STUDIES ON THE ANTIGENIC PROPERTIES OF REDUCED AND CARBOXYMETHYLATED EGG-WHITE LYSOZYME

by

KAREN E. THOMPSON

B.Sc. (Microbiology),
The University of British Columbia, 1966

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Microbiology

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August, 1969

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree tha permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Microbiology

The University of British Columbia
Vancouver 8, Canada

Date 28th September 1969

ABSTRACT

This thesis involved a study of the antigenic properties of reduced and S-carboxymethylated egg-white lysozyme (CM-lysozyme). Since work on the antigenic properties of native lysozyme is currently in progress in two other laboratories, it was thought that a comparative study on CM-lysozyme might elucidate the role of primary and tertiary structure in determining antigenicity in proteins. This molecule was chosen mainly because the complete amino acid sequence, and the X-ray crystallography of the molecule have been completed. This makes it possible to correlate information on antigenic determinants with their orientation on the crystalline structure. The native molecule is relatively resistant to enzymatic digestion, but the reduced and S-carboxymethylated derivative, lacking its disulfide bridges, and consequently its rigid tertiary structure, is readily digested.

The first experiments involved attempts to isolate fragments of the CM-lysozyme molecule exhibiting haptenic activity. Trypsin was used, since this is a specific endopeptidase, cleaving proteins or peptides at the carboxyl of lysine and arginine residues. Only one tryptic peptide thus isolated (T-11) exhibited haptenic activity when various fractions were tested for their ability to

inhibit either precipitation or complement fixation between CM-lysozyme and its homologous antiserum. There was no cross-reactivity observed between CM-lysozyme and antiserum directed against native lysozyme. Likewise, the tryptic digest of CM-lysozyme or any of the peptides isolated from it, did not inhibit the immune reaction of native lysozyme with its homologous antiserum.

Further experiments were carried out in attempts to pinpoint the region of this large 23 amino acid peptide, T-11, which was responsible for haptenic activity. From sequential degradation of the peptide from both the C- and N-terminal, using enzymatic and chemical methods, the antigenic region was narrowed to the N-terminal portion of T-11.

Two synthetic peptides, prepared by the solid phase method, comprising the N-terminal decapeptide of T-11, and the decapeptide plus arginine at the N-terminal, both showed haptenic activity by inhibition of precipitation between CM-lysozyme and its homologous antiserum. The degree of inhibition with the two synthetic peptides were comparable but somewhat less efficient than the whole T-11 peptide.

TABLE OF CONTENTS

Table of Contents (Continued)

Table of Contents (Continued)

LIST OF TABLES

LIST OF FIGURES

List of Figures (Continued)

ABBREVIATIONS

A	=	Angstrom units
α-DNP	=	α-2,4-dinitrophenyl
BAC	=	Bromoacetyl
CM-lysozyme	=	Reduced and carboxymethylated lysozyme
CMC	=	carboxymethylcysteine
CNBr	=	Cyanogen bromide
DCC	=	Dicyclohexylcarbodiimide
DFP	=	Diisopropylfluorophosphate
γ-globulin	=	Gamma-globulin
IgA	=	8-11S immunoglobins, present in secretions such as colostrum, small amounts in serum
IgG	=	7S immunoglobulin; present in serum
IgM	=	19S immunoglobulin; present in serum
LAP	=	Leucine amino peptidase
μc	=	microcurie
μg	=	microgram
μl	=	microliter
μM	=	micromolar
mg	=	milligram
PBS	=	0.02 M sodium phosphate buffer, pH 6.0, 0.15 M NaCl

TAME	=	p-toluenesulfonyl-L-arginine methyl ester
t-BOC	=	t-butyloxycarbonyl
TEA	=	triethylamine
TMVP	=	Tobacco Mosaic Virus Protein
U-ApoMb	=	Apomyoglobin treated with 8 M urea 5% methanol, pH 3.0.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to Dr. Julia Levy for her encouragement, direction and constructive criticism of both the research and writing of this thesis.

