THE PREPARATION AND AMINO ACID SEQUENCE OF CRYSTALLINE TURKEY EGG WHITE LYSOZYME

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
JOHN NELSON LaRUE
1969

THESIS

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Ph.D. degree in Biochemistry

John C. Speck, Jr.
Major professor

Date July 1, 1969

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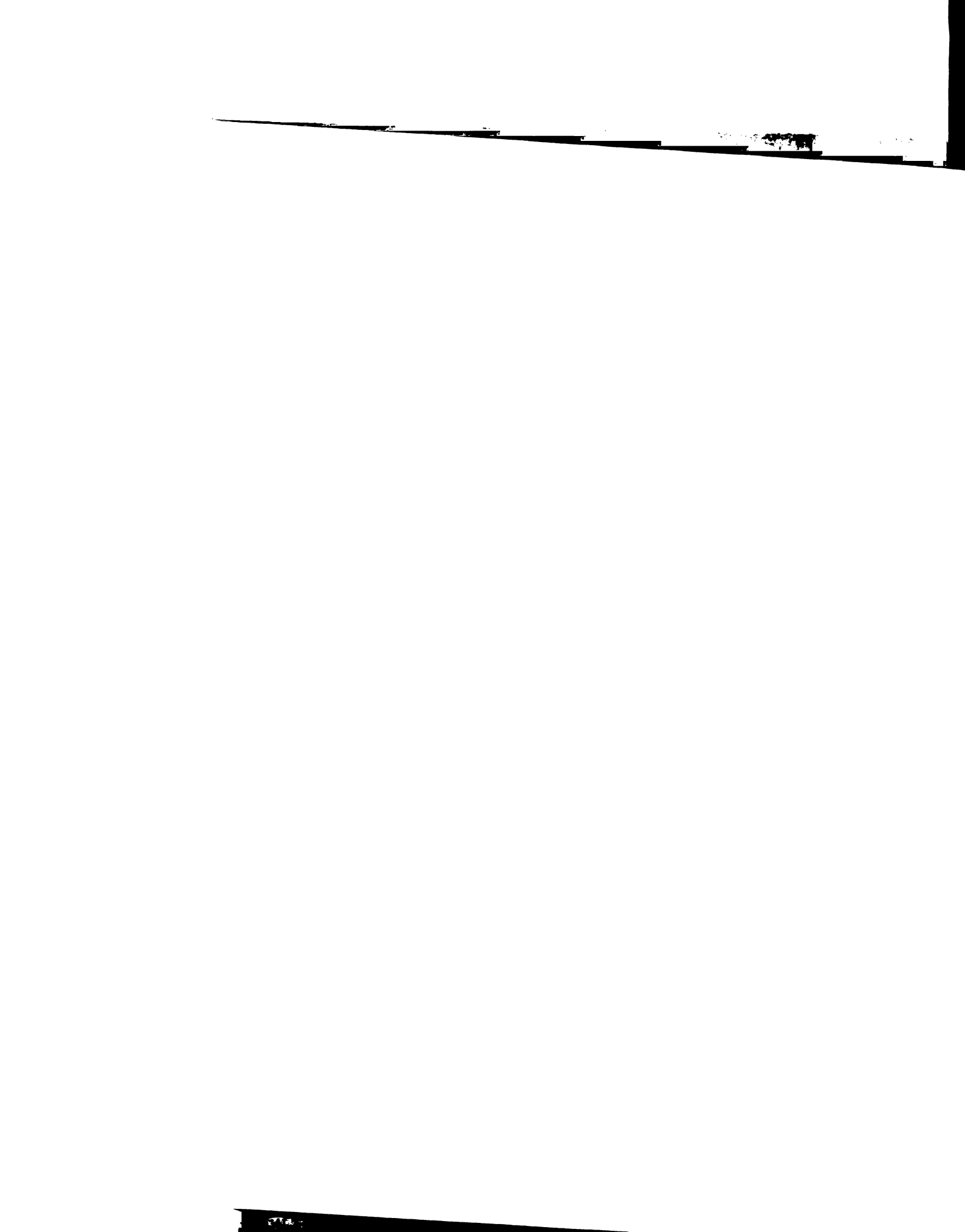
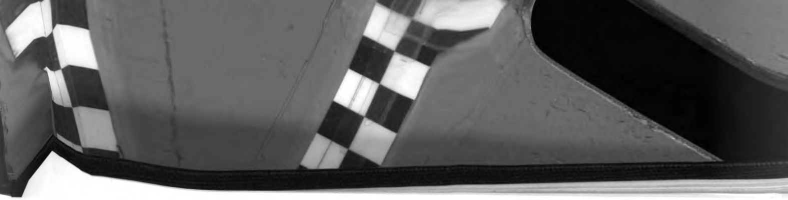
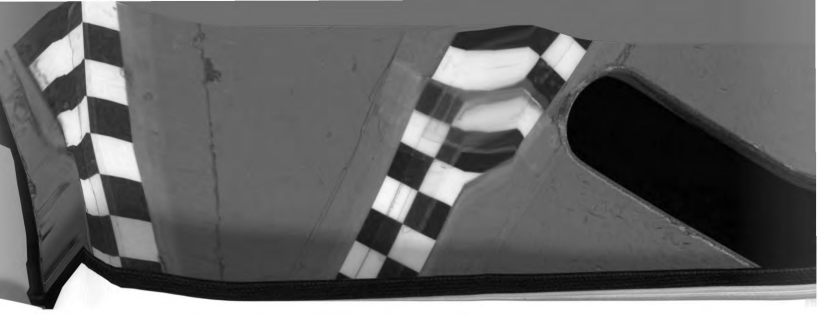






ABSTRACT

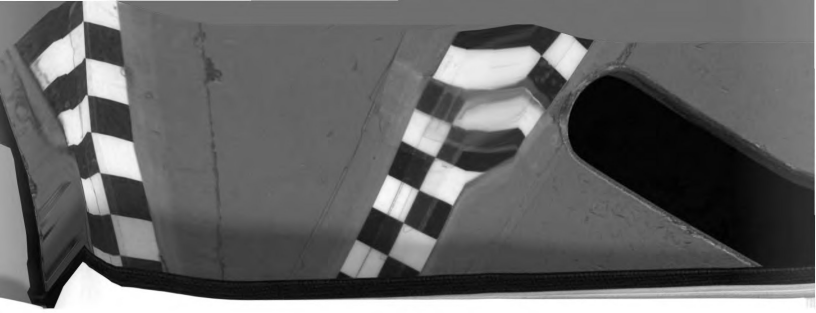
THE PREPARATION AND AMINO ACID SEQUENCE OF
CRYSTALLINE TURKEY EGG WHITE LYSOZYME

By

John Nelson LaRue

The primary and three-dimensional structures of chicken egg white lysozyme have been determined (1,2,3). X-ray crystallographic studies coupled with three-dimensional model building led to predictions which specified the amino acids involved in substrate binding and in the catalytic mechanism (3). It was desirable to examine the primary structure of a closely related avian lysozyme to determine whether or not any differences existed which might have conformational or mechanistic significance in relation to the findings and predictions made for chicken egg white lysozyme.

Crystalline and apparently homogeneous turkey egg white lysozyme was prepared by a simple procedure. The reduced and S-carboxymethylated (RCM) derivative of crystalline turkey egg white lysozyme was prepared. Amino acid analysis of this derivative showed there were a minimum of 6 amino acid differences between the chicken and turkey egg white lysozymes.



John Nelson LaRue

RCM-turkey lysozyme was digested by trypsin. Amino acid analysis of the purified tryptic peptides revealed there were actually 7 amino acid differences between the two lysozymes distributed over 6 tryptic RCM-peptides. A peptide analogue for each RCM-chicken egg white lysozyme tryptic peptide was isolated from the trypsin digest of RCM-turkey egg white lysozyme. This information coupled with sequence analysis of certain peptides disclosed the following differences in primary structure between the turkey and chicken lysozymes respectively: Tyr_3 for Phe_3; Leu_{15} for His_{15}; His_{41} for Gln_{41}; Lys_{73} for Arg_{73}; Ala_{99} for Val_{99}; Gly_{101} for Asp_{101}; His_{121} for Gln_{121}.

The presence of Gly_{101} in turkey egg white lysozyme eliminates the possibility that this residue is involved in hydrogen bonding to the substrate molecule as was predicted for Asp_{101} in chicken egg white lysozyme.

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

THE PREPARATION AND AMINO ACID SEQUENCE OF CRYSTALLINE TURKEY EGG WHITE LYSOZYME

By

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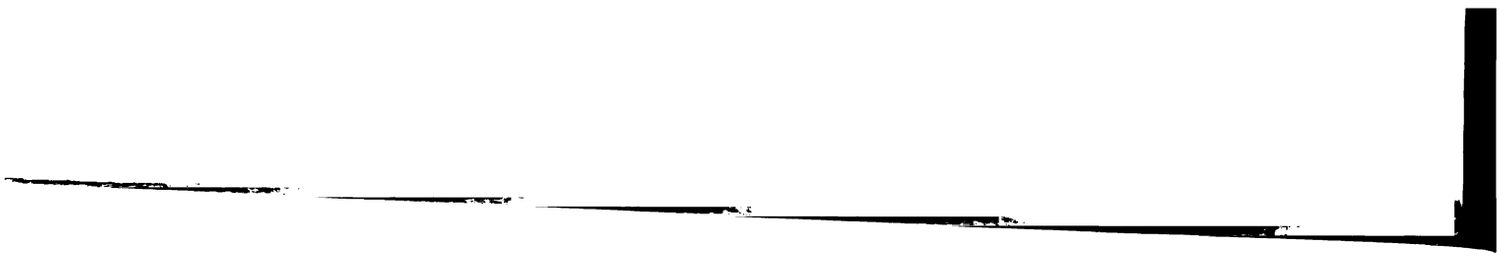

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INTRODUCTION

The term lysozyme has generally been applied to any enzyme capable of hydrolyzing intact bacterial cells regardless of the mechanism by which this lysis is effected. It has been suggested (1) that lysozymes should more correctly refer to that group of basic heat stable proteins which are capable of cleaving the glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine (Figure 1) as is found in bacterial cell walls. This definition would separate lysozymes from some of the other endoacetylmuramidases and all other bacteriolytic carbohydrases, as well as the various bacteriolytic peptidases and acetylmuramyl-L-alanine amidases.

Relatively few investigators have chosen to characterize the products produced upon lysis of bacterial cell walls by suspected lysozymes. Rather, they have simply assayed lytic activity. In this way lysozymes have been reported in phage infected E. Coli cells; various bacterial sources; in the invertebrate annelid, Nephthys hombergi; in different members of the blattid and acridid families of insects; in different plants; in fish as represented by the pike and sturgeon, and in many other invertebrate and vertebrate organs and secretions. Lysozymes are also found in human

Figure 1. A fragment of the peptidoglycan from M. lysodeikticus showing points of cleavage by various enzymes.

The abbreviations used are: NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid. The enzymes involved at the different sites of cleavage are: A, endo-acetylmuramidases (including lysozymes); B, endoacetylglucosaminidases; C, acetyl-muramyl-L-alanine amidases; and D, endopeptidases.

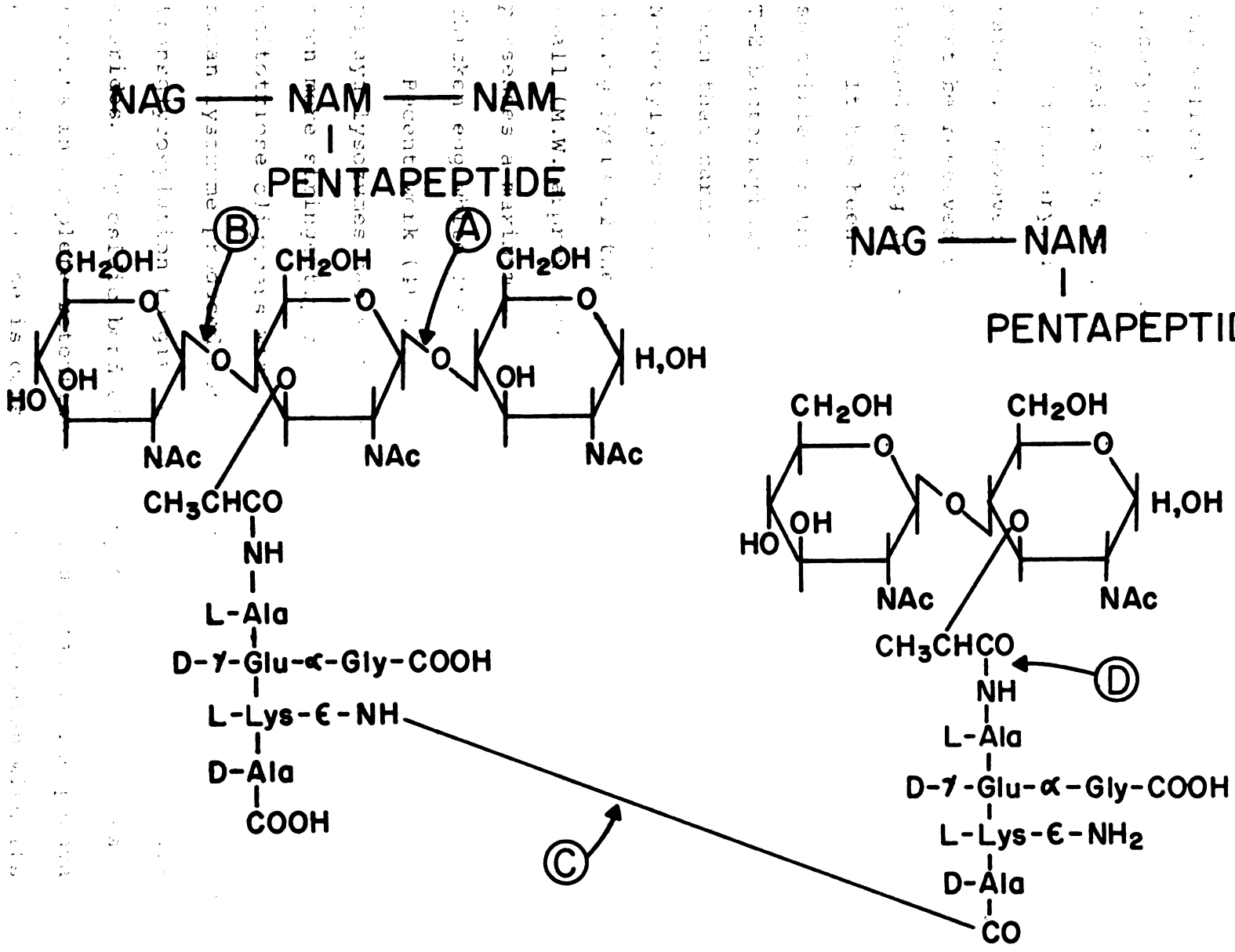
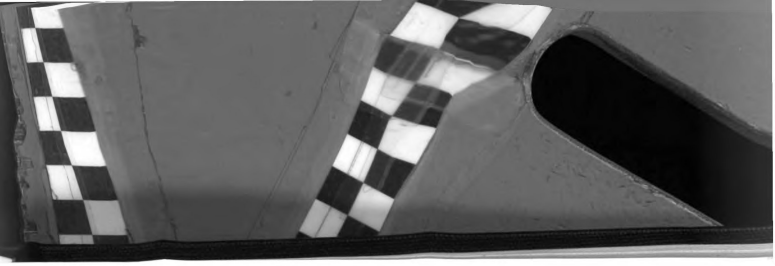

NAG — NAM — NAM
PENTAPEPTIDE
B
A
CH2OH
O
OH
HO
NAc
H,OH
CH3CHCO
NH
L-Ala
D-γ-Glu-α-Gly-COOH
L-Lys-ε-NH
D-Ala
COOH
C
NAG — NAM
PENTAPEPTIDE
CH3CHCO
D
NH
L-Ala
D-γ-Glu-α-Gly-COOH
L-Lys-ε-NH2
D-Ala
CO

Figure 1

NAG — NAM — NAG
PENTAPEPTIDE
NAG — NAM
PENTAPEPTIDE
A
B
C
D
NAM
NAG
L-Ala
D-Glu
L-Lys
D-Ala
D-Ala
Gly
Gly
Gly
Gly
Gly
NH
CO
HOOC
NH
CH3CHCO
CH2OH
HO
OH
O

Figure 1



organs, tissues, and secretions (spleen, kidney, milk, tears, and saliva). Human leucocytes are particularly abundant in lysozyme; however, the richest source of lysozyme to be discovered is the avian egg.

In summary, it appears that lysozymes are ubiquitous in nature. However, judgment concerning each particular lysozyme must be reserved until it is known precisely which bond is cleaved during lysis of the bacterial cell wall.

It has been shown, for instance, that the enzyme responsible for the lysis of E. Coli cells infected with the T-2 bacteriophage is a true lysozyme (2) i.e., by demonstration that carbon atom 1 of N-acetylmuramic acid (and not N-acetylglucosamine) becomes available as a reducing function during lysis of the bacterial cell wall. This enzyme is also small (M.W. approximately 14,000), alkali unstable, and possesses a maximum activity between pH 6 and 7 as does the chicken egg white lysozyme (3).

Recent work (4) with the chicken egg white, human, and papaya lysozymes has shown that lysozymes may have to be even more stringently defined. Treatment of chitobiose and chitotriose oligomers with chicken egg white lysozyme or human lysozyme produced not only hydrolysis but also extensive transglycosylation to give higher molecular weight polysaccharides. Glycosidic bond cleavage by these two lysozymes results in complete retention of configuration. The papaya endoacetylmuramidase is considered a lysozyme even with its

higher molecular weight (M.W. approximately 28,000). However, it does not appear to catalyze transglycosylation reactions and its cleavage of glycosidic bonds proceeds with inversion of configuration. Thus, lysozymes which exclusively catalyze the hydrolysis of β-1,4-N-acetylmuramic acid bonds in M. lysodeikticus cell walls may do so by entirely different mechanisms.

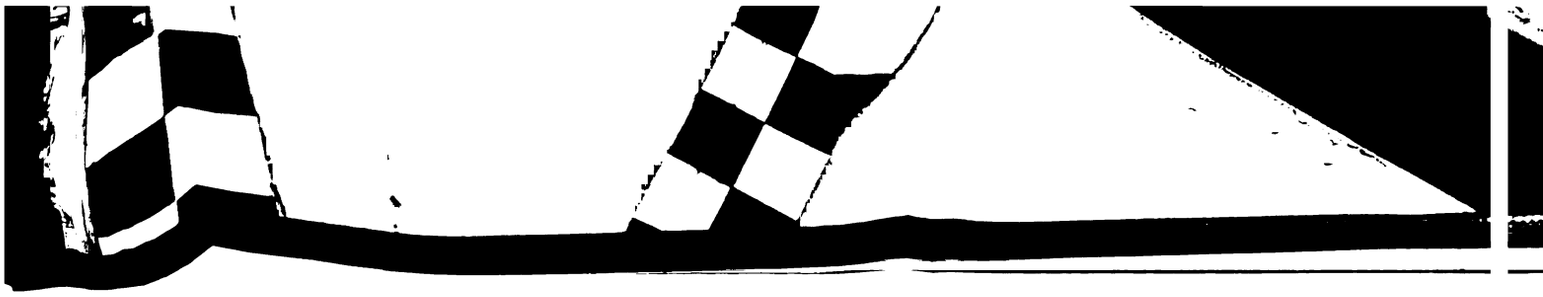
The differences observed in the catalytic mechanisms between the different lysozymes of similar specificity probably reflects the independent nature of their evolution. This is reinforced by the relatively few structural studies which have been carried out on lysozymes of diverse origins.

The primary structure of T-4 bacteriophage lysozyme has been determined (5). This lysozyme was shown to be a muramidase as was the T-2 phage lysozyme. Its specific activity, however, was much higher when assayed with E. Coli cells, its natural host, than with M. lysodeikticus cells (6). It is a basic protein as is the chicken egg white lysozyme and contains 164 amino acids (M.W. equals 18,134) in contrast to 129 for the chicken enzyme (M.W. equals 14,388). The T-4 phage lysozyme is more heat labile than the chicken lysozyme. This has been attributed to the absence of cystine bridges in the phage lysozyme (two cysteine residues are present) whereas the chicken molecule has four disulfide bonds. The results of the sequence determination showed there was no common primary structure between these two lysozymes.

The terminal sequences of the papaya lysozyme have recently been elucidated (7). This proved that the enzyme was actually a single polypeptide chain whose molecular weight was approximately 28,000. Certainly the primary structure of the papaya lysozyme will prove to be very different from the chicken lysozyme whose molecular weight is 14,388.

