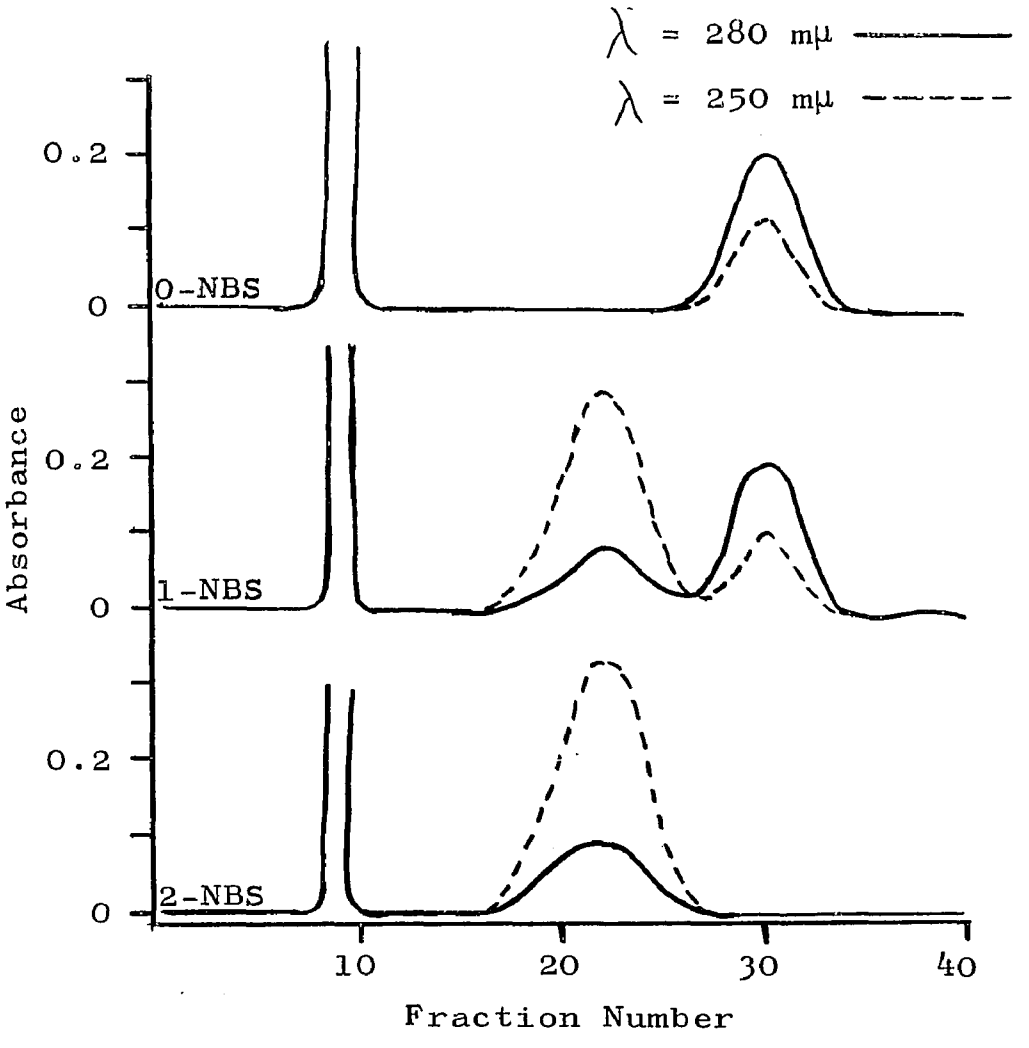

Fig. 21. Sephadex G-25 Separation of the Products of Oxidation of One Mole of Lysozyme and One Mole N-Acetyltryptophan Ethyl Ester by 0, 1, and 2 Moles of N-Bromosuccinimide.