I would also like to thank the members of my committee, Dr. E. Benjamini, Dr. T.H. Blackburn, Dr. J.J.R. Campbell, Dr. D.M. McLean, Dr. G.M. Tener and Dr. J.H. Tremaine for their willing advice and interest in the editing of the thesis.

Also, I would like to thank Mrs. Barbara Mitchell, Mr. John Christensen and Mr. Bob Kezier for their assistance in my research experiments.

Lastly, my thanks go to Mrs. Rita Rosbergen for her patience in the typing of this final manuscript.

INTRODUCTION AND LITERATURE REVIEW

The literature discussed here deals with the most recent observations made in the general area of antigenic characteristics in both synthetic and natural systems, with particular emphasis on the characterization of antigenic determinants in proteins. The research on the historical development of our present knowledge of antigenicity has been copious and divergent. Several good reviews have been published within the past five years dealing with the various specialized aspects (Kabat, 1966; 1968; Sela, 1966; Gill III, Kunz, Friedman and Doty, 1963; Proceedings of The Royal Society, Series B, 1966; Haber and Richards, Sela).

I. The Importance of Molecular Conformation in the Immune Reaction

Beychok and Kabat (1965) and Kabat, Lloyd and Beychok (1969) have shown that the conformation of oligosaccharides can be readily recognized in immune systems. Their work has correlated optical rotary dispersion and circular dichroism measurements as an independent line of evidence for conformation, with immunochemical studies, thus enabling them to explore the role of conformation in antigen-antibody interactions. Optical rotary dispersion measurements

provided estimates of the proportions of α- and β-linked N-acetyl-D-glucosamine residues in staphylococcal teichoic acids in a few hours, whereas the immunochemical studies were extensive and required several weeks of work to achieve similar data. However, it was only by the more sensitive immunochemical method that Torii, Kabat and Bezer (1964) could distinguish mixtures of α- and β-teichoic acids, from both linkages on a single polyribitol phosphate backbone.

Although, to date, studies on the correlation of immunochemical properties with optical rotary dispersion and circular dichroism have been performed principally with oligosaccharide determinants, the same techniques should prove applicable to the study of antigenic determinants of polypeptides and proteins.

Crumpton and Small (1967) demonstrated that a tetradecapeptide from myoglobin, which in the native molecule was predominantly helical, but which became nonhelical in aqueous solution, was able to combine with antibody to metmyoglobin. The peptide, in aqueous solution, may possess a variety of conformations in continuous interchange, but it was presumed that the antibodies would react only with the fraction of molecules whose conformation was the same as that of the corresponding region in the native antigen, namely helical. This presented the possibility that the peptide was stabilized in the helical form in the antibody combining site.

Schlossman, Levine and Yaron (1968) found that the combining sites of antibodies produced against α-2,4-dinitrophenyl-lysine (α-DNP-(Lys)),

were complementary to the determinant α-DNP-$(Lys)_7$, but those produced against α-DNP-$(Lys)_{1200}$ were complementary only to α-DNP-$(Lys)_3$. Kabat suggested that this finding might be related to conformational differences between the two antigens, and that optical rotary dispersion and circular dichroism studies might be beneficial in elucidating this observation, the meaning of which is unclear at this time.

Another study on the structural basis for immune recognition was carried out by Bonavida, Miller and Sercarz (1969) on native hen egg-white lysozyme. The two methionine residues at positions 12 and 105 of hen egg-white lysozyme were cleaved by cyanogen bromide in 70% formic acid. Conversion of the native lysozyme to cyanogen bromide-treated (CNBr-treated) lysozyme caused a red shift in the absorption spectrum. The difference spectrum showed a peak at 2980 Å which has been associated, in some cases, with a shift of tryptophan to a more hydrophobic environment. These workers were interested in establishing how much alteration must take place before one epitope (antigenic determinant) loses its identity and may be recognized as another, by the immune system. Immunochemical studies with the CNBr-treated lysozyme showed that this derivative caused 70% inhibition of the binding of native hen egg-white lysozyme by anti-hen egg-white lysozyme serum, which suggests considerable retention of native conformation. However, from gel diffusion studies, using antiserum to CNBr-treated lysozyme and to native lysozyme, it was shown that CNBr-treated lysozyme had apparently lost some antigenic determinants, and gained new ones not

present on the native (hen egg-white) lysozyme. The explanation proposed was that, near to the points of cleavage, there is relative flexibility in the CNBr-lysozyme, permitting realignment of some of the amino acid residues which, in the parent molecule, were in an unfavourable position for reacting with antibody. This realignment could account for the altered spectral, enzymatic and immunological behaviour of CNBr-treated lysozyme.