It is the chicken lysozyme which is most familiar and about which the most is known concerning its structure. The primary structure has been determined independently by Jolles (8,9) and Canfield (10,11). The three-dimensional structure was established by x-ray crystallographic analysis to 2-angstrom resolution (12) and supports Canfield's primary structure determination rather than Jolles'. The 2-angstrom level of resolution does not show individual atoms as separate maxima. However, the polypeptide backbone appears as a "continuous ribbon of electron density" and many amino acid side chains were easily identified; e.g., the four disulfide bridges and six tryptophan residues. When this information was coupled with the primary structure knowledge, Phillips was able to construct a three-dimensional model of the molecule.

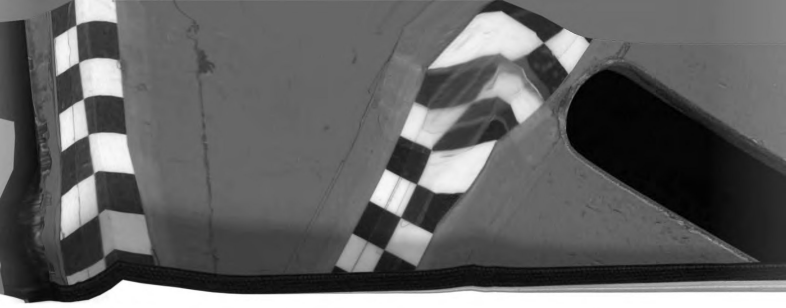
The molecule appears ellipsoidal in nature with approximate dimensions of 45 x 30 x 30 angstroms. Forty-six amino acid residues of 129 total contribute to continuous helical regions of four or more residues. The alpha helix content



is thus 50 per cent lower than found in myoglobin. For the most part, the acidic and basic side chains are distributed over the surface of the molecule while most of the hydrophobic side chains are on the interior of the molecule.

A closer examination reveals that residues 1-40 contain two alpha helical regions (residues 5-15 and 24-34) which fold back on each other burying several hydrophobic residues and in so doing forming one wing of the molecule. Residues 41-45 and 50-54 form an antiparallel pleated sheet structure, enabling the hydrophobic residues 55 and 56 to be buried in the existing hydrophobic pocket. The other wing of the lysozyme molecule is formed by residues 56-86 folding irregularly around the pleated sheet structure. The two wings lie at an angle to each other and the gap between then is partially filled by an irregular helix (residues 88-100) which acts as a hydrophobic backbone. Because the helix only partially fills the gap, the lysozyme molecule is left with a deep cleft on its surface which Phillips has implicated in substrate binding and as the catalytic site.

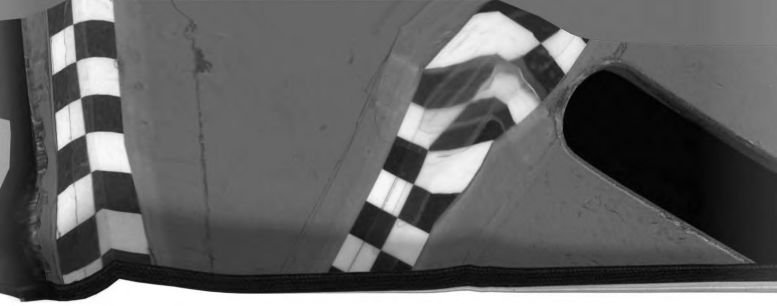
Phillips was able to form a stable N-acetylchitotriose-lysozyme crystal complex. Because of the stability of the complex, Phillips postulated that it represented a partial enzyme-substrate complex in which only a binding function of the enzyme was revealed. X-ray examination of the complex disclosed the possibility of favorable nonpolar interactions and hydrogen bond formation between lysozyme and its



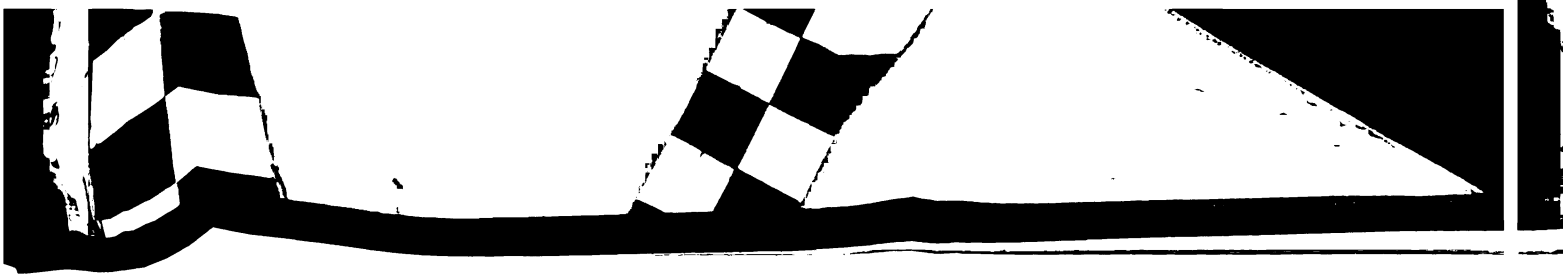
substrate (residues A, B, and C in Figure 11). Through model building, Phillips also demonstrated that the cleft could accommodate a hexasaccharide substrate molecule. The spatial requirements of the model showed that only residues B, D, and F of the substrate could be N-acetylmuramic acid. The proximity of Glu_{35} and Asp_{52} to the glycosidic bond ruptured between residue D and E of the substrate prompted Phillips to propose a cleavage mechanism in which Glu_{35} protonates the glycosidic oxygen and a negatively charged Asp_{52} stabilizes an intermediate carbonium ion at C-1 of substrate residue D.

Support for the involvement of Glu_{35} and Asp_{52} in the catalytic mechanism of lysozyme action has recently come from two sources. By the use of a carboxyl group modification procedure (13) which converts free carboxyl groups to substituted amides, Lin and Koshland (14) were able to show that only these two acidic residues could be essential for catalytic activity. Furthermore, the blocking of Asp_{52} resulted in a concomitant loss of enzyme activity. Similar results were obtained by Parsons et al. (15) who were able to isolate a mono-esterified lysozyme which was able to bind trisaccharide but was catalytically inactive against M. lysodeikticus cells. Unfortunately Parsons and his co-workers have not yet identified the esterified residue.

Although a simplification of Phillips mechanism has been presented, it is apparent from above that some specific amino acid residues are essential for its operation. It is



with this in mind, that we chose to examine the primary structure of a closely related avian enzyme. Using sequence analysis as a tool, we hoped to determine whether or not any differences between the two enzymes existed which might have conformational or mechanistic significance in light of Phillips findings and predictions.



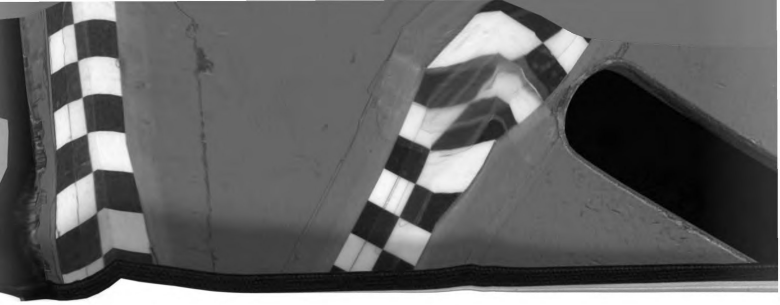
EXPERIMENTAL

Reagents

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

1. beta-Alanine
   General Biochemicals.
2. DL-Aspartic acid, A grade
   California Corporation for Biochemical Research.
3. Benzene, thiophene free
   Mallinckrodt Chemical Works.
4. Bio-Gel P-2, 200 to 400 mesh (wet)
   BioRad Laboratories.
5. Bromine, technical grade
   Dow Chemical Company.
6. n-Butanol
   Mallinckrodt Chemical Works.
7. Carboxymethyl cellulose (Cellex-CM)
   BioRad Laboratories.
8. Carboxypeptidase A, DFP treated
   Worthington Biochemical Corporation.
9. Carboxypeptidase B, DFP treated
   Worthington Biochemical Corporation.

10. alpha-Chymotrypsin, salt free crystalline, A grade
    California Corporation for Biochemical Research.
11. Citric acid, monohydrate
    Mallinckrodt Chemical Works.
12. Cyanogen bromide
    Aldrich Chemical Company.
13. Dialysis tubing, Visking sausage casing (18/32 inch)
    Union Carbide Corporation.
14. p-Dimethylaminobenzaldehyde
    Matheson, Coleman and Bell.
15. Dowex (Aminex) AG 50W-X2, 200 to 325 mesh (wet)
    Dowex AG 50W-X2, 50 to 100 mesh (wet)
    Dowex AG 1-X8, 200 to 400 mesh (wet)
    Dowex 1-X2, 200 to 400 mesh (wet)
    BioRad Laboratories.
16. Ethylenediaminetetraacetic acid, disodium salt
    Sigma Chemical Company.
17. Ethylene dichloride (1,2-dichloroethane)
    Aldrich Chemical Company.
18. N-ethylmorpholine
    Aldrich Chemical Company reagent grade was redistilled through a Vigreaux column; the fraction boiling at 136-137° was collected.
19. L-Glutamic acid, A grade
    California Corporation for Biochemical Research.



20. Iodoacetic acid

    Eastman Organic Chemicals.

21. iso-Amyl alcohol

    Mallinckrodt Chemical Works.

22. Leucine aminopeptidase

    Worthington Biochemical Corporation.

23. Lysozyme, chicken egg white, twice crystallized

    Worthington Biochemical Corporation.

24. 2-Mercaptoethanol, redistilled

    California Corporation for Biochemical Research.

25. Methanol, anhydrous

    Matheson, Coleman and Bell.

26. Methyl Cellosolve (ethylene glycol monomethyl ether)

    Fisher Scientific Company.

27. alpha-Naphthol

    California Corporation for Biochemical Research.

28. Ninhydrin

    Pierce Chemical Company.

29. Papain, twice crystallized suspension

    Worthington Biochemical Corporation.

30. Phenyl isothiocyanate

    Matheson, Coleman and Bell vacumn redistilled through a Vigreaux column; the fraction boiling at 99° at 14 mm pressure was collected.

31. 2-Picoline

    Aldrich Chemical Company reagent grade redistilled

through a Vigreaux column, the fraction boiling at 127° was collected.

32. Polyacrylamide disc gel electrophoresis reagents

    Canal Industrial Corporation.

33. Pyridine

    J. T. Baker Company.

34. Sephadex G-10

    Pharmacia Fine Chemicals, Incorporated.

35. DL-Serine, A grade

    California Corporation for Biochemical Research.

36. Sulfanilic acid

    Matheson, Coleman and Bell.

37. Trifluoroacetic acid

    Aldrich reagent grade; exhaustively oxidized at refluxing temperature with solid CrO_3, distilled off, dried over Na_2SO_4, decanted, and redistilled through a Vigreaux column from fresh Na_2SO_4. The fraction boiling at 65° was collected. The reagent was stored in a desiccator over sodium hydroxide pellets (16).

38. Trimethylamine (anhydrous)

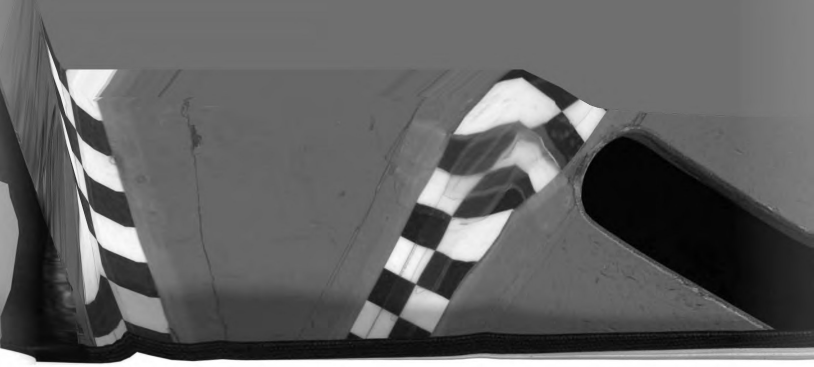
    Eastman Organic Chemicals.

39. Tris(hydroxy methyl)aminomethane

    Sigma Chemical Company.

40. L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)

    California Corporation for Biochemical Research.



41. Trypsin, salt free and twice crystallized
    Worthington Biochemical Corporation.
42. DL-Tryptophan, A grade
    California Corporation for Biochemical Research.
43. Urea
    Mallinckrodt Chemical Works reagent grade; urea solutions were deionized with Amberlite MB-3 mixed bed resin, filtered, concentrated in a rotary evaporator, washed with ethanol and then ether and finally dried in a vacumn desiccator attached to a water aspirator.

Preparation of Turkey Egg White Lysozyme

Preparation of the crude enzyme

A modification of a procedure used for the purification of goose egg white lysozyme was employed (17). A convenient preparation started with 10 turkey egg whites which were separated from the yolk by decantation. The egg whites were suspended in two volumes of 0.05 M NaH_2PO_4 to which CM-cellulose was added (6 to 12 g per ml) with constant stirring. The mixture was stirred at 5° for 18 to 24 hours after which the CM-cellulose was centrifuged. The pellet was washed four times with 150-ml portions of 0.05 M NH_4HCO_3 by alternately mixing and centrifuging. Determination of the residual lysozyme activity of the supernatant established that the adsorption of lysozyme to CM-cellulose had been

quantitative. In addition, no evidence of lysozyme activity was found in the 0.05 M NH_4HCO_3 washings. Therefore both the supernatant and washings were discarded.

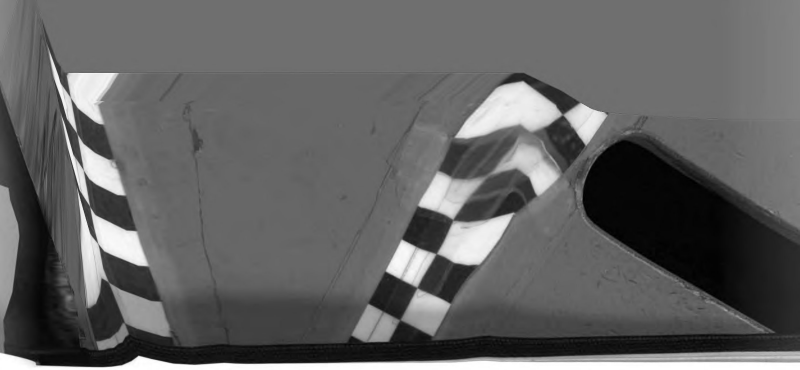
The lysozyme activity was eluted from the CM-cellulose by alternately stirring and centrifuging with three 50-ml aliquots of 0.4 M $(NH_4)_2CO_3$. The eluates were combined, filtered, and lyophilized. To insure that all of the $(NH_4)_2CO_3$ was removed, the lyophilized product was dissolved in 100 ml of redistilled water, adjusted to pH 5.5 with 1 N HCl and re-lyophilized.

Crystallization of turkey egg white lysozyme

The re-lyophilized powder was dissolved in water (50 mg per ml) and adjusted to pH 5.5 with 1 N HCl. The resultant solution was made 5 per cent in NaCl and the pH was readjusted to 8.5 with 1 N NaOH. After standing overnight at 5°, the crystals began to separate. Stirring the solution caused it to become cloudy and crystallization was complete within an hour. Recrystallization was accomplished by centrifuging the crystals, removing the supernatant and repeating the above procedure.

End point assay of lysozyme activity

Lysozyme activity was determined by measuring the decrease in turbidity of a suspension of M. lysodeikticus cells at 550 mμ. Dried M. lysodeikticus cells (13 to 15 mg) were suspended in a solution containing 90 ml of 0.067 M sodium



phosphate buffer, pH 6.8 and 10 ml of 1 per cent NaCl. This gave an initial absorbance of 0.5 to 0.6 at 550 mμ. The assay mixture contained 2.9 ml of the bacterial suspension and 0.1 ml of lysozyme solution. One unit of lysozyme activity was defined as that amount of enzyme which causes a decrease in turbidity of 1 absorbance unit in the time interval between t equals 30 and t equals 180 seconds. This assay was linear in the range of 0.01 to 0.05 absorbance units per time interval.

Initial rate assay of lysozyme activity

This assay was used to compare the lytic activities of chicken and turkey egg white lysozymes. In order to measure precisely the small changes in absorbance observed in the linear range, a Gilford Model 2000 automatic recording spectrophotometer was used. The initial rates of change in absorbance at 550 mμ were determined at 25°. The assay mixture contained 290 μl of the previously described bacterial suspension and 1 to 10 μl of enzyme solution.

Protein determination by the Lowry method

The procedure of Lowry et al. (18) was followed exactly as described. A standard protein curve was prepared from chicken egg lysozyme and is shown in Figure 2.

CM-cellulose column chromatography of crystalline turkey egg white lysozyme

A solution of 200 mg of twice-crystallized turkey egg white lysozyme in 10 ml of 0.05 M $(NH_4)_2CO_3$ was applied to

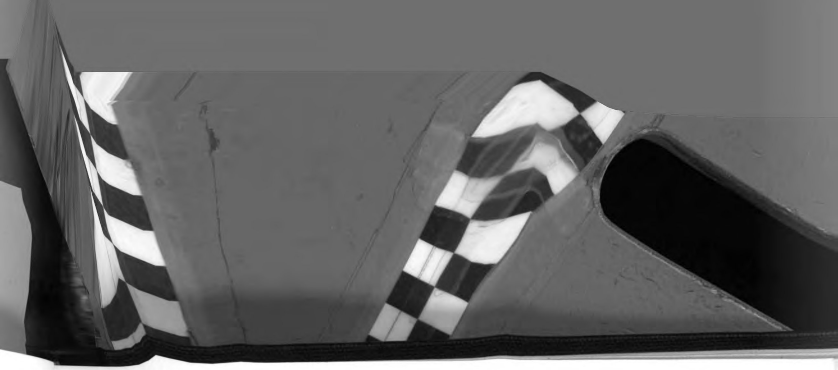


Figure 2. Preparation of a standard curve for the Lowry protein determination (18).

The following reagents were prepared:

1. Reagent A, 2 per cent Na_2CO_3 in 0.10 N NaOH
2. Reagent B, 0.5 per cent $CuSO_4 \cdot 5\ H_2O$ in 1 per cent sodium tartrate
3. Reagent C, alkaline copper solution. Mix 50 ml of Reagent A with 1 ml of Reagent B.
4. Reagent D, Folin-Ciocalteu phenol reagent diluted 1:1 with water

To known amounts of chicken egg white lysozyme (25, 50, 100, 200 μgrams) contained in 1 ml of redistilled water were added 5-ml aliquots of reagent C. After mixing, the samples were allowed to stand for 10 minutes at room temperature. 1.0 ml of reagent E was added rapidly to each tube and mixed quickly. After 30 minutes the samples were read at 550 millimicrons against a blank which contained redistilled water in place of the protein solution.

a 2 x 22 cm column of CM-cellulose previously equilibrated with 0.05 M $(NH_4)_2CO_3$. The column was eluted with a linear gradient delivered from a two-chambered gradient system by means of a positive-displacement piston pump (Milton Roy Co., St. Petersburg, Florida). The mixing chamber contained 500 ml of 0.05 M $(NH_4)_2CO_3$ and the reservoir 500 ml of 0.4 M $(NH_4)_2CO_3$. Fractions of 5 ml were collected at a constant flow rate of 30 ml per hr. The absorbance of the column eluate was monitored at 280 mμ by means of a Beckman [illegible] spectrophotometer attached to a Sargent M Rec SRL [illegible].

Disc electrophoresis on polyacrylamide gels

The procedure of Reisfeld et al. (19) was followed. This is a modification of the original procedure which employs a pH 4.5 [illegible] buffer [illegible] for the separation of basic [illegible] peptide. The gels contained 40 to [illegible] was carried out by applying [illegible] for 45 minutes.