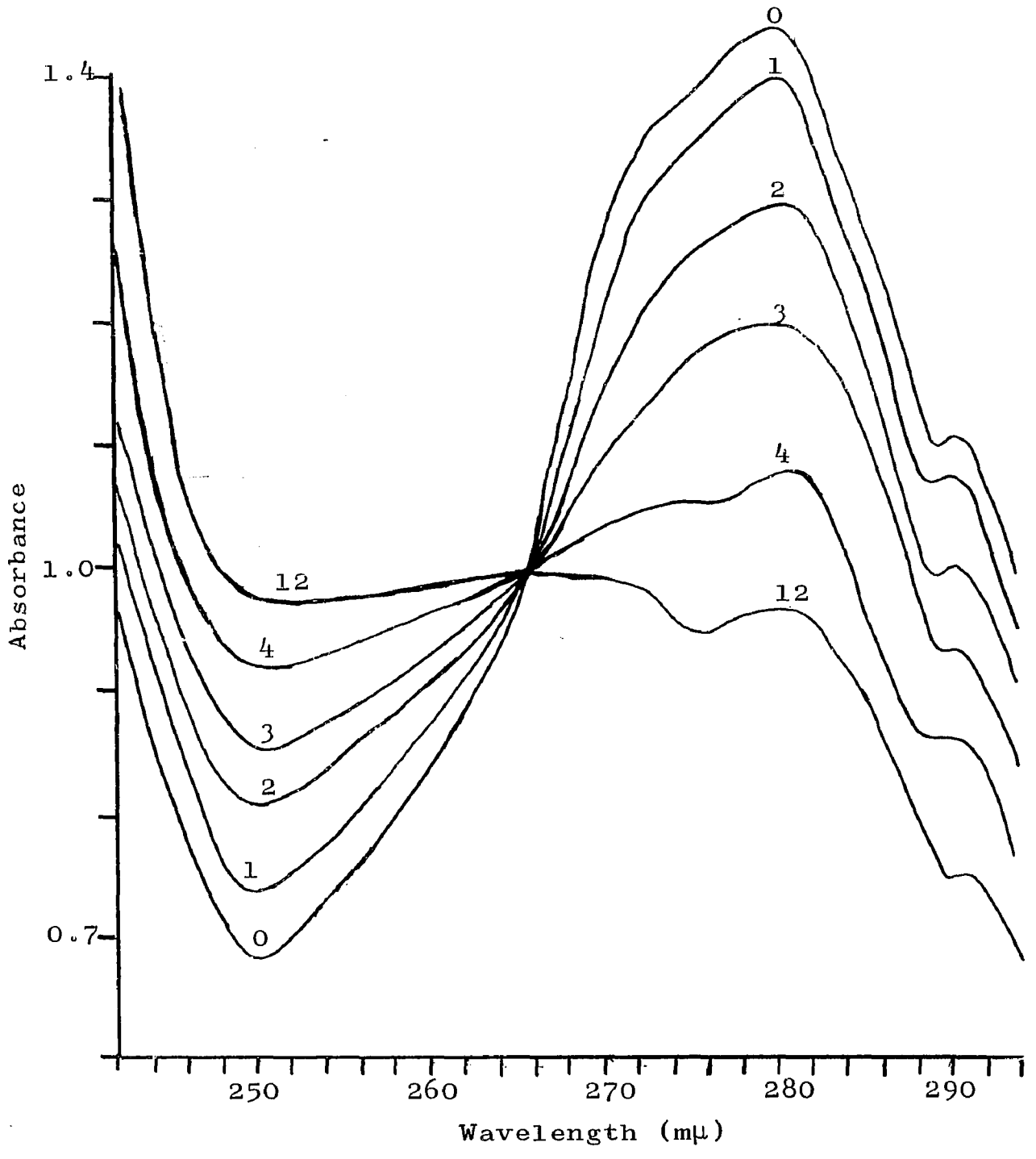

Fig. 22. Spectra of Lysozymes Oxidized in the Presence of N-Acetyltryptophan Ethyl Ester by Successive Additions of N-Bromosuccinimide.

All spectra adjusted to optical density of 1.0 at 266 mμ.

using experimentally determined molar extinction coefficients at these wave lengths for the free amino acids NATEE, oxidized-NATEE, and tyrosine. The ϵmM for tyrosine was 1.21 (280 mμ), 0.00 (250 mμ).

The calculated ratio for lysozyme with one oxidized tryptophan is 1.74; this agrees both with the observed value of 1.75 for pure N-bromosuccinimide-oxidized-lysozyme as determined in this work, and with the spectrum reported by Hayashi *et al.* (1965). The ratios for this and lysozyme with two and three oxidized tryptophans is summarized in Table 14.

Table 14. Absorption Ratios (280/250 mμ) for Oxidized Lysozymes.

Number of Oxidized Tryptophans	Absorption Ratio: 280/250 mμ	
	Calculated	Observed
0	2.40	2.15
1 (by NBS)	1.74	1.75
1 (by I_2)	1.74	1.45
2	1.28	--
3	0.96	--

The 280/250 mμ absorption ratios of lysozyme after each addition of N-bromosuccinimide determined from Fig. 22

is given in Table 15 with the extent of oxidation calculated from the theoretical ratios of Table 14.

Table 15. Extent of Lysozyme Oxidation After Addition of N-Bromosuccinimide.

Moles NBS per Moles Protein	280/250 mμ	Approximate Composition (Calculated)
0	2.16	Pure native
1	1.93	50% native; 50% mono-oxidized
2	1.63	75% mono-, 25% di-oxidized
3	1.41	25% mono-, 75% di-oxidized
4	1.20	100% di-oxidized
12	0.99	100% tri-oxidized

The reaction conditions of the first addition of N-bromosuccinimide, except for the presence of NATEE, are identical to those previously used to oxidize the tryptophan-62 of lysozyme. The foregoing data show that one mole of N-bromosuccinimide oxidizes half of the one mole of NATEE initially present and gives a protein whose spectrum indicates it to be composed of a half mole of native and a half mole of mono-oxidized-lysozyme. Consequently, the rate of oxidation of tryptophan-62 by N-bromosuccinimide is the same as for the free tryptophan ester. Moreover, the second tryptophan must oxidize more slowly than the most

reactive, since oxidation of the protein alone with an equimolar amount of N-bromosuccinimide gives a pure product. After tryptophan-62 and NATEE are both completely oxidized by two moles of N-bromosuccinimide, two other tryptophans of the protein can be oxidized. With a large excess of N-bromosuccinimide (11 fold) only three tryptophans were oxidized. The remaining three residues presumably react at a rate slower than the hydrolysis of the reagent. This is consistent with the observation of Green and Witkop (1964), who found that 3.5 residues of tryptophan in lysozyme are "titrated" with N-bromosuccinimide at a pH of 5.0.