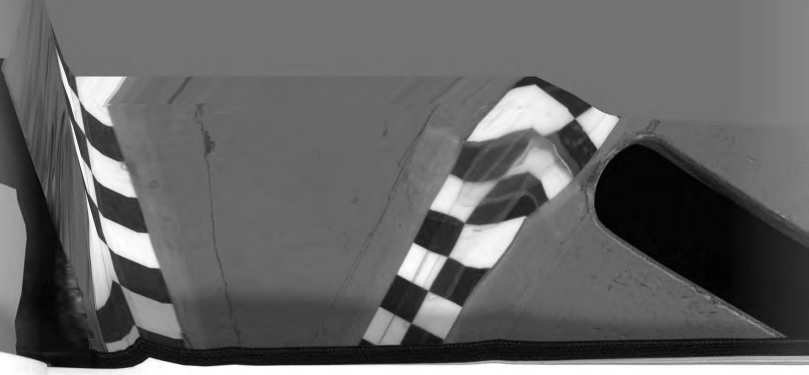
Preparation of [illegible] Turkey E. [illegible]

The reduced and S-[illegible] tive was prepared by a [illegible] by Crestfield, Moore, and Stein (20). The reaction was carried out in Pyrex-[illegible] Rutherford, New Jersey). A 2-[illegible] flask was

Figure 2

Figure 5

micrograms of protein/ml
30
60
90
150
120
180
210
A220
0.60
0.48
0.36
0.24
0.12



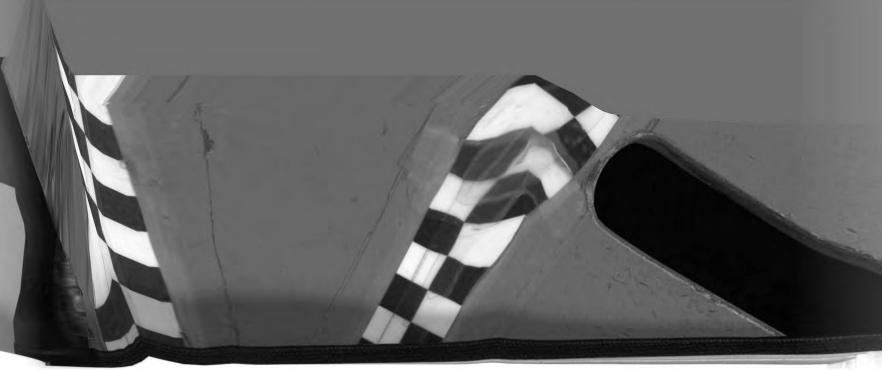
a 2 x 22 cm column of CM-cellulose previously equilibrated with 0.05 M $(NH_4)_2CO_3$. The column was eluted with a linear gradient delivered from a two-chambered gradient system by means of a positive-displacement piston pump (Milton Roy Co., St. Petersburg, Florida). The mixing chamber contained 500 ml of 0.05 M $(NH_4)_2CO_3$ and the reservoir 500 ml of 0.4 M $(NH_4)_2CO_3$. Fractions of 5 ml were collected at a constant flow rate of 30 ml per hr. The absorbance of the column eluate was monitered at 280 mμ by means of a Beckman DB spectrophotometer attached to a Sargent Model SRL recorder.

Disc electrophoresis on polyacrylamide gel

The procedure of Reisfeld et al. (19) was followed. This is a modification of the original procedure which employs a pH 4.5 β-alanine-acetate buffer and makes possible the separation of basic proteins and peptides. The sample gels contained 40 to 500 μg of protein and electrophoresis was carried out by applying a current of 8 mamp per tube for 45 minutes.

Preparation of Reduced and S-Carboxymethylated Turkey Egg White Lysozyme

The reduced and S-carboxymethylated lysozyme derivative was prepared by a modification of the method described by Crestfield, Moore, and Stein (20). The reaction was carried out in Bantam-ware apparatus (Kontes Glass Co., Vineland, New Jersey). A 25 ml round-bottomed flask was



equipped with a Claisen adapter having two 14/20 outer joints. An ebullition tube was inserted through a Teflon adapter in the center joint to the bottom of the flask and was connected to a pre-purified nitrogen source. An outlet tube was placed in the second joint through a Teflon adapter.

Twice crystallized turkey egg white lysozyme (100 mg) was dissolved in a solution containing 3.61 g of deionized urea, 0.3 ml of EDTA solution (50 mg of disodium EDTA per ml), and 3.0 ml of Tris buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1 N HCl diluted to 30 ml with water). The solution volume was made up to 7.5 ml with water and a solution of 8 M urea containing 0.2 per cent EDTA was added to make the final volume 12 ml. The system was flushed gently with nitrogen for 15 minutes before and after the addition of 0.1 ml of redistilled mercaptoethanol; after flushing, the system was closed. The reduction was carried out for 4 hours at room temperature.

At this time a freshly prepared solution of 0.268 g of iodoacetic acid in 1.0 ml of 1 N NaOH was added to the reaction mixture. After 15 minutes at room temperature, in the absence of light, the alkylation mixture was rapidly transferred to 18/32 inch cellulose dialysis tubing and dialyzed against deionized water--also in the dark. Urea and buffer salts were thus removed from the modified protein. The precipitation of RCM[1]-lysozyme began after 15

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[1]The abbreviation used is: RCM, reduced and S-carboxymethylated.

minutes and was usually complete within one hour. After dialysis, the white, precipitated protein was centrifuged in a clinical centrifuge and the pellet was washed three times with 8-ml portions of redistilled water by suspension and centrifugation. The washed protein was then lyophilized. Subsequent amino acid analysis of the RCM-lysozyme indicated that the reduction and S-carboxymethylation had been complete as no cystine could be detected.

Preparation of Trypsin Free from Chymotrypsin Activity

It is possible to inactivate chymotrypsin which is present in trypsin preparations by specifically labeling its active center with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK). This treatment has no effect on trypsin. The procedure of Kostka and Carpenter was followed (21).

Trypsin (100 mg, 4 μmole) was dissolved in 33 ml of 0.001 M $CaCl_2$. To this solution was added 28.4 mg of TPCK dissolved in 0.75 ml of anhydrous methanol. The reaction mixture was titrated to pH 7.0 with 0.5 N NaOH and maintained at that pH for five hours by the automatic addition of 0.5 N NaOH from a pH-stat (Titrator TTT1, Radiometer, Copenhagen). The reaction mixture was adjusted to pH 3.0 with 1 N HCl and excess inhibitor which had precipitated was removed by centrifugation. The filtrate was transferred to 18/32 inch cellulose tubing and dialyzed at 5° against redistilled water. The dialyzed solution was lyophilized and the TPCK-treated trypsin was stored at 5° until use.

Trypsin Digestion of RCM-Turkey Egg White Lysozyme

A finely divided suspension of RCM-turkey lysozyme was produced by briefly sonicating a 1 per cent mixture of the enzyme in redistilled water. The pH of the suspension was adjusted to 8.0 with 0.197 N NaOH. TPCK-treated trypsin was added in an amount equal to 2 per cent of the lysozyme by weight. The pH of the digestion mixture was maintained at 8.0 by the automatic addition of 0.197 N NaOH dispensed from a pH-stat. After digestion at room temperature for 3 hours, the uptake of sodium hydroxide essentially stopped. The addition of more trypsin did not cause consumption of sodium hydroxide, indicating the digestion was finished. A small amount of undigested insoluble material (approximately 1 per cent) remained at the end of digestion and was removed by centrifugation. A similar observation was reported by Canfield in his work with chicken egg white lysozyme (22).

The uptake of sodium hydroxide at the end of the digestion was 50 per cent of the theoretical amount if calculated on the basis of one hydroxide ion per peptide bond cleaved. It is apparent that the pK_a values of the unmasked amino and carboxyl functions of the liberated peptides must be considered in calculating the true theoretical uptake of hydroxide ion.

Amino Acid Analysis of Proteins and Peptides

Preparation of samples for amino acid analysis

Acid hydrolysates of peptides or proteins were prepared by the addition of 2 ml of constant boiling HCl to the sample contained in a small ampule prepared from a drawn-out 18 x 150 mm Pyrex test tube. The solution was cooled in an acetone-dry ice bath and then subjected to repeated evacuations (water pump pressure) and flushings with pre-purified nitrogen. In this manner the dissolved oxygen was removed. After flushing with nitrogen, the tube was sealed under vacumn. Hydrolysis was carried out at 105° in a constant temperature oil bath.

Proteins were hydrolyzed for 24, 48, or 72 hours and peptides, in general, were hydrolyzed from 20 to 24 hours. In some instances, in an attempt to minimize tryptophan degradation, peptides were hydrolyzed only 12 hours. Immediately after hydrolysis the sample was taken to dryness on a rotary evaporator and then dissolved in pH 2.2 sample diluting buffer.

When enzyme digests were analyzed, the mixtures were lyophilized and then dissolved in pH 2.2 sample diluting buffer. Due to the presence of non-volatile buffer salts, it was necessary to adjust the pH of the samples to pH 2.2 before applying them to the amino acid analyzer column.

Amino acid analysis

All amino acid analyses were carried out according to the method of Spackman, Stein, and Moore (23) using a Spinco Model 120 amino acid analyzer.

Tryptophan determinations on intact protein

The method of Spies and Chambers (24) was followed for this determination. An aliquot from a stock solution of turkey egg lysozyme, estimated to contain approximately 100 μg of tryptophan was lyophilized. The concentration of lysozyme present in the stock solution was determined by amino acid analysis of a suitable aliquot. The dried protein was dissolved in 10 ml of 19 N H_2SO_4 which contained 30 mg of dissolved p-dimethylaminobenzaldehyde. The resulting solution was allowed to stand at room temperature for 12 hours at which time 0.1 ml of 0.045 per cent $NaNO_2$ was added. After the color had developed for 30 minutes, the absorbance at 590 mμ was determined in a Beckman Model B spectrophotometer. The blank solution contained 10 ml of 19 N H_2SO_4 and 0.1 ml of 0.045 per cent $NaNO_2$. A standard curve was prepared from free tryptophan and is shown in Figure 3.

Peptide Column Chromatography

Preparation of resins

Dowex (Aminex) AG 50W-X2 (200 to 325 mesh) resin was prepared for column chromatography by thoroughly washing

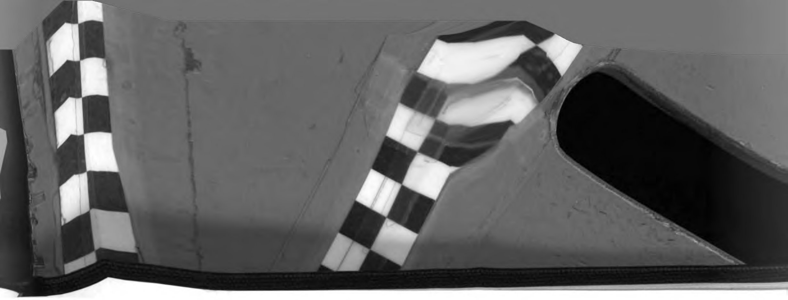


Figure 3. Standard Curve for tryptophan determinations according to the method of Spies and Chambers (24).

Samples of tryptophan (50, 72, 100 μgrams) were dissolved in 10 ml of 19 N H_2SO_4 which contained 30 mg of dissolved p-dimethylaminobenzaldehyde. After standing at room temperature for 12 hours, 0.1 ml of 0.045 per cent $NaNO_2$ was added to each sample. Color development was allowed to proceed for 30 minutes and the absorbance at 590 mμ was determined. The blank solution contained 10 ml of 19 N H_2SO_4 and 0.1 ml of 0.045 per cent $NaNO_2$.

on a sintered glass funnel with 1 M NaOH, followed by 5 M HCl, and then 2 M pyridine to establish the pyridinium ion as the cation. The resin was then equilibrated for use with 0.2 M pyridine-acetic acid buffer, pH 5.. (a).

Dowex AG 50W-X2 (400 to 200 mesh) was prepared for purification of residual peptides in the Edman degradation by washing with 1 N NaOH, followed by 1 M HCl and finally redistilled water. The resin was used in the hydrogen form.

Dowex 1-X2 (200 to 400 mesh) or AG 1-X8 (200 to 400 mesh) resins were prepared for column chromatography by washing on a sintered glass funnel with water at 60°, followed by 0.5 N NaOH, water, 1 N HCl, and finally water. The resin was stored wet in the chloride form until [illegible]. Before packing a column the resin was washed in the [illegible] with water, 0.5 N [illegible] acetic [illegible]. The resin was then [illegible]

Packing of ion exchange [illegible]

The jacketed ion [illegible] sections at the operating [illegible] quantity of resin slurry [illegible] was then carefully filled [illegible] section was packed by [illegible] the column at a reasonable flow rate. After the excess buffer, another section could be packed in a similar fashion. It was especially important to pack the Dowex 1 columns as quickly as possible to prevent formation of air bubbles.

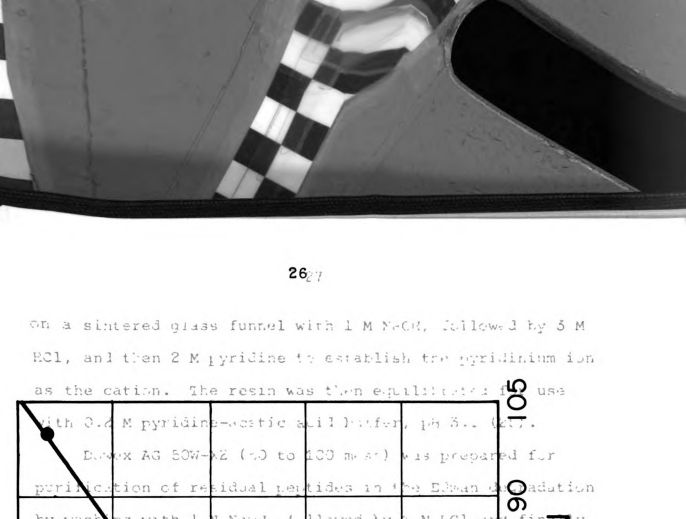
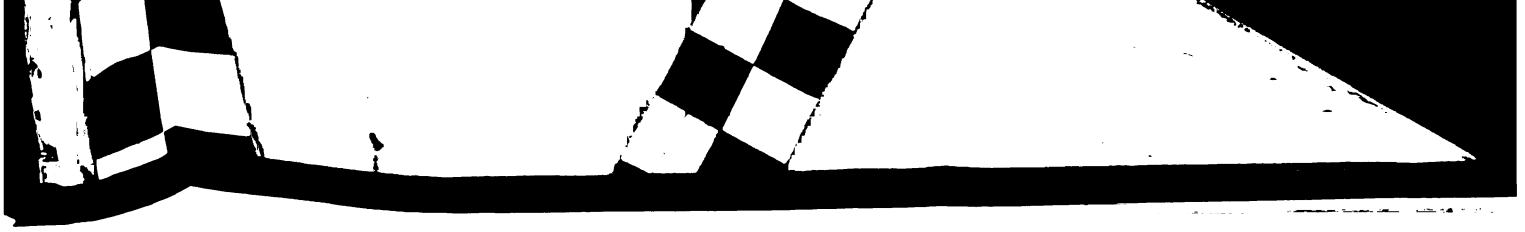


Figure 3

Figure 3



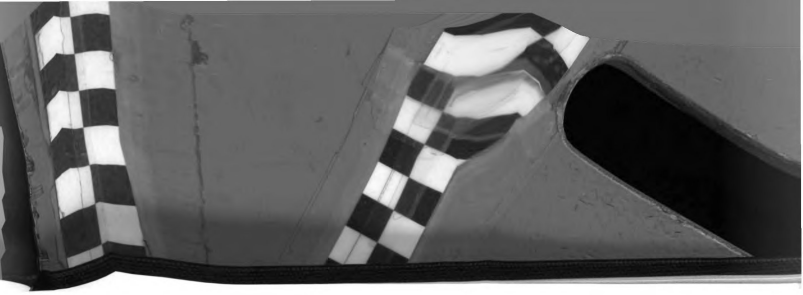
on a sintered glass funnel with 1 M NaOH, followed by 3 M HCl, and then 2 M pyridine to establish the pyridinium ion as the cation. The resin was then equilibrated for use with 0.2 M pyridine-acetic acid buffer, pH 3.1 (25).

Dowex AG 50W-X2 (50 to 100 mesh) was prepared for purification of residual peptides in the Edman degradation by washing with 1 N NaOH, followed by 1 M HCl and finally redistilled water. The resin was used in the hydrogen form.

Dowex 1-X2 (200 to 400 mesh) or AG 1-X8 (200 to 400 mesh) resins were prepared for column chromatography by washing on a sintered glass funnel with water at 60°, followed by 0.5 N NaOH, water, 1 N HCl, and finally water. The resin was stored wet in the chloride form until needed. Before packing a column, the resin was washed in this order with water, 0.5 N NaOH, water, 1 N acetic acid, and water. The resin was then equilibrated with pH 9.4 buffer (26).

Packing of ion exchange resin columns

The jacketed ion exchange columns were poured in sections at the operating column temperature. A suitable quantity of resin slurry was poured in the column which was then carefully filled with the initial buffer. The section was packed by pumping starting buffer through the column at a reasonable flow rate. After removal of the excess buffer, another section could be packed in a similar fashion. It was especially important to pack the Dowex 1 columns as quickly as possible to prevent formation of air bubbles.



Description of buffers used in the elution of peptides from ion exchange columns

Gradient elution from Aminex AG 50W-X2 employed two pyridine-acetic acid volatile buffers (25).

First buffer: pH 3.1, 0.2 M in pyridine (64.5 ml pyridine and 1114 ml of glacial acetic acid diluted to a volume of 4 liters).

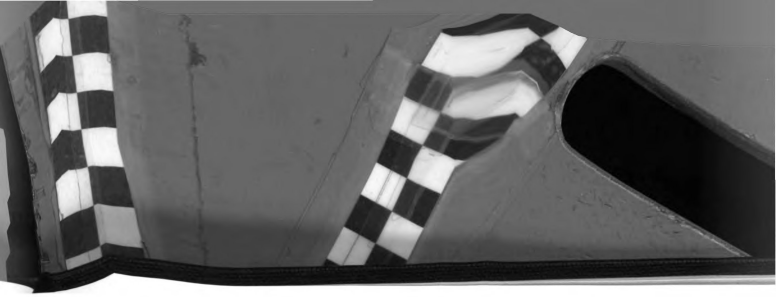
Second buffer: pH 5.0, 2 M in pyridine (645 ml pyridine and 573 ml of glacial acetic acid diluted to a volume of 4 liters).

Dowex 1-X2 or AG 1-X8 peptide columns were eluted with an N-ethylmorpholine, α-picoline, pyridine, acetic acid system of buffers (26).

First buffer: pH 9.4 (60 ml N-ethylmorpholine, 80 ml α-picoline, 40 ml pyridine, and approximately 0.5 ml glacial acetic acid to give a pH of 9.4 when diluted to 4 liters with water). Carbon dioxide was removed from the redistilled water used in preparing both the pH 9.4 and 8.4 buffers by bubbling with nitrogen. Both of these buffers were protected from the air by means of an Ascarite tube attached to the dispensing bottle.

Second buffer: pH 8.4 (60 ml N-ethylmorpholine, 80 ml α-picoline, 40 ml pyridine, and about 3 ml of acetic acid diluted to 4 liters).

Third buffer: pH 6.5 (60 ml N-ethylmorpholine, 80 ml α-picoline, 40 ml pyridine and approximately 37 ml of acetic acid diluted to 4 liters).



Fourth solution: acetic acid, 0.5 N.

Fifth solution: acetic acid, 2.0 N.

Description of systems used in column chromatography on ion exchange resins

In general, eluting buffer, from a two-chambered gradient system was delivered by means of a positive-displacement piston pump (Milton Roy Co., St. Petersburg, Florida) to a column equipped with a water jacket. The column was maintained at a constant temperature (usually 40°) by attaching the jacket to a circulating water bath. Suitable fractions were collected in a fraction collector. A description of the gradients and flow rates employed for the various resins and columns is shown in Table 1.