IV. DISCUSSION AND CONCLUSIONS

Carboxymethylation of the Single Histidine.--Concurrently with these experiments, Kravchenko et al. (1962) published the first evidence which demonstrated histidine was not a residue important for the enzymic activity of lysozyme. Their work involved the same modification considered in this study; the foregoing results confirm their conclusions, and extend them as described in this section.

The major product of the carboxymethylation reaction showed a single change from the native protein, i.e., the conversion of histidine-15 to 3-carboxymethyl histidine. The modified lysozyme retained 40% of its activity when measured by the rate of cell-wall lysis; Kravchenko et al. (1962) reported full activity in their modification, a discrepancy which is unexplained but which perhaps follows from the difficulties inherent in the activity assay using cell-walls. In particular, the lytic activity is sensitive to electrostatic effects, suggesting that a change in the charge on the enzyme might alter the rate differently in different conditions of assay.

The monotonic increase in the rate of inactivation by carboxymethylation between pH 3.0 and 10.6 (Fig. 2) is in agreement with the noninvolvement of the histidine in

the activity. In contrast, the rate of inactivation of ribonuclease reaches a maximum at pH 5.5, the pH of maximum enzymic activity (Gundlach et al., 1959; Bernard et al., 1959), consistent with the participation of histidine-119 of ribonuclease in the bond-breaking process.

In this work and that of Kravchenko et al. (1962), measurements revealing at least partial activity were made on a singly-modified lysozyme which was isolated and characterized. Reports which implicated the histidine in the enzymic process have all concerned unseparated reaction products, rendering it difficult to separate the contribution of each residue modified to the inactivation.

Fraenkel-Conrat (1950) reacted lysozyme at pH 7.6 with a 35 molar excess of iodine and obtained a product containing 3.5 equivalents of bound iodine per mole of protein, and which retained only 38% of the original activity. Addition of thioglycol reduced the bound iodine to 2.4 equivalents and raised the activity to 78%. Since regeneration of activity was accompanied by the loss of bound iodine, it was concluded that iodination, rather than oxidation had caused inactivation. Since tyrosine could not be regenerated from mono or di-iodotyrosine by thioglycol, but the unstable N-iodohistidine (but not C-iodohistidine) could be reduced to free histidine, it was concluded that the inactivation had been caused by reaction of histidine; this was confirmed by the paper chromatographic

isolation of mono-iodohistidine from the reaction product. As further evidence that iodination rather than oxidation was responsible for inactivation, the reaction product had lost less than 10% of its tryptophan, determined by spectral analysis. Therefore, Fraenkel-Conrat (1950) concluded histidine, but not tryptophan, was essential for the catalytic activity of lysozyme.

Since the carboxymethylation results conflict with this conclusion, the experiments of Fraenkel-Conrat (1950) were repeated; however, all attempts to regenerate activity were unsuccessful.

The difficulties in interpreting these results can be summarized as follows:

1. Reaction by a large excess of iodine resulted in a product retaining 38% of the initial activity. However, it was shown that pure carboxymethyl-histidine-lysozyme is only 40% active. Therefore, the activity loss reported by Fraenkel-Conrat (1950) could indeed have been due to complete iodination of the histidine.
2. Although the presence of N-iodohistidine was shown in the product of iodine reaction, the molar amount could not be determined; it could only be inferred to be slightly less than one residue, based upon the difference between the amounts of bound iodine and iodotyrosine. It is obviously difficult to

evaluate the importance of a specific residue in chemical modification studies when neither the amounts or nature of all the reactions are known.

It is also of interest that Fraenkel-Conrat (1950) found only a 10% loss of tryptophan by reaction of lysozyme with excess iodine at pH 7.6, whereas at pH 5.5 a half-molar amount of iodine results in a 25% oxidation of tryptophan.

The unessential nature of the histidine of lysozyme can be confirmed by the work of Weil et al. (1952) who showed by photo-oxidation studies leading to histidine and tryptophan destruction, that when histidine was completely oxidized, the protein was still 30% active. Unfortunately, since the initial (less than 30%) histidine oxidation rate parallels the activity loss, they concluded that this residue was possibly also involved with tryptophan in the active site, a conclusion based mainly on their belief in the work of Fraenkel-Conrat (1950).

Ferrini (1964) concluded from photo-oxidation studies that histidine but not tryptophan is essential for lysozyme activity. The unseparated oxidation product, retaining at least 20% activity, was analyzed for amino acid composition after pronase hydrolysis, and showed complete histidine loss but full tryptophan recovery. In the study reported here, pronase gave only 70% hydrolysis

of lysozyme, and had to be supplemented by carboxypeptidase A and B and leucine amino peptidase to achieve complete lysozyme hydrolysis; Ferrini (1964) did not report amino acid analyses for residues other than histidine and tryptophan, and lack of complete enzymic hydrolysis by pronase would render the results suspect.

Iodine Inactivation of Lysozyme.--Since it was found that histidine was not an essential residue of lysozyme, reinvestigation of the iodine reaction, in particular the involvement of other amino acid side chains, became important. Using milder conditions than Fraenkel-Conrat's (1950) (i.e., a concentration of iodine half-molar with respect to lysozyme and one which did not lead to protein precipitation at pH values from 5.5 to 10.0), a pure mono-modified, enzymically-inactive species was obtained.

The oxidation of a single tryptophan generated the complete loss in enzymic activity, shown by amino acid and spectral analyses. However, the inactivation of enzymes after chemical modification can result either directly from a reaction occurring at the active site, or indirectly from reactions occurring distant from this region but which induce extensive changes. To distinguish between these alternatives, lysozyme was reacted with iodine in the presence of inhibitors which are also substrate analogs.

Since the inhibitor presumably binds at the active site, a tryptophan there would be protected from oxidation. Reaction in the presence of N-acetylglucosamine or its dimer at concentrations 2 and 100 times greater, respectively, than that required for 50% inhibition decreased the amount of inactive species formed by 75% and 100% respectively. In agreement with the conclusion that the reactive tryptophan is an essential element of the active site, the optical rotation of lysozyme showed a slight positive shift upon modification; a large negative shift would be expected if the inactivation were the result of a gross denaturation rather than a specific modification.

A peptide of iodine-inactivated-lysozyme (M-Ea) having an oxindole spectrum was isolated and purified; its amino acid composition showed it to be composed of residues 98 to 108 of the lysozyme chain, requiring that the oxidized residue be tryptophan-108.

Another peptide (M-G) with an oxindole spectrum was found, but it represented such a small amount of the total sample it could not be analyzed. To show that peptide M-Ea contained essentially all the oxindole present in the derivative, it is necessary to consider the yields of the tryptophan peptides. The percentage of the total optical density represented by each of the tryptophan peptides of iodine-inactivated-lysozyme is shown in Table 8. The percentage of one tryptophan that each peak contains was

calculated assuming that each tryptophan (or oxidized-tryptophan) contributes 15.1% of the total optical density at 266 mμ (the isosbestic point of the lysozyme, oxidized-lysozyme system. Table 16 shows this percentage based upon both total initial sample (before chromatography on Dowex-1) and recovered sample.

Table 16. Recoveries of Tryptophan Containing Peptides From Dowex-1 Chromatography of Reduced Carboxymethyl-Iodine-Inactivated-Lysozyme.

		Percentage of One Tryptophan Recovered	
Peptide	Tryptophan	Based on Column Yield	Based on Initial Sample
M-B	111	95.5	81.0
M-Ea	108	113	95.5
M-F	62, 63	208	182
M-G	(108)	9.3	7.9

The recoveries of tryptophans 62, 63, 108, and 111 were similar and in high absolute yields (above 80%). It will be shown that peptide M-G, containing less than 10% of a residue of tryptophan, also contains oxindole-tryptophan-108. Consequently, tryptophan-108 accounts for the full oxindole content of the inactive derivative, and its modification must cause the loss in enzymic activity.

The product of iodine oxidation of lysozyme was also heat-denatured instead of reduced and carboxymethylated before tryptic hydrolysis, yielding four peptides with an oxindole spectrum. From the elution positions, HD-1 corresponded to M-Ea and HD-C to M-G. Non-oxindole peptides were not analyzed since disulfide interchange would lead to a large number of them. The amino acid analyses of three of these oxindole peptides were identical to that obtained for M-Ea, except for full recovery of methionine, which was not destroyed by heat-denaturation. The fourth peptide could not be purified under the conditions used. No oxindole spectrum was found for a peptide not containing tryptophan-108. This experiment, confirming the results with the reduced-carboxymethyl-protein, was possible only because the tryptic peptides of interest, those containing tryptophan-108, contain no cysteine.

The generation of multiple peptides of tryptophan-108 with the same amino acid composition is not understood; however, several explanations may be considered:

1. Isomerization of the α-carbon of tryptophan. There is no apparent reason for an unusually rapid racemization of oxindole-tryptophan.
2. Oxidation of methionine to the sulfone. Reduction of any sulfone present was attempted by the addition of 25 mg of mercaptoethanol to 2 μmoles of protein hydrolysate in 3.0 ml. of 0.2 M acetic acid and

reacting for 72 hours at 40°, the method of M. Doscher (unpublished results), but no change in the peptide pattern was observed.

3. Formation of the oxindole introduces a new center of asymmetry at the γ-carbon. The restraints of the protein structure should strongly favor one isomer, consistent with the observed pattern. However, this process would account for only two of the three or four peptides.
4. Deamination of asparagine-106.