Detection of peptides eluted from column chromatography on ion exchange resins

Alkaline hydrolysis: A suitable aliquot (usually 0.05 to 0.2 ml) was removed from each or every other fraction and carefully pipetted into the bottom of polypropylene test tubes (16 x 100 mm). The samples were evaporated to dryness in a ventilated oven at 110° in the hood. The polypropylene tubes melt if heated at higher temperatures. Each tube received 0.15 ml of 13.5 N NaOH (71.5 ml of 50 per cent NaOH solution diluted to 100 ml with water) and was then covered with a Kap-ut (Bellco) culture tube cap. Alkaline hydrolysis of the samples was carried out in the autoclave with steam at 15 psi for 20 minutes. After hydrolysis, 0.25

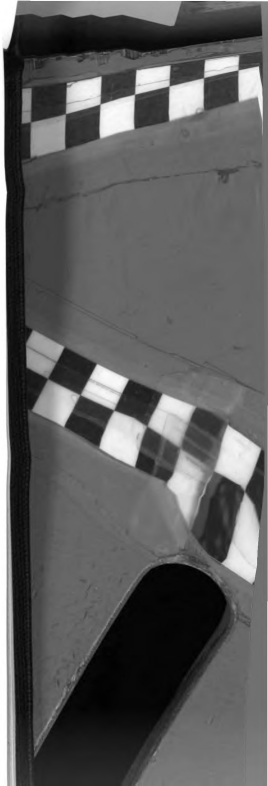
TABLE 1. PARAMETERS OF ION EXCHANGE COLUMN CHROMATOGRAPHY

Aminex AG 50W-X2 Column Chromatography

Column Dimensions (cm)	Flow Rate (ml/hr)	Type of Gradient	Diameter of Mixing Chamber	Diameter of Reservoir	Developer Volume (ml) pH 3.1 Buffer before Gradient	pH 3.1 Buffer in Mixer	pH 5.0 Buffer in Reservoir
2.0 x 150	60	Linear	D	D	500	3500	3500
0.9 x 100	20	Linear	D	D	100	900	900
0.5 x 50	10	Convex	D	1.4 D	25	80	160

Dowex 1-X2 Or AG 1-X8 Column Chromatography

Column Dimensions (cm)	Flow Rate (ml/hr)	Type of Gradient	Volume of Mixer (ml)	Developer Volume (ml) Buffer pH 9.4	Buffer pH 8.4	Buffer pH 6.5	Acetic Acid 0.5 N	Acetic Acid 2.0 N
0.5 x 70	10	Constant Volume	40	10	30	40	60	100
0.9 x 100	20	Constant Volume	135	40	120	160	240	400



ml of glacial acetic acid and was added to each tube for neutralizing the alkali (27)

Reaction of hydrolyzed peptides with ninhydrin

The base-hydrolyzed samples were reacted with ninhydrin according to the method of Yemm and Cocking (28). The simplicity of this procedure and stability of the reagents are its main advantages.

Reagents

Citrate buffer: pH 5.0, 0.2 M (21.0 g of citric acid monohydrate and 16.0 g of 50 per cent NaOH were dissolved and diluted to 500 ml with redistilled water). The buffer was stored at 5^{o} with a crystal of thymol.

Potassium cyanide solution: 0.01 M (0.1628 g of potassium cyanide was dissolved in 250 ml of redistilled water). This solution is stable for 3 months at room temperature.

Ethanol solution: 60 per cent by volume.

Potassium cyanide-methyl cellosolve solution: (5 ml of 0.01 M potassium cyanide was diluted to 250 ml with methyl cellosolve).

Methyl cellosolve-ninhydrin solution: 5 per cent weight per volume solution of ninhydrin in methyl cellosolve. This solution is stable for 6 months at room temperature.

Potassium cyanide-methyl cellosolve-ninhydrin solution: (50 ml of the methyl cellosolve-ninhydrin solution was mixed with 250 ml of the potassium cyanide-methyl cellosolve

solution). This reagent is stable for one week at room temperature.

Procedure

To each neutralized alkaline hydrolysate tube was added 0.2 ml of citrate buffer followed by 0.4 ml of the potassium cyanide-methyl cellosolve-ninhydrin solution. The tubes were covered with culture tube caps and heated in a boiling water bath for 15 minutes, after which they were removed and cooled. The addition of 2.0 ml of 60 per cent ethanol brought the final volume of each tube to 3.0 ml. The absorbance of each tube was determined at 570 mμ against the appropriate blank. A plot of absorbance at 570 mμ versus tube number indicated the positions of the eluted peptide peaks.

Treatment of peptide peak fractions eluted from ion exchange chromatography

The individual tubes comprising a peak were combined and diluted with water. Fractions were evaporated at 40° on a rotary evaporator until a small volume remained. Repetition of this process two or three times served to remove the volatile buffers. In some instances, non-volatile buffer products from Dowex 1 column chromatography were removed by column chromatography on Bio-Gel P-2 or Sephadex G-10. The fraction was then lyophilized and dissolved in a known volume of water or 50 per cent pyridine.

Characterization of Individual Peptide Peak Fractions Eluted from Ion Exchange Column Chromatography

The concentrated peak fractions were examined for homogeneity by paper chromatography or high voltage electrophoresis (29,30). Aliquots from each peak were spotted at 1 to 4 origins depending on the number of stains to be used.

Paper chromatography was carried out for 12 to 18 hours on Whatman No. 1 or Whatman 3mm chromatographic paper. One of two solvent systems was used: (1) n-butanol-acetic acid-water (4:1:5). The organic upper phase was used for chromatography and the lower aqueous phase was placed in the bottom of the chamber. If this system gave poor resolution, the second system was used. (2) pyridine-isoamyl alcohol-0.1 N NH_4OH (6:3:5). This system was described by Harris and Hindley (31).

High voltage electrophoresis was carried out at 2500 V on Whatman 3mm paper for 45 to 90 minutes in one of two buffer systems: (1) pH 3.6 buffer, pyridine-acetic acid-water (1:10:289). (2) pH 6.5 buffer, pyridine-acetic acid-water (25:1:225).

After either paper chromatography or high voltage electrophoresis, the chromatograms were dried at room temperature in the hood and then cut into strips containing one origin per strip. Each strip was stained with one of four different reagents given below.

Ninhydrin dip for amino acids and peptides: A 0.5 per cent solution of ninhydrin in acetone was prepared. The paper was dipped in this solution and dried in a ventilated oven at 110°. Amino acids and peptides stain various shades of blue.

Pauly reagent for histidine and tyrosine: The diazonium salt of sulfanilic acid was prepared according to Fieser (32). A mixture of 9.55 g (0.05 mole) of sulfanilic acid, 2.65 g (0.025 mole) of anhydrous sodium carbonate, and 50 ml of water was heated and stirred until all of the sulfanilic acid was dissolved. The solution was cooled to 15° in an ice bath, after which a solution of 3.7 g (0.054 mole) of sodium nitrite in 10 ml of redistilled water was added. The resultant solution was immediately poured onto a mixture of 10.6 ml of concentrated HCl and 60 g of ice in a 250-ml beaker. Upon stirring, the p-benzenediazonium sulfonate separated as a white precipitate. The mixture was washed with two volumes of cold water and stored in a moist state at 5°.

For staining, 0.1 g of this reagent was dissolved in 100 ml of 10 per cent aqueous sodium carbonate and the paper was sprayed lightly. Tyrosine containing peptides stain reddish brown to orange and histidine peptides stain cherry-red (30).

Sakaguchi reagent for arginine (30,33): Solution A was prepared by dissolving 10 mg of α-naphthol in a solution of

5 per cent urea in ethanol. Solution B contained 2 g of bromine dissolved in 100 ml of 5 per cent NaOH. For staining, 5 pellets of sodium hydroxide were added to 10 ml of solution A and the paper was sprayed. After drying at room temperature, the paper was sprayed lightly with solution B. Arginine containing peptides stain pink to red.

Ehrlich stain for tryptophan (34): The paper was sprayed with a solution containing 2.5 g of p-dimethylaminobenzaldehyde in 500 ml of 95 per cent ethanol to which 10 ml of concentrated HCl had been added. Tryptophan containing peptides develop a blue color after a few minutes at room temperature.

Purification of Peptides

If examination of a peptide fraction revealed purification was necessary, one or more of the following techniques was employed: (1) preparative paper chromatography on Whatman 3mm paper with n-butanol-acetic acid-water (4:1:5) as the developer, (2) preparative paper electrophoresis on Whatman 3mm paper at pH 3.6 (2500 V for 45 to 90 minutes), (3) column chromatography on Aminex AG 50W-X2, (4) column chromatography on Dowex 1-X2 or AG 1-X8. A summary of the procedures used in the purification of the RCM-turkey lysozyme tryptic peptides[2] is shown in Table 2.

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[2]All tryptic peptides derived from reduced and S-carboxymethylated turkey egg white lysozyme have been given the same designation as their corresponding analogues from chicken egg white lysozyme (22).

TABLE 2. PURIFICATION AND CHARACTERISTICS OF RCM-TURKEY EGG WHITE LYSOZYME TRYPTIC PEPTIDES

Peptide	Method of Purification	Distinguishing Characteristics
T-1+2	No purification required	Sakaguchi positive and Pauly positive (orange)
T-2	Paper chromatography (n-BuOH:HAc:H_2O, 4:1:5) or column chromatography, Dowex 1-X2, 0.9 x 100 cm	Sakaguchi positive and Pauly positive (orange)
T-3	Paper electrophoresis at pH 3.6	
T-4	Paper chromatography (n-BuOH:HAc:H_2O, 4:1:5)	Sakaguchi positive
T-5	Column chromatography, AG 1-X8, 0.5 x 70 cm followed by gel filtration on Sephadex G-10	Sakaguchi positive and Pauly positive (orange)
T-6	No purification required	Ehrlich positive
T-7	Paper chromatography (n-BuOH:HAc:H_2O, 4:1:5) or column chromatography, Dowex 1-X-2, 0.9 x 100 cm followed by gel filtration on Bio-Gel P-2	Sakaguchi positive and Pauly positive (cherry-red)
T-8	Paper electrophoresis at pH 3.6	Ninhydrin negative, Sakaguchi positive and Pauly positive (orange)

T-9	No purification required	Ehrlich positive and Sakaguchi positive
T-10	Paper chromatography (n-BuOH:HAc:H_2O, 4:1:5)	
T-11	No purification required	
T-1,12	Column chromatography, AG 1-X8, 0.5 x 50 cm	
T-13	No purification required	Ehrlich positive and Sakaguchi positive
T-14	Column chromatography, AG 1-X8, 0.5 x 70 cm	Sakaguchi positive
T-15	No purification required	
T-16	No purification required	Sakaguchi positive and Pauly positive (cherry-red) and Ehrlich positive
T-17	Paper chromatography (n-BuOH:HAc:H_2O, 4:1:5)	Sakaguchi positive
T-17+18	Paper chromatography (n-BuOH:HAc:H_2O, 4:1:5)	Sakaguchi positive

Figure 4. Elution of Whatman 3mm paper strips.

The strip to be eluted was cut to fit inside a 12-ml conical centrifuge tube with a tapered point on one end (A). After moistening the strip with 50 per cent pyridine, it was wrapped in two folds of aluminum foil--tapered end protruding (C) and inserted into the centrifuge tube--tapered end first (D). The square end of the strip was folded over the lip of the centrifuge tube and held in place with an aluminum foil-covered cork. A brief centrifugation in the clinical centrifuge served to elute the paper. The eluate was removed and the process repeated two more times.

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follwing t[illegible]
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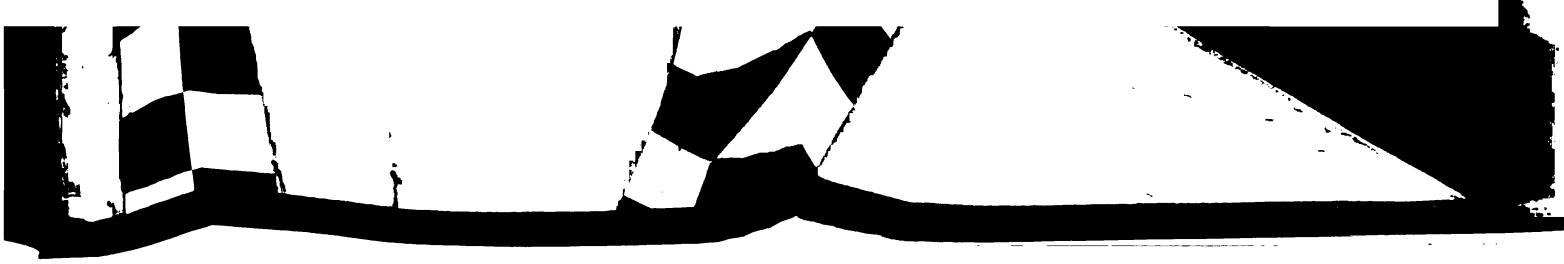
Subtractive Brain Dem[illegible]

The procedure [illegible]
for all but one of the pa[illegible]
The coupling mixture co[illegible]
0.8 ml of distilled water, [illegible]
triocyanate in pyridine and [illegible] per cent [illegible]-
adine. The mixture was placed in a 12-m[illegible] conical [illegible]
tube fitted with a cork stopper which had two [illegible], one
for a steinless steel ebullition tube and the other for an
outlet tube. The reaction mixture was [illegible] for two

Figure 4

Figure 4

(A)
Paper strip
(B)
Aluminum Foil
(C)
Wrapped strip
(D)
Inserted strip



Peptides were eluted from Whatman 3mm paper by the following time-saving procedure. The strip to be eluted was cut to fit inside a 12-ml conical centrifuge tube with a tapered point on one end. After moistening the strip with 50 per cent pyridine, it was wrapped in two folds of aluminum foil (tapered end protruding) and inserted into the centrifuge tube (tapered end first). The square end of the strip was folded over the lip of the centrifuge tube and held in place with an aluminum foil-covered cork. A brief centrifugation in the clinical centrifuge served to elute the paper. The eluate was removed and the process repeated twice more, after which no ninhydrin positive material remained on the paper. A representation of the method is shown in Figure 4.

Peptide Sequencing Techniques

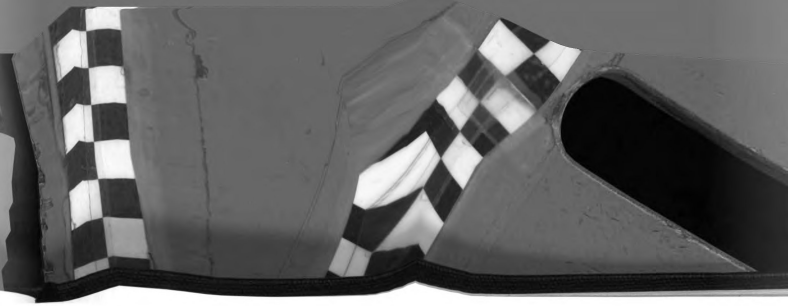
Subtractive Edman degradation

The procedure of Taniuchi and Anfinsen (35) was followed for all but one of the peptides degraded by the Edman method. The coupling mixture contained 2 to 5 μmole of peptide in 0.5 ml of distilled water, 0.5 ml of 2 per cent phenyl isothiocyanate in pyridine, and 50 μl of 25 per cent trimethylamine. The mixture was placed in a 12-ml conical centrifuge tube fitted with a cork stopper which had two openings; one for a stainless steel ebullition tube and the other for an outlet tube. The reaction mixture was flushed for two

minutes with pre-purified nitrogen after which the tube was sealed and incubated at 40° for two hours. The mixture was extracted five times with 4 to 5 ml of thiophene-free benzene by mixing in a Vortex mixer, followed by centrifugation in a clinical centrifuge. The upper layer was removed with a Pasteur pipette and discarded, taking care not to disturb any emulsion which might have formed. After the final extraction, the mixture was lyophilized.

Cyclization and subsequent cleavage of the phenylthiocarbamyl derivative was accomplished by dissolving the lyophilized product in 1.0 ml of trifluoroacetic acid. The solution was flushed with nitrogen for 1 minute; the tube was sealed and incubated at 37° for 30 minutes. The reaction mixture was taken to dryness in a vacumn desiccator over NaOH and $CaCl_2$ pellets; the dried residue was extracted four times with 4 to 5 ml of ethylene chloride. The residual peptide residue remained as a film, layered on the inside surface of the conical centrifuge tube, as the extracts containing the thiazoline and phenylthiohydantoin amino acid derivatives were removed by Pasteur pipette. The residual peptide residue was lyophilized and dissolved in a known quantity of 50 per cent pyridine. A suitable aliquot was withdrawn for amino acid analysis and the remainder was lyophilized prior to the next subtractive step.

A modification of this procedure (36,37), in which the residual peptide product was purified after each cyclization

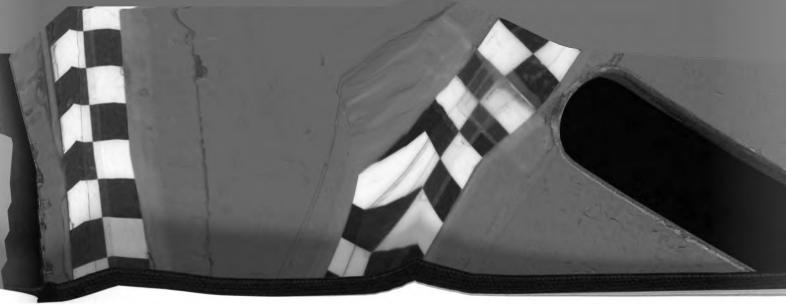


step, was applied to the Edman degradation of the N-terminal cyanogen bromide fragment from peptide T-13. After removal of the trifluoroacetic acid from the cyclization mixture, the residue was dissolved in 4 ml of 0.2 M acetic acid and extracted twice with an equal volume of benzene. The aqueous layer containing the residual peptide was adsorbed on a 0.3 x 6 cm column of Dowex AG 50W-X2 (50 to 100 mesh) and the resin was washed with three 4-ml portions of 0.2 M acetic acid. The peptide was then eluted with 4 ml of 1.07 M pyridine-acetic acid buffer, pH 5.4 (86 ml of pyridine and 30 ml of acetic acid diluted to a volume of 1 liter with redistilled water). The eluate containing the peptide was lyophilized and treated as above.

Carboxypeptidase digestion

The procedure of Dolpheide, Moore, and Stein was followed (38). Carboxypeptidase A solution was prepared by washing 50 μl of the suspension of crystalline enzyme twice with cold redistilled water by suspending it in the water and then centrifuging it in the clinical centrifuge. The precipitate was dissolved in 1 M potassium bicarbonate to a concentration of 1 mg per ml. Carboxypeptidase B was used as a solution in 0.1 M sodium phosphate, pH 7.8. All digestions were carried out in 0.1 M sodium phosphate, pH 7.8 at either 25° or 37°.

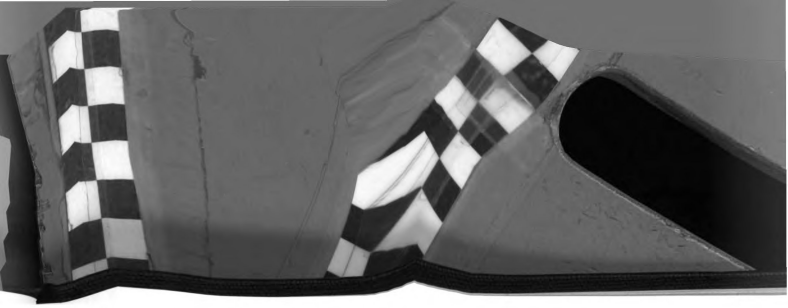
Peptide T-5 (1.3 μmole) was dissolved in 1.0 ml of phosphate buffer and incubated with 2.0 μl of carboxypeptidase



B (6 mg per ml) for 5.75 hours at room temperature. At this time 50 µl of carboxypeptidase A (1.0 mg per ml) was added so that the molar carboxypeptidase A to substrate ratio equaled 1:1000. At the end of one hour's incubation, an additional 450 µl of carboxypeptidase A solution was added to the digest, increasing the molar carboxypeptidase A to substrate ratio to 1:100. Digestion at the increased carboxypeptidase A concentration was carried out for 20.5 hours at 37°. Aliquots for amino acid analysis were removed at 6.75, 8.25, 12.25, and 27.75 hours after initiation of the digestion with carboxypeptidase B.

The N-terminal pentapeptide derived from chymotrypsin hydrolysis of T-7 (T-7-Cht-1) was digested with carboxypeptidase A. The reaction mixture contained 0.45 µmole of peptide, 400 µl of phosphate buffer and 100 µl of carboxypeptidase A (10 mg per ml). Amino acid analysis was performed on the entire digest after 2.5 hours at 37°. The molar enzyme to substrate ratio was 1:15.

Carboxypeptidase digestion of the N-terminal octapeptide from T-7 (T-7-Cht-2) was also carried out. The reaction mixture contained 0.56 µmole of peptide, 2.0 ml of phosphate buffer, and 150 µl of carboxypeptidase A (4 mg per ml). The digestion was carried out for 11 hours at 37° with aliquots removed for amino acid analysis at 0.67 and 11 hours. The molar enzyme to substrate ratio was 1:30.

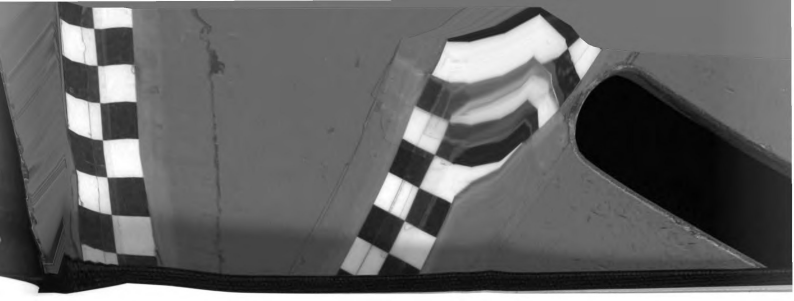


Cyanogen bromide cleavage of T-13

Cyanogen bromide digestion of T-13 was carried out according to Taniuchi and Anfinsen's procedure (35) which is a modification (39) of the original procedure (40). Approximately 4 μmole of peptide T-13 was dissolved in 0.75 ml of 70 per cent formic acid to give a 1 per cent solution of the peptide. A 30-fold molar excess of cyanogen bromide (120 μmole, 12.7 mg) per methionine residue was added. The reaction mixture was kept at room temperature for 24 hours at which time 2 volumes of redistilled water were added. The solution was lyophilized to remove methyl thiocyanate and excess cyanogen bromide. The cleavage products were separated by column chromatography on a 0.5 x 50 cm column of Aminex AG 50W-X2.