Cleavage of Peptide T-5.--The oxidation of tryptophan-108 was identified by the isolation of a peptide (T-5[108]) that resulted from a split which was C-terminal to tryptophan-108 and was not expected for trypsin. The split occurred in all hydrolyses of iodine-inactivated-lysozyme, unfolded by either reduction or heat-denaturation, but not in hydrolyses of native lysozyme. The split produced a tetrapeptide (T-5[111]) which occurs in an easily identifiable region in both the Dowex-1 (Fig. 7) and Sephadex G-25 (Fig. 17) patterns for iodine-inactivated-lysozyme, but which is clearly absent in these patterns for native-lysozyme (Figs. 8 and 17, respectively).

If the cleavage after tryptophan-108 occurred during the initial iodine oxidation, it could have been the cause of the enzymic inactivation. This possibility can be eliminated for two reasons:

1. The protein samples were exhaustively dialyzed after reduction and carboxymethylation. If the split had occurred during the iodine oxidation, two peptides would be obtained upon reduction: one of 108 residues and one of 21 residues; the smaller one would be expected to pass through the dialysis tubing significantly faster than the larger. Amino acid analysis of the product, however, showed it to contain the same amino acids as the initial sample. Also, the recoveries of peptides M-B and M-Ea (containing tryptophans-111 and 108, respectively) were similar (14.4% and 17.7% of the total optical density).
2. Samples of reduced carboxymethyl-lysozyme, both native and iodine-inactivated were each homogeneous as determined by ultracentrifugal analysis.

The split therefore occurred during or subsequent to the tryptic hydrolysis, for reasons not determined. Possible explanations are:

1. N-bromosuccinimide can cause cleavage C-terminal to tryptophan during oxidation (Patchornik et al., 1960), suggesting that a peptide bond with a carbonyl contributed by oxindole-tryptophan is unusually labile. Digestion by trypsin may remove restraints imposed by the polymeric structure and

so induce cleavage. A plausible mechanism is shown in Fig. 23, in which the oxindole-oxygen of the oxidized-tryptophan (I) attacks the relatively positive carbonyl-carbon of the peptide bond forming a six member cyclic intermediate (II) which is more labile than the peptide bond. Cleavage of the peptide chain results in a new C-terminal residue and a lactone (III) which easily hydrolyzes to give the C-terminal oxidized-tryptophan (IV).

2. An oxindole peptide may be more susceptible than one containing tryptophan to a trace chymotryptic impurity in the trypsin. However, cleavage has not been observed after oxindole-tryptophan-62, indicating that the nature of the amino acids near the involved tryptophan must either specifically enhance or repress the cleavage rate. The situation may be parallel to the well-known unusual lability of serine or aspartic acid bonds, or the stability of isoleucine bonds.

Oxidation of Tryptophan-62.--Hayashi et al. (1965) have shown that the N-bromosuccinimide reaction leads to the oxidation of tryptophan-62, which is in contrast to the results obtained with iodine. To verify this difference, peptides from N-bromosuccinimide and iodine oxidized lysozyme and also the native protein were separated on

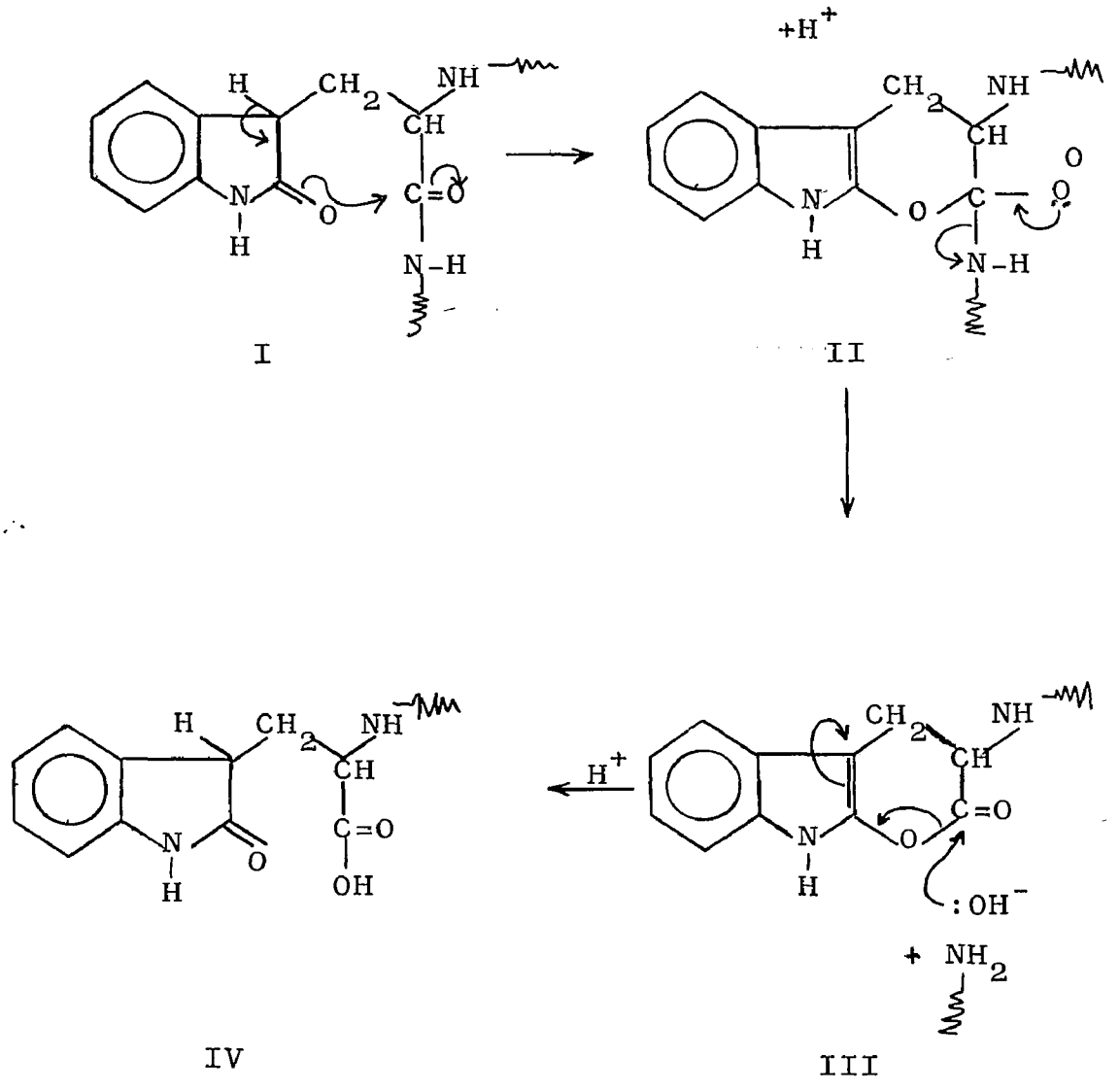

Fig. 23. Proposed Mechanism for the Cleavage of the Peptide Bond of Oxidized-Tryptophan-108

Sephadex G-25. A peptide from the N-bromosuccinimide sample (peptide P-NBS) was isolated and purified on Dowex-1; it had an oxindole-indole spectrum, and amino acid analysis showed it to be peptide T-4 (which contains tryptophan-62 and 63). This peptide was isolated in full molar amount from the peptides of iodine-inactivated-lysozyme, and it had a normal tryptophan spectrum.

The specific oxidation by N-bromosuccinimide of tryptophan-62 is explainable in terms of the three dimensional structure of lysozyme. D. C. Phillips (unpublished results) has shown that this is the most exposed tryptophan in the molecule (its position is in fact not crystallographically defined due to excessive movements of the residue), and it is reasonable that it reacts at a rate equal to that of the free tryptophan ester. With an excess of N-bromosuccinimide, two other tryptophans are also oxidized at a rate too fast to measure directly, but significantly more slowly than tryptophan-62 or the model compound, indicating they are at least partially buried.

These results point up the difficulty in the iodine reaction, that a tryptophan buried or partially buried by the N-bromosuccinimide criterion is the most reactive with iodine. There is no evidence available to resolve this problem, but a plausable explanation is that tryptophan-108 is "activated" in some manner, i.e., by the proximity of an electron donor enhancing the nucleophilic character of the

β-carbon and consequently the sensitivity to oxidation by the I^+ electrophile. Although this residue reacts with a rate half that of a tryptophan ester, there is no way of knowing how slowly it would react were it not activated. As an additional complication, it must also be assumed either that tryptophan-108, which lies in a cleft in the surface of the molecule, although activated, is not accessible to the larger N-bromosuccinimide molecule, or that the mechanism of N-bromosuccinimide and iodine oxidation are markedly different.

The crystallographic data of D. C. Phillips (unpublished results) show two interactions with the ring of tryptophan-108 (Fig. 24) which might activate the β-carbon. In the first, the γ-carboxylate group of glutamic acid-35 is in close proximity to the indole ring; in fact, an adjacent carboxyl has been shown to affect the spectrum of one of the tryptophan chromophores in lysozyme (J. A. Rupley, unpublished results). In the second, the carbonyl oxygen of leucine-56 forms a hydrogen bond with the indole nitrogen. The interaction of either of these oxygens would lead to an increase in the nucleophilic character of the β-carbon of the indole, resulting in activation with respect to iodine oxidation. It is of interest that a similar activation of the ring may be significant in the oxidative reaction catalyzed by a