Chymotrypsin digestion of peptide T-7

The initial digestion mixture contained 3.8 μmole of peptide T-7, 1.25 ml of 0.1 M NH_4HCO_3 buffer, and 200 μl of a 1 per cent chymotrypsin solution. The molar enzyme to substrate ratio was 1:50. Digestion was carried out at 37° for 10 hours after which the digest was lyophilized and then dissolved in pH 9.4 buffer. The peptides were separated by column chromatography on a 0.9 x 100 cm column of Dowex 1-X2. Because of the low yield of the N-terminal pentapeptide (T-7-Cht-1) and difficulty in eluting this peptide from Dowex 1-X2, the reaction conditions were changed; also changed was the method of separation.

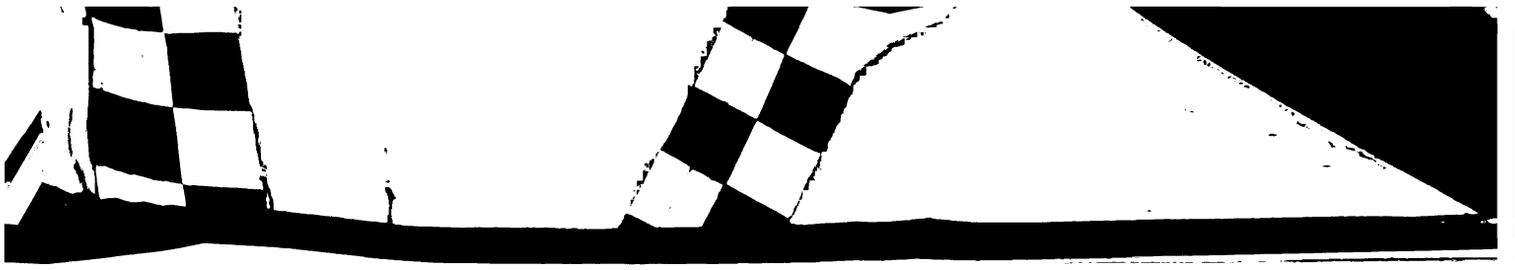


The digestion mixture contained 5 μmole of peptide T-7, 0.2 μmole of chymotrypsin, and 2.25 ml of 0.1 M NH_4HCO_3 buffer. The molar ratio of enzyme to substrate was 1:25. Digestion was carried out at 40° for 12.75 hours. The pH was adjusted to 2.7 with formic acid and the digest was chromatographed on a 0.5 x 50 cm column of Aminex AG 50W-X2.

Enzymatic hydrolysis of peptides

Peptides which gave a positive Ehrlich test were subjected to enzymatic digestion in an attempt to quantitate their tryptophan content. Peptides T-6, T-13, and T-16 were digested by the combined action of papain and leucine aminopeptidase. Peptide T-9 was treated with leucine aminopeptidase only.

Papain digestion of peptides was performed according to the procedure of Smyth, Stein, and Moore (41). The papain hydrolyzing mixture contained 0.3 to 0.4 μmole of peptide, 2400 μl of 0.02 M sodium phosphate buffer, pH 7.0, 300 μl of 0.01 M Na_2EDTA in phosphate buffer, 200 μl of freshly prepared 0.1 M NaCN in phosphate buffer, and 100 μl of papain solution (1.1 mg per ml) activated prior to use by incubation for 2 hours at room temperature in the above EDTA-NaCN-phosphate buffer system. The molar enzyme to substrate ratio was 1:50. The papain digest was maintained at 40° for 18 hours at which time the pH of the solution was adjusted to 2.0 with 1 N HCl; the solution was lyophilized.

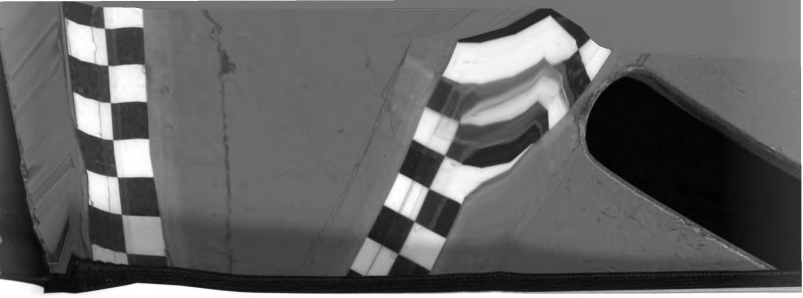


Peptides (0.2 to 0.4 μmole) to be digested by leucine aminopeptidase were dissolved in 0.5 ml of 0.005 M $MgCl_2$ in 0.005 M Tris buffer, pH 8.5. To the dissolved peptide was added a solution containing 24 μl of a leucine aminopeptidase suspension (5 mg per ml) previously activated by incubation for 30 minutes at 40° with 0.5 ml of the Tris-$MgCl_2$ buffer system (42). Hydrolysis with leucine aminopeptidase was carried out for 18 to 24 hours at 40°. If present, denatured papain precipitated during this time and was removed by centrifugation at the end of the digestion. The digest was diluted to 5 ml with pH 2.2 sample diluting buffer before amino acid analysis.

A better procedure for the enzymatic hydrolysis of peptides and proteins has been described by Hill and Schmidt (43).

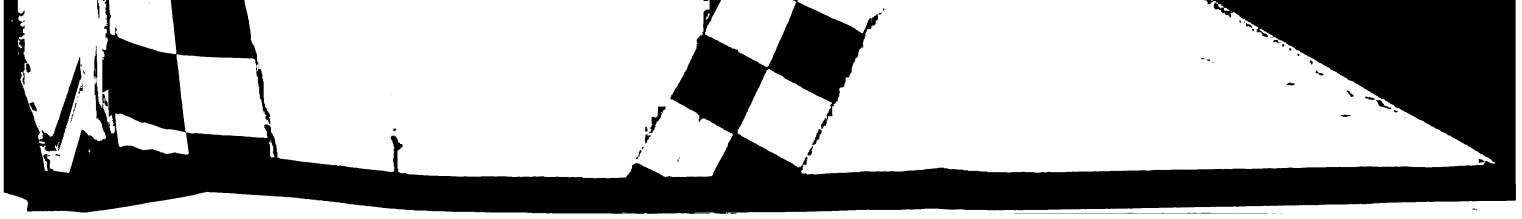
Determination of the net charge on peptides

The assignment of amide or free carboxylic acid function to an acidic amino acid within a peptide can often be made on the basis of the net charge on the peptide. If this assignment is to be based solely on the net charge, the peptide must contain only a single unknown amide or acidic function. The relative net charge of peptides was determined from the mobility of the peptides compared to aspartic acid, glutamic acid, and serine after high voltage electrophoresis at pH 6.5. The peptides were located by means of the ninhydrin dip.



Initial Separation of RCM-Turkey Egg White Lysozyme Tryptic Peptides

The digest from 600 mg of the RCM-turkey lysozyme was adjusted to pH 2.8 with formic acid and applied with air pressure to a 2 x 150 cm jacketed column of Aminex AG 50W-X2, previously equilibrated with 0. 2 M, pH 3.1 starting buffer. The column was eluted first at 40° with 500 ml of starting buffer and the elution then was continued at 40° with a linear gradient provided by a two-chambered gradient apparatus containing 3500 ml of starting buffer in the mixing chamber and 3500 ml of 2.0 M, pH 5.0 pyridine-acetic acid buffer in the reservoir. After completing the gradient elution, 500 ml of 2 M NH_4OH was passed through the column at 50°. The flow rate during all stages of the elution was 60 ml per hour and the volume of fractions collected was 10 ml.



RESULTS

Purification of Turkey Egg White Lysozyme

The purification of egg white lysozymes, in general, takes advantage of the basic nature of these proteins and of their abundance in egg whites compared to other basic proteins. Therefore, a 90 fold purification of turkey egg white lysozyme was achieved by batch adsorption of the basic egg white proteins to CM-cellulose, washing with ammonium bicarbonate, and finally elution of the lysozyme activity with an ammonium carbonate solution. As seen in Table 3, this was the principal step in the purification procedure. The crystallization and recrystallization of turkey lysozyme served to remove minor basic protein contaminants without a significant increase in specific activity. This was demonstrated by both CM-cellulose column chromatography and polyacrylamide disc electrophoresis of the enzyme before and after crystallization (Figures 5 and 6 respectively). The twice crystallized turkey lysozyme gave a single peak on CM-cellulose column chromatography and a single band on disc electrophoresis. By these two criteria the enzyme was homogeneous. The appearance of the twice crystallized enzyme is shown in Figure 7.

TABLE 3. ENZYME PURIFICATION SUMMARY SHEET

Step	Volume	Activity			Protein	Purification	
		Units	Total	Per Cent	Mg	Total	Specific	Fold Purification
	Ml	Ml	Units	Recovery	Ml	Mg	Activity	
Dilution	840	26	21,800	100	55.0	46,300	00.47	--
CM-cellulose eluate	150	130	19,500	90	3.1	460	42.50	90
Twice crystallized enzyme	2.25	4545	10,100	46	86.5	195	52.00	110

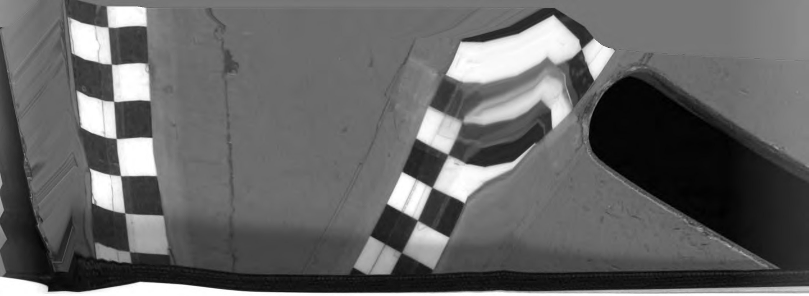


Figure 5. CM-cellulose chromatography of turkey egg white lysozyme.

A solution of 200 mg of turkey egg lysozyme in 10 ml of 0.05 M $(NH_4)_2CO_3$ was applied to a 2 x 22 cm column of CM-cellulose previously equilibrated with 0.05 M $(NH_4)_2CO_3$. The column was eluted with a linear gradient delivered from a two-chamber varigrad. The mixing chamber contained 500 ml of 0.05 M $(NH_4)_2CO_3$ and the reservoir 500 ml of 0.4 M $(NH_4)_2CO_3$. Fractions of 5 ml were collected at a constant flow rate of 30 ml per hour. The absorbance of the column eluate was monitored continuously at 280 mμ.

ENZYME BEFORE CRYSTALLIZATION
TWICE CRYSTALLIZED ENZYME
A280
FRACTION NUMBER
M OF (NH4)2CO3 GRADIENT
7.5
6.0
4.5
3.0
1.5
0.5
0.4
0.3
0.2
0.1
25
50
75
100
125
150
175

Figure 5

FRACTION NUMBER
25
50
75
100
125
150
175
A280
0.1
0.2
0.4
0.6
0.7
1.0
2.0
3.0
4.0
5.0
M OF $(NH_4)_2SO_4$ GRADIENT
CRYSTALLIZATION BEFORE ENZYME
ENZYME TWICE CRYSTALLIZED

Figure 2

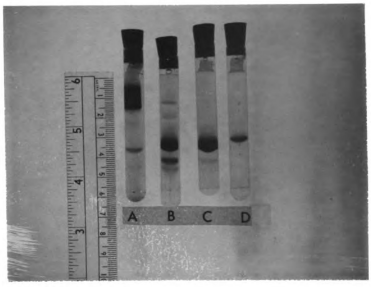


Figure 6. Polyacrylamide disc gel electrophoresis of turkey egg white lysozyme at different stages of the purification.

Electrophoresis was carried out by applying a current of 8 mamps per tube for 45 minutes in pH 4.5 β-alanine-acetate buffer. Turkey egg white lysozyme appears approximately 3.7 cm from the spacer gel in all tubes.

Tube A: approximately 500 μgrams of crude turkey egg white proteins

Tube B: 240 μgrams of the lyophilized eluate from carboxymethyl cellulose (overloaded)

Tube C: 300 μgrams of turkey egg white lysozyme--first crystals (overloaded)

Tube D: 40 μgrams of twice crystallized turkey egg white lysozyme.

Figure 7. Crystals of turkey egg white lysozyme.

The crystals were obtained from a 5 per cent sodium chloride solution at pH 8.5 in which turkey egg lysozyme was dissolved to a concentration of 50 mg/ml. They were photographed under an AO Spencer Phasestar microscope fitted with an Ortho-illuminator. The photograph was taken with Kodak 35 mm Panatomic-x film under phase contrast using a shutter speed of 1.0 second. The 97x objective was employed giving a magnification of 1940x on film. The final magnification on the photograph is 14,000x.

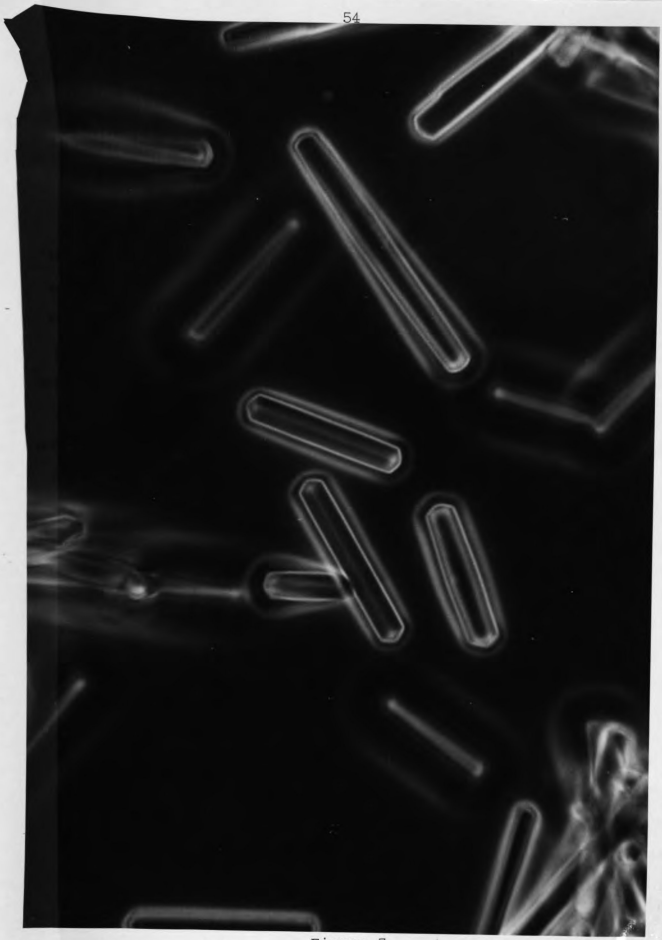
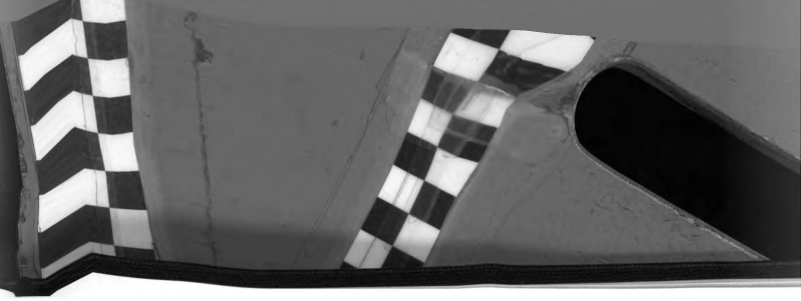


Figure 7



Amino Acid Content of Turkey Egg White Lysozyme

Amino acid analysis of RCM-turkey lysozyme derived from the apparently homogeneous crystalline preparation served two purposes. It provided a third criterion of purity for the crystalline enzyme and indicated the degree of similarity between chicken and turkey lysozymes.

The results of the amino acid analysis of turkey egg white lysozyme are shown in Table 4. With the exception of S-carboxymethylcysteine, aspartic acid, and tryptophan, the molar ratios of amino acids per mole of enzyme were within 0.2 residues of interger numbers. In addition to strongly supporting the homogeneous nature of the crystalline enzyme, these results revealed there were a minimum of six amino acid differences between the chicken and turkey lysozymes as indicated in Table 4. The significant number of apparent amino acid differences between the two lysozymes prompted sequence investigations.

Purification of the Tryptic Peptides from RCM-Turkey Egg White Lysozyme

The RCM-turkey lysozyme tryptic peptides were eluted from a column of Dowex AG 50W-X2 as shown in Figure 8. No peak contained more than two peptides and some contained a single peptide which required no further purification. The methods by which these peptides were purified were previously described in Table 2.

TABLE 4. AMINO ACID COMPOSITION OF RCM-TURKEY EGG WHITE LYSOZYME

Amino Acid	Residues per Mole of RCM-Lysozyme		Difference from Chicken Lysozyme
	Average Value	Integral Value	
Lysine	7.00	7	+1
Histidine	1.90	2	+1
Ammonia	17.5	17-18	
Arginine	10.02	10	-1
S-Carboxymethylcysteine	8.36	8	0
Aspartic Acid	19.52	20	-1
Threonine	7.00	7	0
Serine	10.10	10	0
Glutamic Acid	2.98	3	-2
Proline	1.94	2	0
Glycine	12.88	13	+1
Alanine	13.00	13	+1
Valine	4.92	5	-1
Methionine	1.88	2	0
Isoleucine	5.80	6	0
Leucine	8.98	9	+1
Tyrosine	3.78	4	+1
Phenylalanine	1.92	2	-1
Tryptophan	6.5	6-7	

Note: Tryptophan determinations were performed on the unmodified protein according to Spies and Chambers (24). The average molar values of all other amino acids were calculated by averaging six determinations--two each of the 24, 48, and 72 hour acid hydrolysates; these values were normalized from lysine and alanine for the short and long columns respectively. Serine and threonine were extrapolated to zero hydrolysis time.

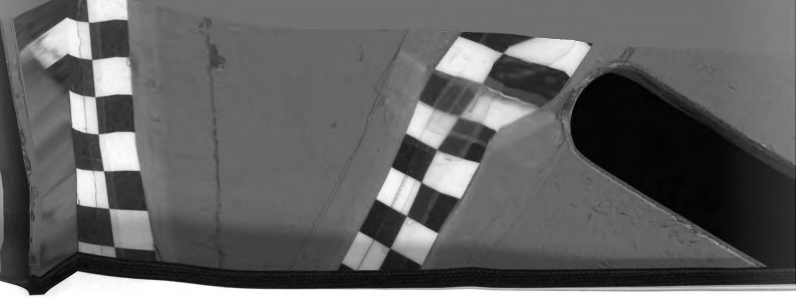


Figure 8. Initial separation of RCM-turkey egg white lysozyme tryptic peptides.

The trypsin digest from 600 mg of RCM-turkey lysozyme was applied to a 2 x 150 cm column of Aminex AG 50W-X2. The column was eluted with 500 ml of 0.2 M pyridine-acetic acid starting buffer, pH 3.1 at 40°. This was followed by a linear gradient from a two-chambered gradient apparatus which contained 3500 ml of starting buffer in the mixing chamber and 3500 ml of 2.0 M pyridine-acetic acid buffer, pH 5.0 in the reservoir. After completing the gradient elution, 500 ml of 2 M NH_4OH were passed through the column at 50°. The flow rate during all stages of the elution was 60 ml per hour and 10-ml fractions were collected. The peaks were located by reacting base-hydrolyzed aliquots from each fraction with ninhydrin and determining the absorbance at 570 mμ. The tryptic peptides are identified by their numerical designation.