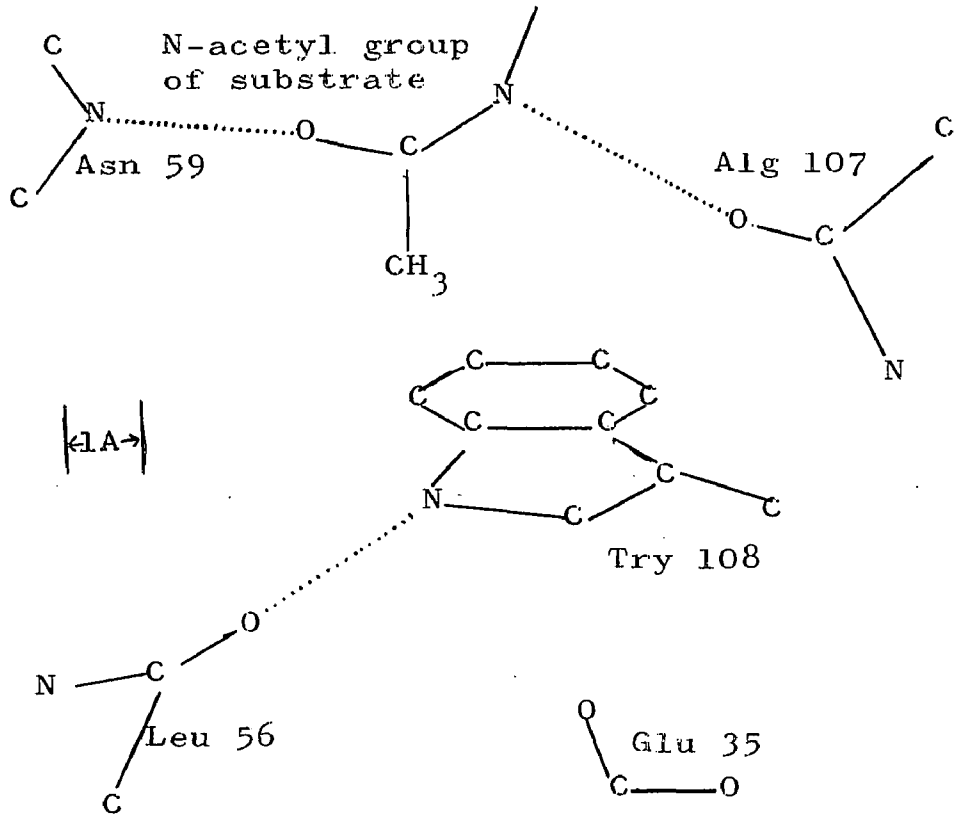

Fig. 24. Binding of Substrate by Lysozyme and Activation of Tryptophan-108 by Neighboring Groups.

Projection on a plane of the atoms of the substrate N-acetyl group together with those of the indole ring of tryptophan-108, the -carbonyl of glutamic acid-35 and the peptide bonds of leucine-56, alanine-107 and asparagine-59. Based upon atomic coordinates determined by D. C. Phillips (unpublished results) from the crystal structure.

Hydrogen bonds are shown by dashed lines. The carboxyl oxygen of glutamic acid-35 is 3.2 A from the indole 2-carbon.

tryptophan side chain in yeast alcohol dehydrogenase (Schellenberg, 1965).

Mechanism of Tryptophan Oxidation.--The mechanism of the oxidation by N-bromosuccinimide of tryptophan and β-substituted indoles has been studied by Green and Witkop (1964), who found that above pH 5.5 all oxidations proceed via a bromohydrin intermediate, which can lose HBr in at least three ways depending on the pH and the nature of the substituent in the 3-position. At a lower pH (such as the ones used here), the oxindole is formed in less than one second and no intermediate could be detected.

A plausible mechanism for the oxidation of tryptophan-108 by iodine is shown in Fig. 25. The iodinium ion attacks the nucleophilic β-carbon of tryptophan (I) to form the unstable iodinium species (II) which adds a hydroxyl ion to form the iodohydrin intermediate (III). This species loses HBr to form the hydroxytryptophan (IV) which by a tautomeric shift results in the oxindole (V).

Comparison of Chemical and Crystallographic Data.--The chief value of experiments elucidating the amino acid residues of the active site of lysozyme is derived from the fact that this enzyme is the only one whose crystallographic structure has been determined. The results of all these chemical studies can be explained with reference to the