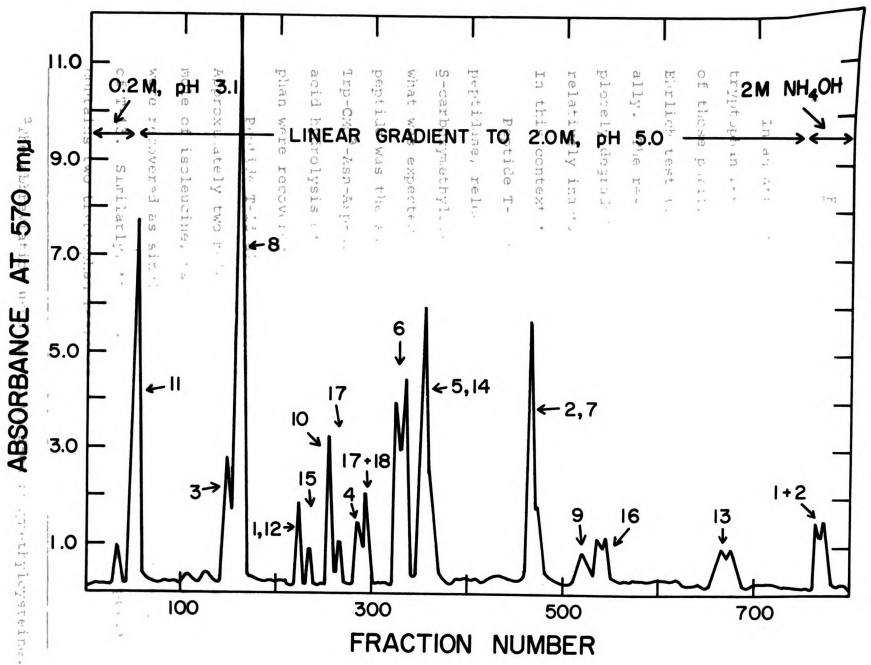
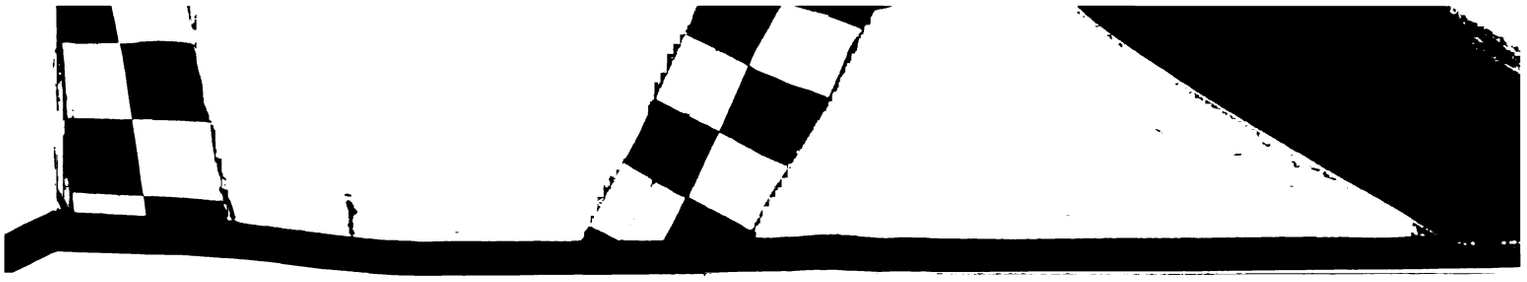

ABSORBANCE AT 570 mμ
11.0
9.0
7.0
5.0
3.0
1.0
0.2 M, pH 3.1
LINEAR GRADIENT TO 2.0M, pH 5.0
2M NH4OH
11
8
3
1,12
10
15
17
4
17+18
6
5,14
2,7
9
16
13
1+2
100
300
500
700
FRACTION NUMBER

Figure 8

Figure 8

ABSORBANCE AT 210 mμ
FRACTION NUMBER
LINEAR GRADIENT TO 0.05M, pH 2.0
0.05M, pH 1.3
5M NH4OH



Enzymatic Hydrolysis of Tryptophan Containing Tryptic Peptides

In an attempt to resolve whether there were six or seven tryptophan residues in the turkey lysozyme molecule, aliquots of those purified tryptic peptides which gave a positive Ehrlich test (T-6, T-9, T-13, T-16) were hydrolyzed enzymatically. The results are shown in Table 5. No peptide was completely degraded by this treatment--perhaps because of a relatively inactive preparation of leucine aminopeptidase. In this context the results are not wholly satisfactory.

Peptide T-9, which was digested only with leucine aminopeptidase, released two moles of tryptophan for each mole of S-carboxymethylcysteine and asparagine. This is precisely what was expected if the sequence of the turkey lysozyme peptide was the same as its chicken lysozyme analogue: Trp-Trp-CMC[3]-Asn-Asp-Gly-Arg. This confirmed the results from acid hydrolysis of the peptide in which 1.6 moles of tryptophan were recovered per mole of peptide.

Peptide T-13 also contained two tryptophan residues. Approximately two moles of tryptophan were released for each mole of isoleucine, valine, and arginine--amino acids which were recovered as single residues from the acid hydrolysate of T-13. Similarly, the analogous chicken lysozyme peptide contains two tryptophan residues.

---

[3]The abbreviation used is: CMC, S-carboxymethylcysteine.

TABLE 5. ENZYMATIC HYDROLYSIS OF THE TRYPTIC PEPTIDES FROM RCM-TURKEY LYSOZYME WHICH CONTAINED TRYPTOPHAN

Amino Acid	Peptide			
	T-9	T-13	T-16	T-6
Trp	1.90	2.00	1.16	1.24
Lys				0.31 (1)
His			-- (1)	
Arg	0.11 (1)	1.07 (1)	1.02 (1)	
S-Carboxymethyl-cysteine	1.00 (1)			0.82 (1)
Asp	0.26 (2)	0.22 (2)	0.10 (1)	-- (1)
Thr			0.22 (1)	
Ser (Asn)	0.90 (0)	1.47 (1)		1.21 (1)
Gly	0.12 (1)	0.95 (3)	0.30 (1)	1.45 (2)
Ala		2.61 (3)	1.00 (1)	1.14 (2)
Val		1.00 (1)	0.06 (1)	0.75 (1)
Met		0.36 (1)		
Ileu		0.87 (1)	0.97 (1)	
Leu				1.00 (1)
Tyr				0.63 (1)

Note: The amino acids released by enzymatic hydrolysis are expressed as molar ratios based on the moles of S-carboxymethylcysteine, valine, alanine, and leucine for peptides T-9, T-13, T-16, and T-6 respectively. Following in parenthesis, is the integral value as determined from acid hydrolysis.

Peptide T-16 quantitatively released a single tryptophan residue for each alanine, isoleucine, and arginine residue. Also, approximately one mole of tryptophan was recovered for each mole of leucine in peptide T-6. Therefore it seemed likely that each of these two peptides contained a single tryptophan residue as do their chicken lysozyme counterparts.

These results indicate that the four Ehrlich positive peptides isolated from trypsin digestion of RCM-turkey lysozyme most likely contain six tryptophan residues which comprise all of the tryptophan present in turkey lysozyme.

Amino Acid Composition of the Tryptic Peptides from RCM-Turkey Egg White Lysozyme

Amino acid analysis of the purified tryptic peptides from RCM-turkey lysozyme was a powerful tool in this investigation. The results as shown in Table 6 served as (1) a test of homogeneity for purified peptides, (2) an unequivocal means of confirming the amino acid composition of the intact protein, and (3) a method by which amino acid sequence differences between the turkey and chicken lysozymes could be detected.

An inspection of Table 6 shows that the molar ratios of amino acids in the purified peptides are nearly intergers, confirming the homogeneity of these peptides. This was the most stringent criterion of purity available because neither single peaks from column chromatography nor single bands

TABLE 6. AMINO ACID COMPOSITION* OF THE RCM-TURKEY LYSOZYME TRYPTIC PEPTIDES

Amino Acid	Peptides				
	T-1	T-1+2	T-3	T-4	T-5
Lys	+(1)	0.93 (1)	0.99 (1)		
His					
NH_3					1.47 (1)
Arg		1.01 (1)		+(1)	1.00 (1)
CMC			1.02 (1)		
Asp					2.07 (2)
Thr					
Ser					
Glu			1.01 (1)		
Pro					
Gly		1.17 (1)			1.01 (1)
Ala			3.00 (3)		
Val		1.01 (1)			
Met			0.96 (1)		
Ileu					
Leu			1.02 (1)		1.94 (2)
Tyr		0.87 (1)			0.99 (1)
Phe					
Trp					

continued

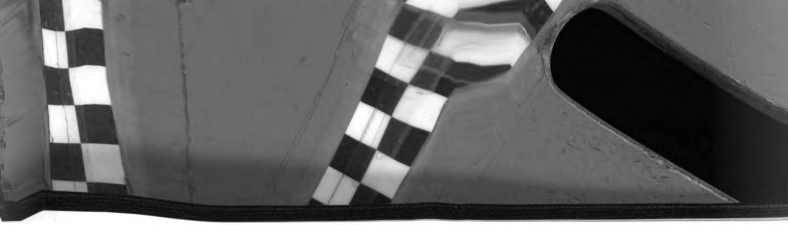


TABLE 6 - continued

Amino Acid	Peptides			
	T-6	T-7	T-8	T-9
Lys	0.97 (1)			
NH_3	1.54 (1)	3.76 (3)	3.96 (3)	1.51 (1)
Arg		1.05 (1)	1.04 (1)	1.04 (1)
CMC	1.00 (1)			1.05 (1)
Asp	0.74 (1)	3.00 (3)	4.10 (4)	1.91 (2)
Thr		1.79 (2)	2.01 (2)	
Ser	0.98 (1)	0.94 (1)	1.92 (2)	
Glu		1.08 (1)	1.00 (1)	
Pro				
Gly	2.03 (2)		2.01 (2)	1.01 (1)
Ala	2.06 (2)	1.08 (1)		
Val	0.94 (1)			
Met				
Ileu			1.98 (2)	
Leu	1.03 (1)		1.01 (1)	
Tyr	0.99 (1)		0.96 (1)	
Phe		1.80 (2)		
Trp	(1)			1.6 (2)

continued

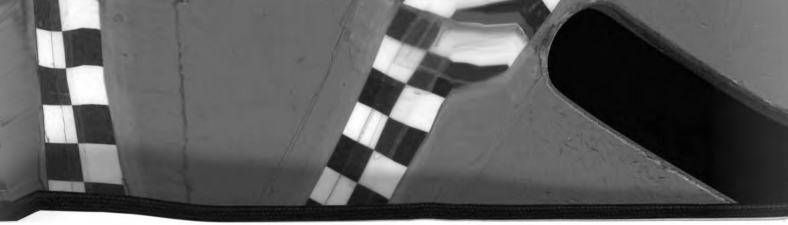


TABLE 6 - continued

Amino Acid	Peptides				
	T-10	T-11	T-12	T-13	T-14
Lys	1.01 (1)	1.00 (1)	+(1)		
His					
NH_3		3.48 (3)			0.82 (1)
Arg				1.00 (1)	1.00 (1)
CMC		3.09 (3)			
Asp		3.92 (4)		2.04 (2)	1.00 (1)
Thr	0.87 (1)	0.99 (1)			
Ser	1.00 (1)	3.90 (4)		0.90 (1)	
Glu					
Pro	0.88 (1)	1.03 (1)			
Gly	1.09 (1)			2.97 (3)	
Ala		3.14 (3)		2.96 (3)	
Val		1.03 (1)		1.02 (1)	
Met				1.02 (1)	
Ileu		2.18 (2)		1.03 (1)	
Leu		3.08 (3)			
Tyr					
Phe					
Trp				1.4 (2)	

continued

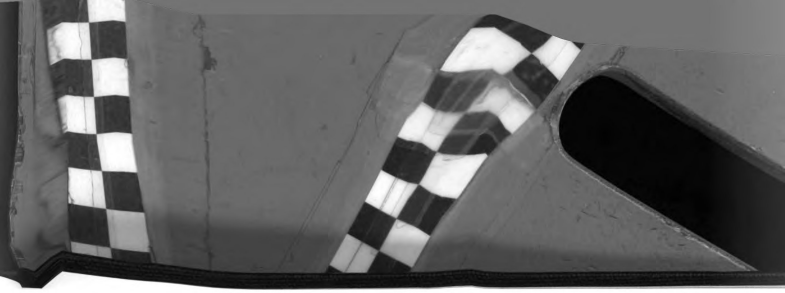
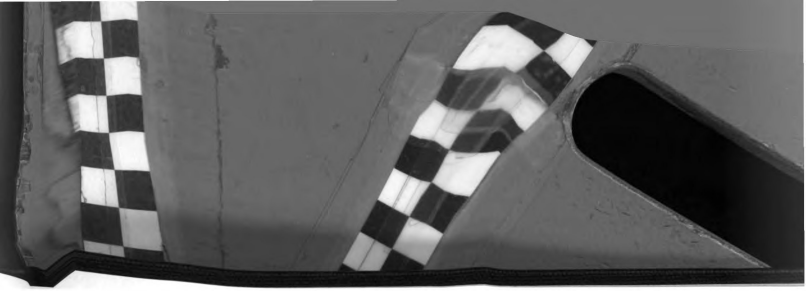


TABLE 6 - continued

Amino Acid	Peptides			
	T-15	T-16	T-17	T-17+18
Lys	0.95 (1)			
His		0.85 (1)		
NH_3		1.86 (1)		
Arg		0.89 (1)	1.03 (1)	1.00 (1)
CMC	1.05 (1)		0.90 (1)	0.99 (1)
Asp		1.16 (1)		
Thr		1.06 (1)		
Ser				
Glu				
Pro				
Gly		1.22 (1)	1.07 (1)	1.00 (1)
Ala		1.19 (1)		
Val		0.81 (1)		
Met				
Ileu		0.81 (1)		
Leu				1.00 (1)
Tyr				
Phe				
Trp		(1)		

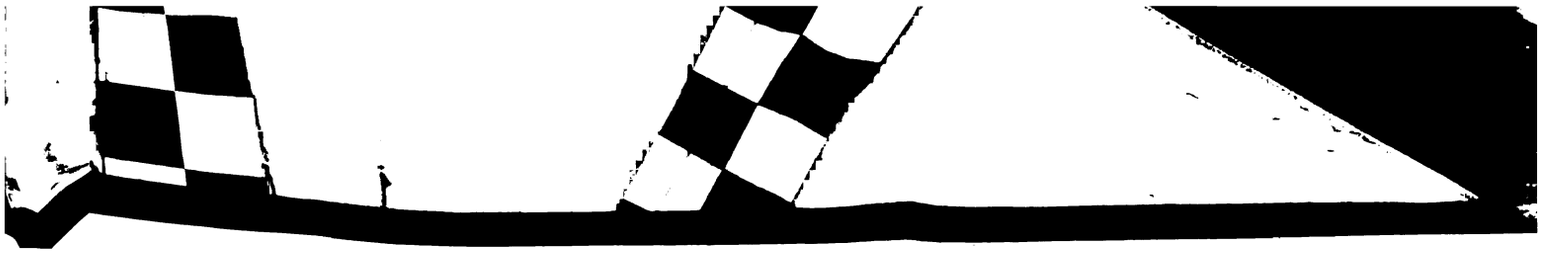
*The amino acid compositions for the purified tryptic peptides are expressed as molar ratios based upon the average number of μmoles per residue as determined from amino acid analysis of their acid hydrolysates. The integral number of amino acid residues per peptide is given in parenthesis. Serine and threonine are uncorrected for destruction during acid hydrolysis. The integral tryptophan values were determined from enzymatic hydrolysis (Table 5).



from paper electrophoresis or paper chromatography are reliable or sufficient proof of homogeneity.

The ambiguities arising from amino acid analysis of the reduced and S-carboxymethylated turkey lysozyme were resolved. Summation of the individual amino acid residues over all tryptic peptides (Table 6) confirmed the presence of 8 S-carboxymethylcysteine residues and 20 aspartic acid residues per mole of protein. All other amino acid values previously determined for the intact protein (Table 4) were verified by this procedure. Coupled with the results from the enzymatic hydrolysis of the tryptophan containing peptides, these results establish that turkey lysozyme contains 129 amino acid residues--the same number found in the chicken lysozyme.

The amino acid compositions of the tryptic peptides from RCM-turkey egg white lysozyme and RCM-chicken egg white lysozyme were compared. This revealed that an analogue for each of the reduced and S-carboxymethylated chicken egg white lysozyme tryptic peptides had been isolated. In addition, two tryptic dipeptide analogues, T-1+2, and T-17+18 were also separated from the trypsin digest of RCM-turkey lysozyme. Furthermore, as shown in Table 7, this comparison demonstrated there were a minimum of seven amino acid sequence differences between the primary structures of these two lysozymes and that these differences were distributed over six tryptic peptides.



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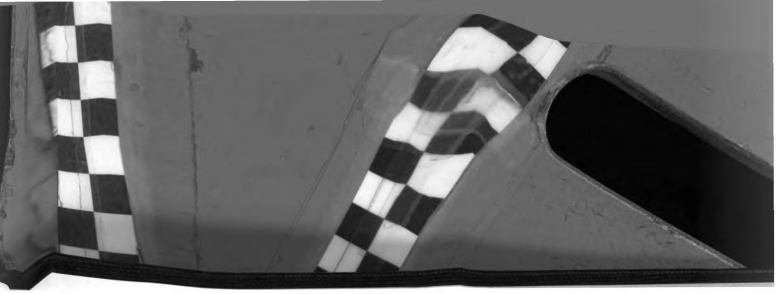
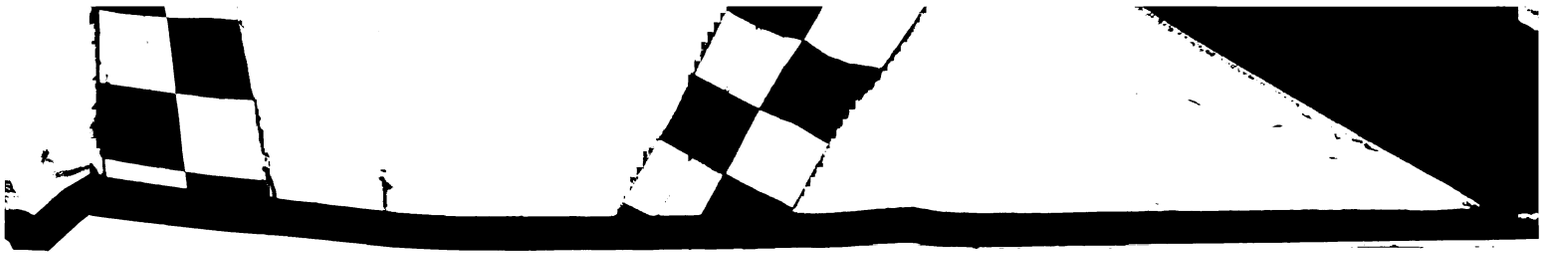


TABLE 7. OBSERVED DIFFERENCES IN AMINO ACID COMPOSITION BETWEEN THE ANALOGOUS TRYPTIC PEPTIDES FROM RCM-CHICKEN AND RCM-TURKEY LYSOZYMES

Tryptic Peptide	Amino Acid Present in Turkey Lysozyme	Amino Acid Present in Chicken Lysozyme
T-1+2	Tyrosine	Phenylalanine
T-5	Leucine	Histidine
T-7	Histidine	Glutamine
T-10	Lysine	Arginine
T-13	Glycine and Alanine	Valine and Aspartic Acid
T-16	Histidine	Glutamine

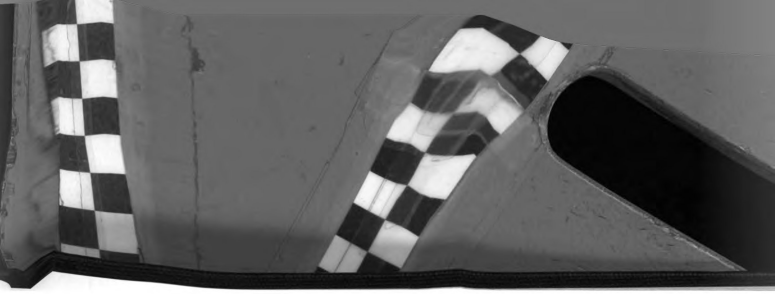


It is to be emphasized that certain sequence differences between analogous peptides cannot be detected by comparison of their amino acid contents as determined from acid hydrolysates. Acid hydrolysis destroys the ability to recognize differences in amide content between peptide analogues whose composition is otherwise the same. For example, the substitution of aspartic acid for asparagine would not be detected. Also, this comparative technique does not distinguish between peptide analogues of identical amino acid composition and varying sequence. The C-terminal tetrapeptide sequence of cytochromes c is known to vary in this way (44).

Sequence Determinations on Selected RCM-Turkey Egg White Lysozyme Tryptic Peptides

To show homology between two proteins, it is first necessary to establish their primary structures independently. In the case of turkey lysozyme this would involve: (1) determining the sequence of each tryptic RCM-peptide--including those whose amino acid compositions were the same as their tryptic RCM-chicken lysozyme analogues and (2) correct alignment of the tryptic peptides through a set(s) of overlapping peptides. This exhaustive study was not undertaken and only certain of the tryptic RCM-turkey lysozyme peptides, known to differ in amino acid composition from their chicken lysozyme counterparts, were sequenced.

Peptide T-1+2 (corresponds to residues 1 through 5 from chicken egg white lysozyme): The sequence of this



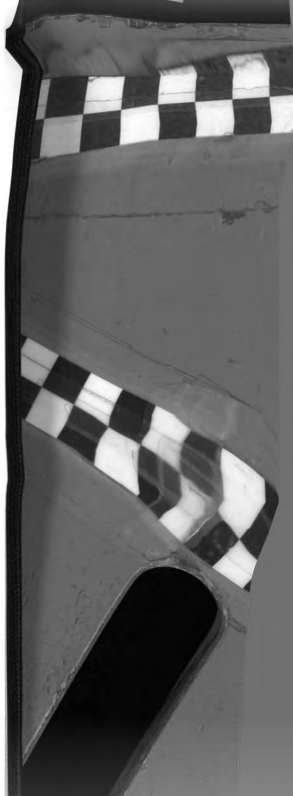
pentapeptide was established by three steps of the Edman degradation (Table 8). The specificity of trypsin placed arginine as the C-terminal residue after lysine had been shown to be N-terminal. The sequence is: Lys-Val-Tyr-Gly-Arg. Thus it is shown that tyrosine, rather than phenylalanine, is the third residue in tryptic peptide T-1+2 from RCM-turkey lysozyme.

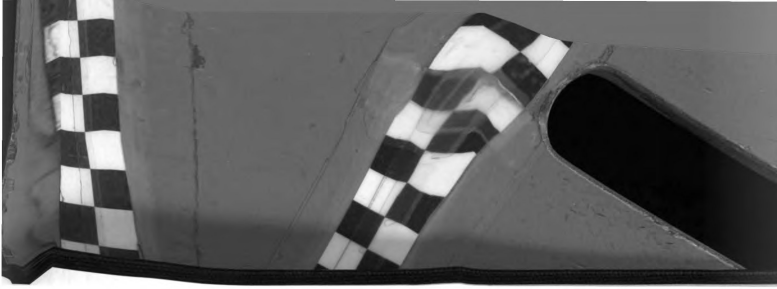
Peptide T-5 (corresponds to residues 15 through 21 from chicken egg white lysozyme): The sequence of the four N-terminal residues of this peptide was shown to be Leu-Gly-Leu-Asx-peptide by successive Edman degradations (Table 8). Treatment of peptide T-5 with carboxypeptidase B and A released 0.94 moles of arginine and 0.96 moles of tyrosine per mole of peptide (aliquot removed for analysis after 5.75 hours digestion with carboxypeptidase B and 1.00 hours digestion with carboxypeptidase A). A trace of asparagine was also present at this time and small but progressively larger quantities were released as the digestion continued to 27.75 hours. The ammonia content of the peptide as determined from acid hydrolysis (Table 6), indicated that only one of the two aspartic acid residues was present as asparagine. No aspartic acid was released by carboxypeptidase treatment. Thus the sequence of peptide T-5 is: Leu-Gly-Leu-Asp-Asn-Tyr-Arg. This places leucine, instead of histidine, at residue 15 of tryptic peptide T-5 from RCM-turkey lysozyme.

TABLE 8. EDMAN DEGRADATION OF PEPTIDES T-1+2 AND T-5

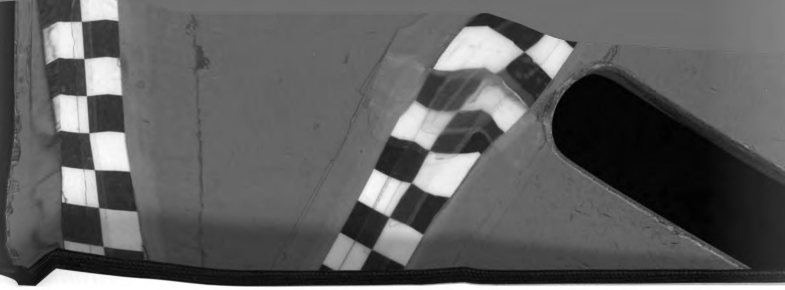
Analysis Performed	Amino Acid							
	Lys	NH_3	Arg	Asp	Gly	Val	Leu	Tyr
Composition of T-1+2	0.93		1.01		1.17	1.01		0.87
Edman Degradation of T-1+2								
Stage 1	0.09		1.07		1.10	0.91		0.94
Stage 2	0.08		1.09		1.06	0.11		0.86
Stage 3	0.05		1.02		0.98	0.11		0.15
Composition of T-5		1.47	1.00	2.07	1.01		1.94	0.99
Edman Degradation of T-5								
Stage 1			N.D.	2.04	0.99		1.05	0.98
Stage 2			0.84	2.01	0.17		1.01	0.97
Stage 3			0.87	2.04	0.13		0.34	0.96
Stage 4			0.86	1.55	0.15		0.31	1.00

Note: All values are expressed as molar ratios based upon the average number of μmoles per residue as calculated from the stable constituents. Those amino acid residues which were removed at different stages of the Edman degradation are underlined. Amino acids not determined are indicated by N.D.





Peptide T-7 (corresponds to residues 34 through 45 from chicken egg white lysozyme): A summary of the steps involved in the sequence determination of peptide T-7 is given in Figure 9. Three stages of the Edman degradation performed on tryptic peptide T-7 showed the N-terminal sequence to be: Phe-Glu-Ser-peptide (Table 9). When the amino function of glutamine is unmasked by an Edman degradation, it undergoes a facile deamidation and cyclization to pyrrolidonecarboxylic acid, thereby losing its ability to couple with phenylisothiocyanate. The successful removal of the penultimate N-terminal residue was the basis for its designation as free glutamic acid rather than glutamine. The remainder of the sequence was elucidated from sequence determinations carried out on the peptides produced by chymotryptic digestion of T-7. Treatment of the N-terminal pentapeptide, T-7-Cht-1 (Table 9), with carboxypeptidase A released 0.81 moles of phenylalanine and 0.73 moles of asparagine per mole of peptide after 2.5 hours digestion at 37°. Although the serine-asparagine peak was calculated entirely as asparagine, it is likely it contained some serine since the third stage of the Edman degradation of peptide T-7 removed serine. The sequence of the first five N-terminal residues is: Phe-Glu-Ser-Asn-Phe-peptide. Carboxypeptidase A treatment of the N-terminal octapeptide, T-7-Cht-2 (Table 9), released 0.60 moles of histidine, 0.29 moles of threonine and 0.20 moles of asparagine per mole of peptide after 40



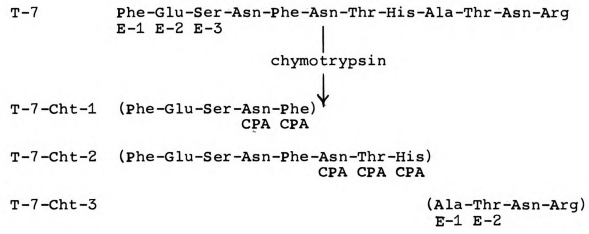


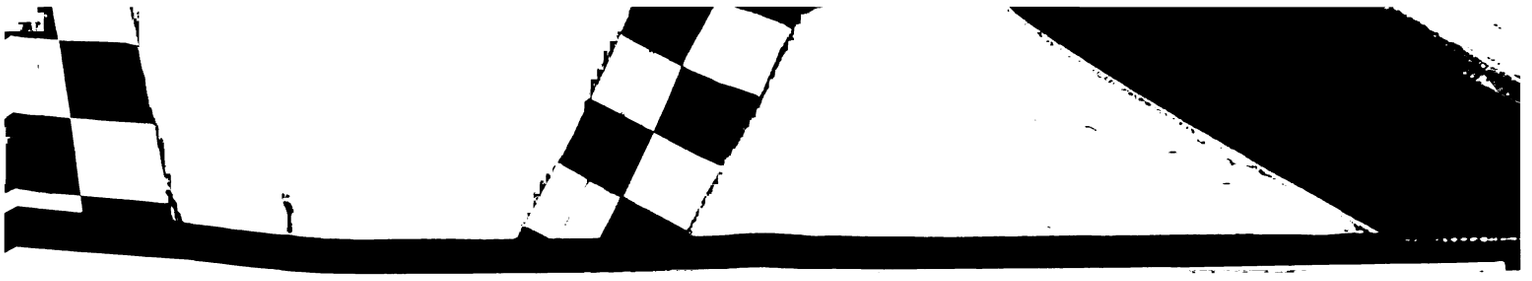
Figure 9. Sequence diagram of peptide T-7.

Subtractive Edman degradations are represented by: E-(number of stage). Amino acid residues released by carboxypeptidase A treatment are designated CPA. Peptides derived from chymotrypsin digestion are referred to as: (parent peptide)-Cht-(numerical designation).

TABLE 9. EDMAN DEGRADATION OF PEPTIDES T-7 AND T-7-Cht-3

Analysis Performed	Amino Acid								
	His	NH_3	Arg	Asp	Thr	Ser	Glu	Ala	Phe
Composition of T-7	1.00	3.76	1.05	3.00	1.79	0.94	1.08	1.08	1.80
Edman Degradation of T-7									
Stage 1	0.98		1.10	2.98	1.90	0.90	0.98	0.98	0.97
Stage 2	0.86		1.00	3.15	2.01	0.91	0.21	1.02	1.04
Stage 3	1.01		1.00	3.00	1.97	0.35	0.20	1.00	1.00
Composition of T-7-Cht-1				1.17		1.02	1.01		1.79
Composition of T-7-Cht-2	0.99			2.16	1.12	0.96	0.96		1.84
Composition of T-7-Cht-3			1.05	1.08	1.01			0.84	
Edman Degradation of T-7-Cht-3									
Stage 1			N.D.	1.04	0.96			0.16	
Stage 2			1.05	0.96	0.38			0.26	

Note: All values are expressed as molar ratios based upon the average number of μmoles per residue as calculated from the stable constituents. Those amino acid residues which were removed at different stages of the Edman degradation are underlined. Amino acids not determined are indicated by N.D.



minutes digestion at 37°. This extended the sequence of T-7 through the first eight N-terminal residues to: Phe-Glu-Ser-Asn-Phe-Asn-Thr-His-peptide. Also isolated from the chymotryptic digestion of peptide T-7 was the C-terminal tetrapeptide fragment, T-7-Cht-3 (Table 9). Electrophoresis of this peptide at pH 6.5 showed that it had a net positive charge with an R_f of 4.5 compared to serine. If aspartic acid had been present, the net charge should have been zero. Therefore, T-7-Cht-3 contains asparagine and not aspartic acid. Two stages of the Edman degradation showed the sequence of this peptide to be: Ala-Thr-Asn-Arg (Table 9). Combined with the previous results, the sequence of peptide T-7 is: Phe-Glu-Ser-Asn-Phe-Asn-Thr-His-Ala-Thr-Asn-Arg. Thus it is established that residue 41 is histidine, rather than glutamine, in peptide T-7 from RCM-turkey lysozyme.

Peptide T-13 (corresponds to residues 98 through 112 from chicken egg white lysozyme): The Edman degradation of peptide T-13 suggested the N-terminal tetrapeptide sequence was: Ileu-Ala-Ser-Gly-peptide (Table 10). However, difficulty in removing serine at the third stage caused the results from the fourth stage of the Edman degradation to be less than satisfactory. Therefore, the peptide was cleaved with cyanogen bromide to isolate smaller fragments which might be more amenable to the Edman degradation. The N-terminal octapeptide, T-13-CNBr-1 (Table 11), was isolated by this procedure and six stages of the Edman degradation

TABLE 10. EDMAN DEGRADATION OF PEPTIDE T-13

Analysis Performed	Amino Acid								
	Trp	Arg	Asp	Ser	Gly	Ala	Val	Met	Ileu
Composition of T-13	1.63	1.00	2.04	0.90	2.97	2.96	1.02	1.02	1.03
Edman Degradation of T-13									
Stage 1	N.D.	N.D.	2.08	0.95	3.00	2.91	0.81	0.75	0.06
Stage 2	N.D.	N.D.	2.05	0.85	2.95	2.14	0.91	0.76	0.04
Stage 3	N.D.	N.D.	2.05	0.37	2.94	2.10	0.93	0.72	0.00
Stage 4	N.D.	N.D.	2.05	0.33	2.54	2.14	0.89	0.88	0.00

Note: All values are expressed as molar ratios based upon the average number of μmoles per residue as calculated from the stable constituents. Those amino acid residues which were removed at different stages of the Edman degradation are underlined. Amino acids not determined are indicated by N.D.

TABLE 11. EDMAN DEGRADATION OF CYANOGEN BROMIDE CLEAVAGE FRAGMENTS FROM PEPTIDE T-13

Analysis Performed	Amino Acid								
	Trp	Arg	Asp	Ser	Homo Ser	Gly	Ala	Val	Ileu
Composition of T-13-CNBr-1			1.08	0.93	+	3.00	1.00		0.98
Edman Degradation of T-13-CNBr-1									
Stage 1			0.98	0.94	+	3.02	1.01		0.00
Stage 2			1.01	0.90	+	2.99	0.00		0.00
Stage 3			1.00	0.04	+	3.00	0.00		0.00
Stage 4			1.02	0.04	+	1.98	0.00		0.00
Stage 5			1.0		+	1.0			
Stage 6			0.3		+	1.0			
Composition of T-13-CNBr-2	1.37	1.00	1.00				2.00	0.90	
Edman Degradation of T-13-CNBR-2									
Stage 1	N.D.	N.D.	0.13				1.95	1.04	
Stage 2	N.D.	N.D.	--				1.07	0.93	

Note: All values are expressed as molar ratios based upon the average number of μmoles per residue as calculated from the stable constituents. Those amino acid residues which were removed at different stages of the Edman degradation are underlined. Amino acids not determined are indicated by N.D.

were performed on it with purification of the residual peptide at each stage (Table 11). These results coupled with the specificity of cyanogen bromide cleavage showed the sequence of T-13-CNBr-1 to be: Ileu-Ala-Ser-Gly-Gly-Asx-Gly-Met-peptide. Edman degradation of the C-terminal cleavage fragment, T-13-CNBr-2 (Table 11), extended the partial sequence of T-13 to: Ileu-Ala-Ser-Gly-Gly-Asx-Gly-Met-Asx-Ala-(Trp-Val-Ala-Trp-Arg). The residues in parenthesis are the chicken lysozyme sequence. Therefore, it is shown that residue 99 is alanine instead of valine and that residue 101 is glycine rather than aspartic acid in peptide T-13 from RCM-turkey lysozyme.

Comparison of the Lytic Activities of Turkey and Chicken Egg White Lysozymes

The lytic activities of both chicken and turkey egg white lysozymes towards M. lysodeikticus cells were measured (Figure 10). From this, it is seen that the turkey lysozyme is approximately 1.5 times more active than the chicken lysozyme in this assay system.

● TURKEY EGG WHITE LYSOZYME
▲ CHICKEN EGG WHITE LYSOZYME
0.30
0.15
0.10
0.05
ΔO.D. / MIN
0.14
0.29
0.42
0.57
0.72
0.86
1.00
μGRAMS OF ENZYME

Figure 10. Comparison of the lytic activities of turkey and chicken egg white lysozymes.

Each assay mixture contained 290 μl of a M. lysodeikticus cell suspension (13 to 15 mg of dried cells suspended in 90 ml of 0.067 M sodium phosphate buffer, pH 6.8 and 10 ml of 1 per cent NaCl) and 1 to 10 μl of the enzyme solution. The initial rates of change in absorbance at 550 mμ were determined at 25° in a Gilford Model 2000 automatic recording spectrophotometer. The protein concentrations were determined by the Lowry method (18).

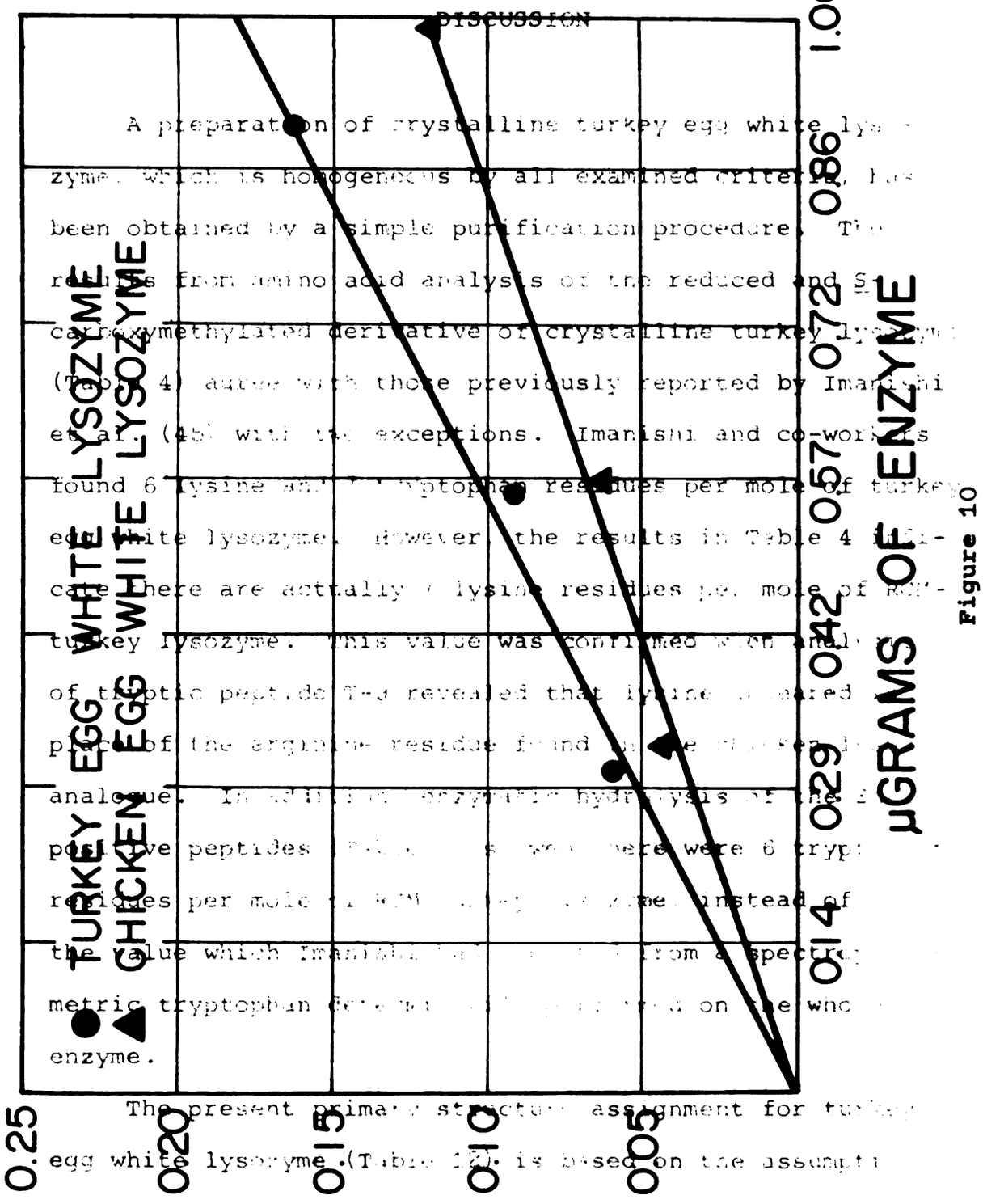


Figure 10

DISCUSSION

A preparation of crystalline turkey egg white lysozyme, which is homogeneous by all examined criteria, has been obtained by a simple purification procedure. The results from amino acid analysis of the reduced and S-carboxymethylated derivative of crystalline turkey lysozyme (Table 4) agree with those previously reported by Imanishi et al. (45) with two exceptions. Imanishi and co-workers found 6 lysine and 3 tryptophan residues per mole of turkey egg white lysozyme. However, the results in Table 4 indicate there are actually 7 lysine residues per mole of turkey lysozyme. This value was confirmed when an analysis of tryptic peptide T-3 revealed that lysine appeared in place of the arginine residue found in the chicken lysozyme analogue. In addition, enzymatic hydrolysis of the [illegible] positive peptides [illegible] were 6 tryptophan residues per mole [illegible] enzyme instead of the value which Imanishi [illegible] from a spectrophotometric tryptophan [illegible] on the whole enzyme.