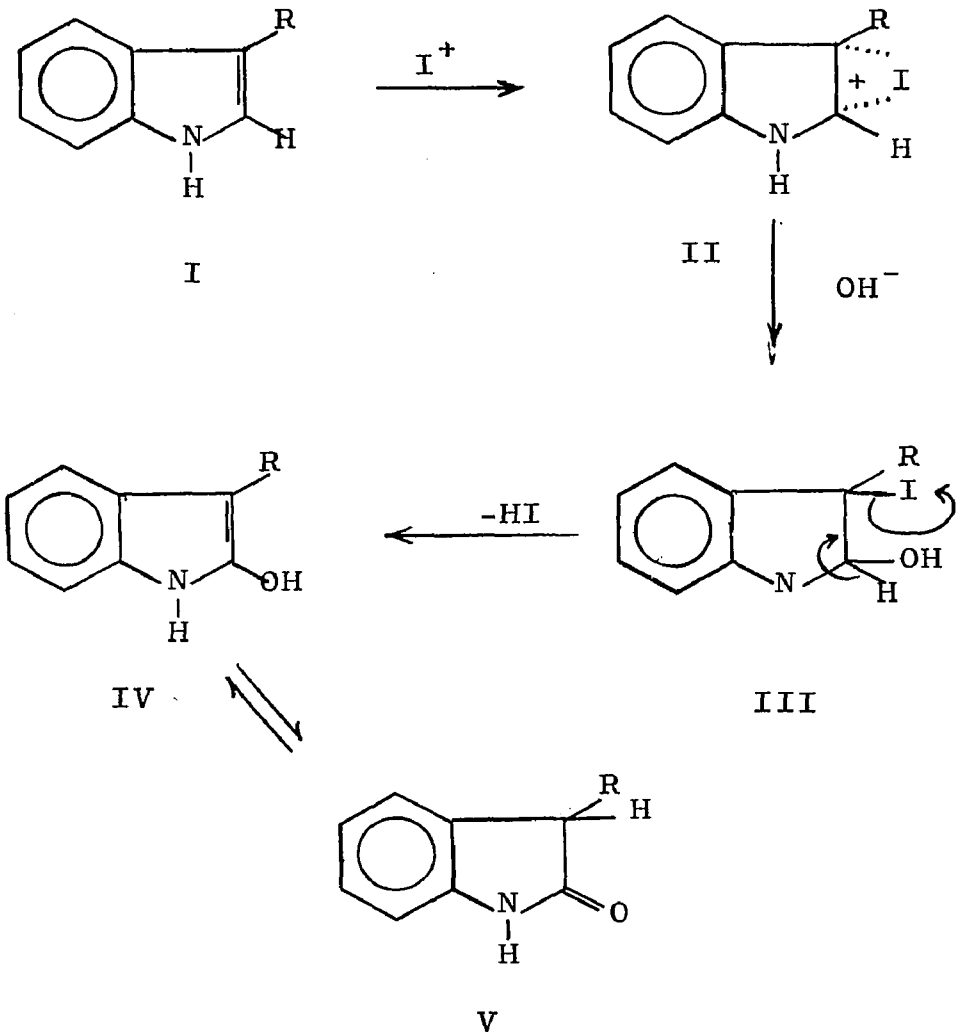

Fig. 25. Proposed Mechanism for the Oxidation of Tryptophan-108 by Iodine.

molecular structure, but could not have been completely predicted from a knowledge of the structure alone.

Histidine-15 is located on the side of the lysozyme molecule opposite to the active site, and its modification should not drastically affect enzymic activity; this is in accord with the carboxymethylation studies. It is interesting that both imidazole nitrogens are hydrogen bonded (N-1 to the oxygen of the peptide bond of alanine-11; N-3 to the hydroxyl oxygen of threonine-89), and its abnormal ionization is therefore expected. Preliminary experiments (R. Pecoraro, unpublished results) have indicated this is true.

Crystallographic studies (D. C. Phillips, unpublished results) of lysozyme have shown there is a deep cleft in the center of the molecule which divides it into two parts of different character; one part contains a high proportion of non-polar residues, which form a hydrophobic core and presumably impart considerable structural stability; the active site is this cleft, and contains among other residues, three tryptophans (62, 63, and 108) and four carboxyl groups. Of these carboxyls, two are involved in cleavage, one in binding and one is not directly involved. Recent chemical studies (J. A. Rupley, unpublished results) have shown the binding site of lysozyme requires an amide group (glucosamine and glucose are not bound to a measurable extent), and the saccharide

ring makes a significantly smaller contribution to the binding energy (about 1000 calories of a total monomer binding energy of 4700 calories). Although N-acetylglucosamine and its homologs are all bound by the enzyme, hydrolysis occurs at a significant rate only with the pentamer or higher polymers. Studies of the binding energy and cleavage products indicate there are several possible enzyme-substrate complexes with different stabilities, and it has been concluded that the active site can interact with at least six saccharide units. A model built according to the atomic coordinates has shown a hexamer can completely fit in the cleft in the molecular surface. The trimer when bound is not in the bond-breaking region, and it is an adjacent region that is likely responsible for catalysis.

The oxidation of tryptophan-108 by iodine abolishes the trimer binding; there is a greater than 4000 calorie loss out of a 9000 calorie total unitary free energy of interaction. This can be explained by recent crystallographic studies (C. C. F. Blake, unpublished results) which have shown that iodine oxidation of crystalline lysozyme lead to changes in the positions of the side chains of tryptophan-108 and glutamic acid-35, which is in contact with it (Fig. 24). The changes were local and small, as would be expected for this oxidation, but they were at a critical point in the molecule, since glutamic acid-35

participates in the cleavage reaction and tryptophan-108 forms the bottom of a non-polar pocket into which the amide-methyl group of the trimer fits. Tryptophan-62 oxidation by N-bromosuccinimide also reduces binding, but only by 2600 calories. It is understandably not as drastic an effect as tryptophan-108 oxidation, as tryptophan-62 is located on top of the molecule, and although its displacement would destroy two interactions with the saccharide (one hydrogen bond and one hydrophobic bond), it would not prevent an element of the substrate from "fitting in," as is the case with tryptophan-108 oxidation.

The agreement between the reaction of iodine with crystalline and solution lysozyme suggest that their conformations are identical. This conclusion is borne out more forcefully by the identity of the binding of monomer and dimer in the crystal and in solution (L. Butler, unpublished results).

The complications in the chemistry of the tryptophans of lysozyme (in particular, the presence of three in the active site, the preferential activity of tryptophan-108 with iodine and tryptophan-62 with N-bromosuccinimide, and the probable activation of tryptophan-108 to iodine oxidation) serve as an excellent illustration of the advantages of collaboration between protein chemistry and crystallography; neither type of data alone can yield reasonable conclusions about the properties of lysozyme.

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