The present primary structure assignment for turkey egg white lysozyme (Table 12) is based on the assumption

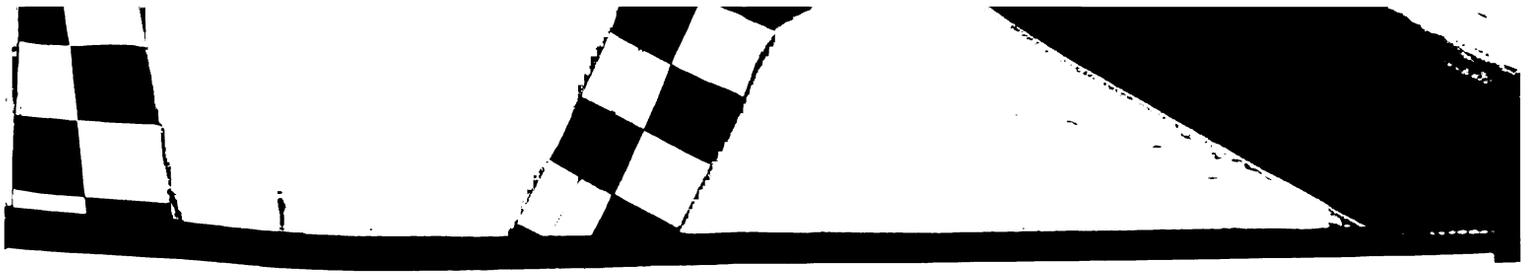
Figure 10

ΔO.D.
MIN
0.25
0.20
0.15
0.10
0.05
0.14
0.28
0.42
0.57
0.71
0.85
1.00
CHICKEN EGG WHITE LYSOZYME
TURKEY EGG WHITE LYSOZYME

DISCUSSION

A preparation of crystalline turkey egg white lysozyme, which is homogeneous by all examined criteria, has been obtained by a simple purification procedure. The results from amino acid analysis of the reduced and S-carboxymethylated derivative of crystalline turkey lysozyme (Table 4) agree with those previously reported by Imanishi et al. (45) with two exceptions. Imanishi and co-workers found 6 lysine and 7 tryptophan residues per mole of turkey egg white lysozyme. However, the results in Table 4 indicate there are actually 7 lysine residues per mole of RCM-turkey lysozyme. This value was confirmed when analysis of tryptic peptide T-9 revealed that lysine appeared in place of the arginine residue found in the chicken lysozyme analogue. In addition, enzymatic hydrolysis of the Ehrlich positive peptides (Table 5) showed there were 6 tryptophan residues per mole of RCM-turkey lysozyme, instead of 7--the value which Imanishi had reported from a spectrophotometric tryptophan determination performed on the whole enzyme.

The present primary structure assignment for turkey egg white lysozyme (Table 12) is based on the assumption



that the turkey and chicken lysozymes are homologous; i.e., that these two proteins are derived from a common ancestor. The assumption of homology was supported by the isolation of an analogue for each of the RCM-chicken lysozyme tryptic peptides from the tryptic digestion of RCM-turkey lysozyme. Thus, the RCM-turkey lysozyme tryptic peptides were aligned according to the positions occupied by their respective analogues in the primary structure of chicken egg white lysozyme as determined by Canfield (10). In addition, tryptic peptides from RCM-turkey lysozyme and RCM-chicken lysozyme, whose amino acid compositions were identical, were assumed to have the same sequence. The results of this investigation have provided good evidence that the turkey enzyme is identical with the chicken lysozyme in the total number of amino acid residues. Sequence analysis has established differences between the turkey and chicken lysozymes at residues 3, 15, 41, 73, 99, and 101. The presence of histidine and absence of glutamine in peptide T-16 from RCM-turkey lysozyme strongly indicates that residue 121 is the site of the seventh difference.

The differences in the turkey and chicken lysozyme primary structures may give rise to changes in conformation as well as related variations in binding and catalytic mechanism. The magnitudes of these effects cannot be assessed from the data presented here. However, the results from optical rotatory dispersion measurements indicate the

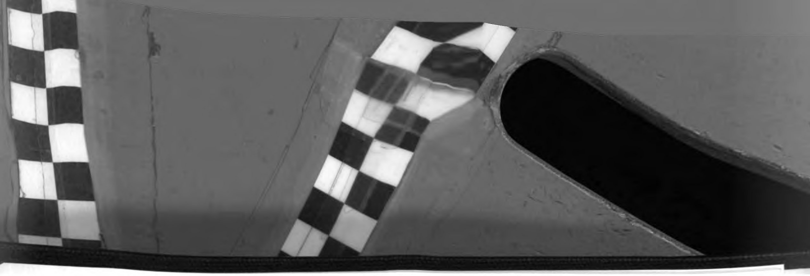
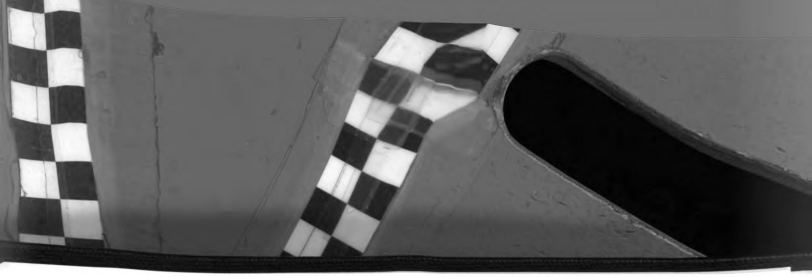


TABLE 12. PROPOSED AMINO ACID SEQUENCE OF RCM-TURKEY EGG WHITE LYSOZYME TRYPTIC PEPTIDES

Peptide	Sequence	Number of Residues
T-1	Lys	1
T-2	Val-Tyr-Gly-Arg	4
T-3	(CMC-Glu-Leu-Ala-Ala-Ala-Met-Lys)	8
T-4	(Arg)	1
T-5	Leu-Gly-Leu-Asp-Asn-Tyr-Arg	7
T-6	(Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-CMC-Ala-Ala-Lys)	12
T-7	Phe-Glu-Ser-Asn-Phe-Asn-Thr-His-Ala-Thr-Asn-Arg	12
T-8	(Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg)	16
T-9	(Trp-Trp-CMC-Asn-Asp-Gly-Arg)	7
T-10	(Thr-Pro-Gly-Ser-Lys)	5

T-11	(Asn-Leu-CMC-Asn-Ile-Pro-CMC-Ser-Ala-Leu-Leu-Ser-Ser-Asp- 80 Ile-Thr-Ala-Ser-Val-Asn-CMC-Ala-Lys) 90	23
T-12	(Lys)	1
T-13	Ile-Ala-Ser-Gly-Gly-Asx-Gly-Met-Asx-Ala(Trp-Val-Ala-Trp-Arg) 100 110	15
T-14	(Asn-Arg)	2
T-15	(CMC-Lys)	2
T-16	(Gly-Thr-Asp-Val-His-Ala-Trp-Ile-Arg) 120	9
T-17	(Gly-CMC-Arg)	3
T-18	(Leu)	1

Note: The proposed sequence is based on the isolation of an analogue for each RCM-chicken lysozyme tryptic peptide from the trypsin digest of RCM-turkey lysozyme. Sequence analysis established the sequence of those amino acid residues not enclosed within parenthesis. The sequences within parenthesis are those determined by Canfield (10,11) for chicken egg white lysozyme; the exceptions are at residues 73 and 121 where amino acid analysis of the tryptic peptide analogue revealed the designated differences. The amino acids which are different in the turkey egg white lysozyme are underlined.



conformations of the two proteins are not radically different (46). Figure 11 is an attempt to show where these differences would appear in the chicken lysozyme three-dimensional structure. It is interesting that all of the residues which are different, except residue 99, seem to occupy peripheral or surface positions. These are well removed from the Glu_{35} and Asp_{52} carboxyl groups which Phillips has presumed to function in the chicken lysozyme catalytic mechanism (12). They are also removed from several residues such as Trp_{62}, Trp_{63}, and Arg_{114}, that have been presumed to participate in substrate binding.

Four of the differences occur within conformationally regular regions of the chicken lysozyme molecule. The replacement of histidine by leucine at position 15 occurs at the end of the helix extending from residue 5 through 15. Histidine instead of glutamine appears at position 41 which marks the beginning of an antiparallel pleated sheet structure formed by residues 41 through 45 and 50 through 54. Alanine replaces valine at the end of an irregular helix extending from residue 88 through 100. Histidine again replaces glutamine in an isolated turn of helix extending from residue 119 through 122. It may be significant that three of these four differences occur at the extremes of regular structures in the chicken lysozyme molecule and hence they may not bring about an appreciable conformational change.

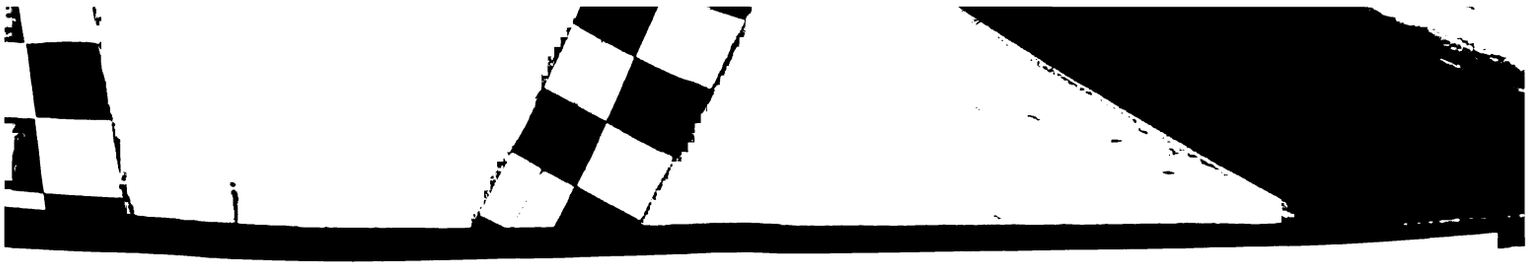
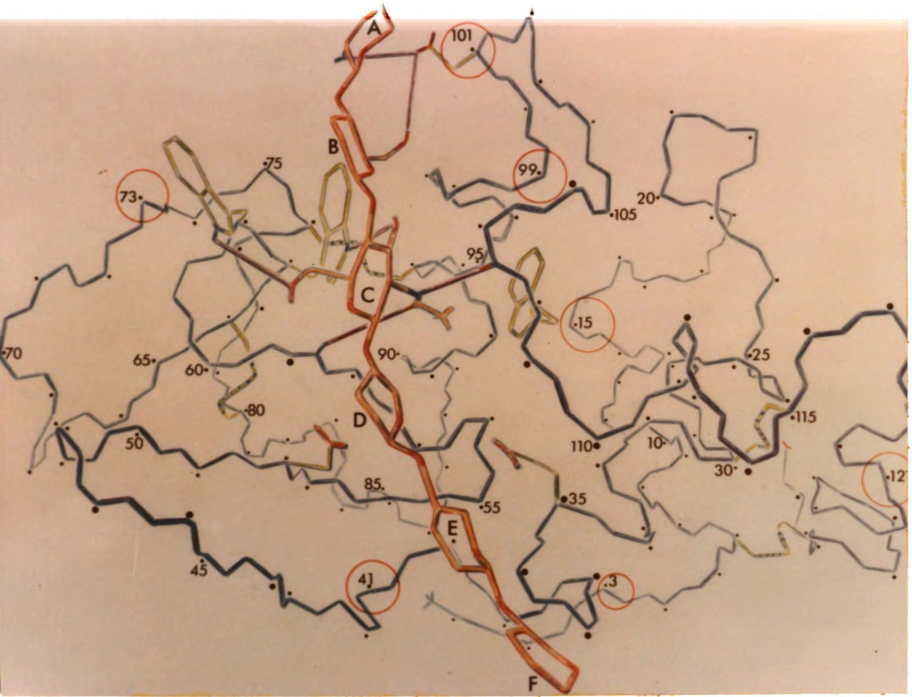


Figure 11. Three-dimensional diagram of crystalline chicken egg white lysozyme.

This figure is adapted from a drawing by Phillips (47) in which the interaction of chicken egg white lysozyme and a substrate is depicted. The polypeptide chain of the lysozyme molecule is shown in blue. The side chains have been omitted except for those that produce the four disulfide bonds and certain ones postulated to be involved in the catalytic mechanism and in hydrogen bonding to the substrate molecule. These are shown in yellow. The six monosaccharide residues comprising the hexasaccharide substrate are labeled A, B, C, D, E, and F. Cleavage by lysozyme occurs between substrate residues D and E.

The amino acids shown to be different in the turkey egg white lysozyme molecule have been circled.


A
B
C
D
E
F
101
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73
75
105
20
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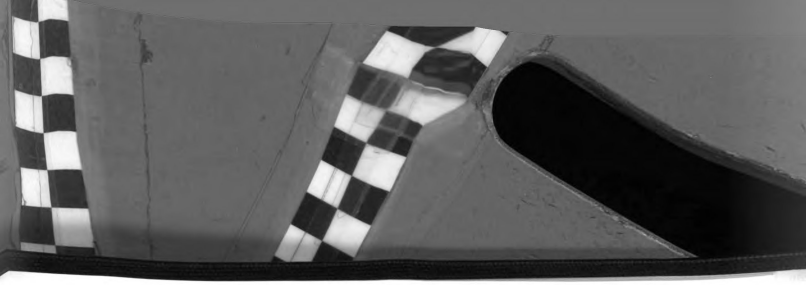
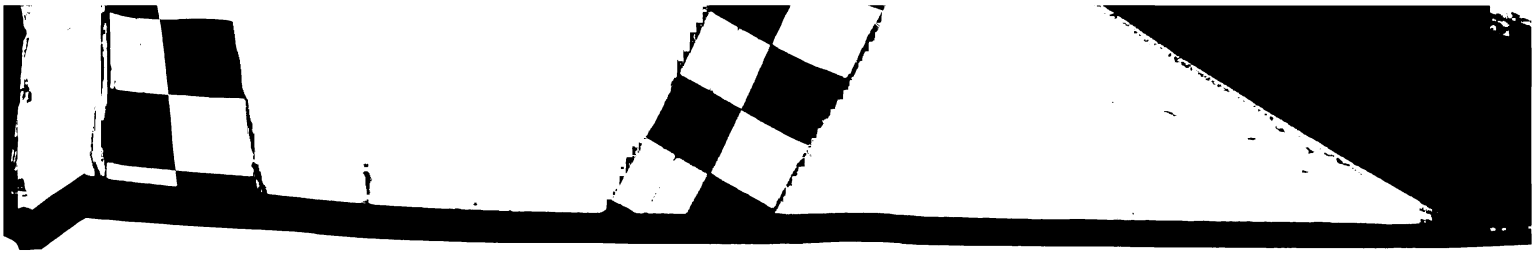
3

Figure 11



The most noteworthy of the apparent amino acid sequence differences occurs at residue 101. X-ray crystallographic examination of chicken lysozyme complexes with certain competitive inhibitors has implicated the Asp_{101} carboxyl group in substrate binding through hydrogen bonds to either substrate residue A or B (Figure 11). Substrate binding by turkey lysozyme has not been examined. Nevertheless, this enzyme is a better catalyst for hydrolyzing M. lysodeikticus cell walls than is the chicken enzyme and therefore must bind some substrates efficiently. The effect of replacing Asp_{101} by glycine on substrate binding can be viewed in two ways: (1) the carboxyl group of Asp_{101} is involved in binding substrate to the chicken lysozyme, and the turkey lysozyme binds substrates somewhat differently; or (2) Asp_{101} is not important for binding substrate to the chicken lysozyme. Certainly glycine cannot participate in this type of binding regardless of whether the conformations of the two enzymes are the same or not. We cannot know which of these possibilities is correct without further knowledge of the turkey lysozyme structure.

It is evident that structural investigations of this nature on lysozymes from closely related species may have considerable significance, not only for lysozyme evolution, but also for deciding between various possible catalytic mechanisms.



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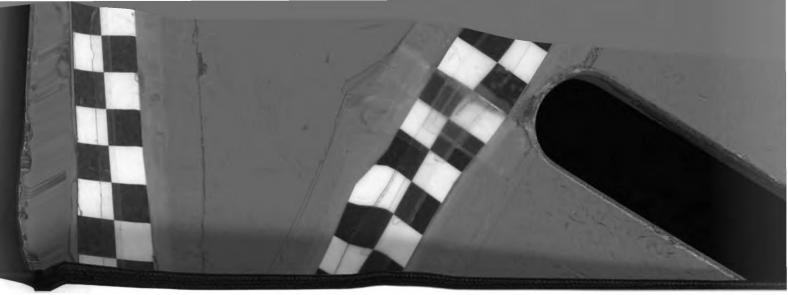

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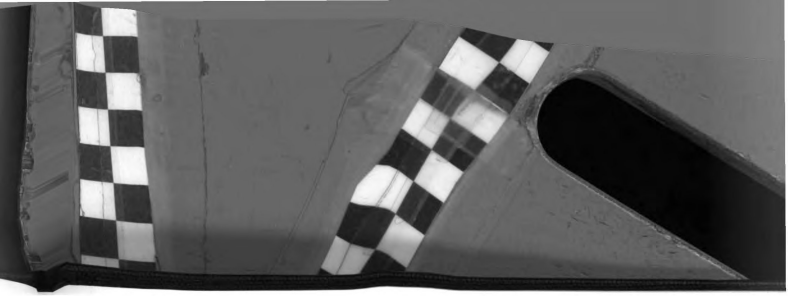

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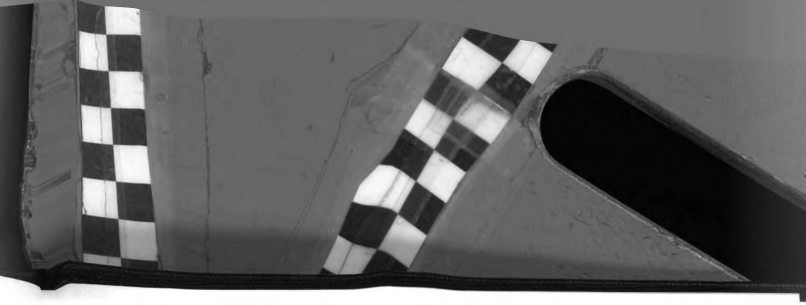

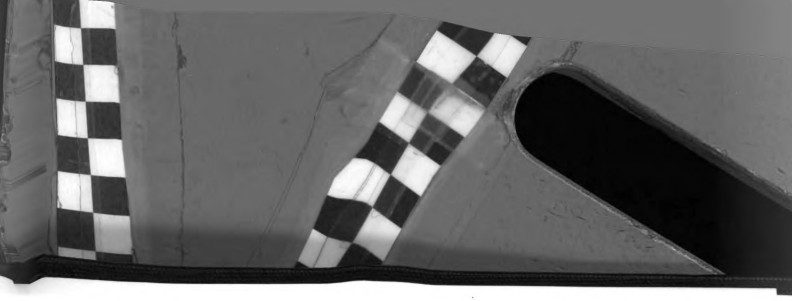

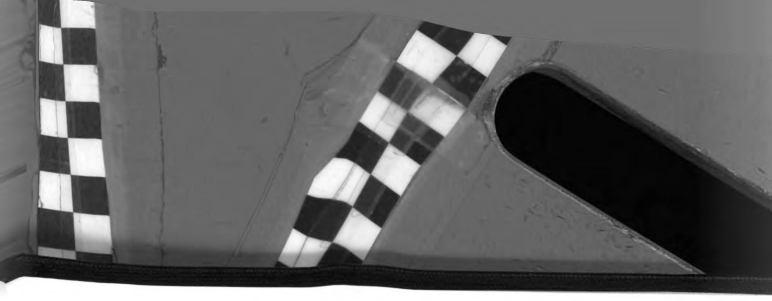

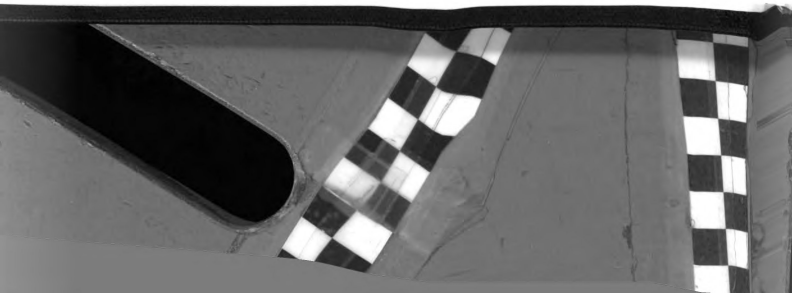

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