Atassi and Habeeb (1969) demonstrated that the nature of a chemical modification at a given site determines its effect on biological activity (enzymatic or antigenic). Native hen egg-white lysozyme and the derivative prepared by nitration of the tyrosines at positions 20 and 23 were employed. This nitrated derivative exhibited conformational changes as shown by its increased susceptibility to tryptic hydrolysis, and the availability of one disulfide group for reduction which was not reactive in the native molecule. The antigenic reactivity was only slightly affected (namely, 77-90% effectiveness relative to the homologous reaction), whereas the enzymic activity was decreased by 50%. When nitrotyrosine residues were reduced to aminotyrosine most of the antigenic reactivity was recovered (namely, 95-99% effectiveness relative to the homologous reaction), despite the fact that conformational changes still existed. This was demonstrated by its similar susceptibility to tryptic hydrolysis, and availability of the one disulfide group for reduction, as with the nitrotyrosine derivative. The loss in enzymic activity resulting from nitration was not recovered on reduction of

the nitrotyrosyl residues. This suggested that the loss of enzymic activity was the result of these conformational changes which appeared to be similar in both the nitrotyrosyl and aminotyrosyl derivatives. It also suggested that the amino acid sequences around these two tyrosines (Tyr_{20} and Tyr_{23}) were important for enzymic activity. The nitro group, in the ortho position to the phenolic hydroxyl of tyrosine, through its electron withdrawing effect, increased the acidity of the phenolic hydroxyl (pK_a values: tyrosine, 10.1; 3-nitro-tyrosine, 7.2). Because the reduction of nitrotyrosyl residues to aminotyrosine (thereby increasing the pK_a to 10.0) resulted in almost complete recovery of antigenic activity, while maintaining the altered conformation, it was suggested that one or both of tyrosines 20 and 23 were present in an antigenic region of lysozyme. It should be noted that this recovery in antigenic activity was only between 5 to 22%, which did not provide conclusive evidence for the presence of the two tyrosines in an antigenic region. Further, these workers found 3 nitrotyrosine, or Gly-3-nitrotyrosine failed to inhibit the reaction of $(NO_2)_2$-lysozyme with its homologous purified antibodies. This indicated that the antigenic specificity was not directed to nitrotyrosyl residues alone, but more probably to the nitrotyrosyl residues in conjunction with its neighbouring residues.

II. Cross-Reacting Antigens

A comparison of the antigenic structure of the mutually related enzymes, papain and chymopapain, was reported by Arnon and Shapira (1968). By the method of cross-immunoadsorption, the antibodies to chymopapain were fractionated using specific papain cross-immunoadsorbent, and the antibodies to papain were similarly fractionated with chymopapain cross-immunoadsorbent. These two antibody species were directed to antigenic determinants common to both enzymes, although it was uncertain as to whether they were directed to identical amino acid sequences on the two proteins. With respect to the quantitative precipitin reaction, their capacity to bind antigens, and their inhibitory capacity toward each of the two enzymes regarding their catalytic activity on a large substrate, the two antibody species were identical. It was also found that both antibody species had a similar inhibitory effect on the catalytic activity of chymopapain on a low molecular weight substrate. However, a dissimilarity between the two species was demonstrated when the inhibition of papain on a low molecular weight substrate was tested. In this case, the antibody species prepared from anti-chymopapain serum by cross-immunoadsorbent was less efficient as an inhibitor than those from anti-papain. This phenomenon might be due to a difference in the affinities of the two species, resulting from the same antigenic determinants being present on different carriers.

From this method of selective fractionation of the antibody species by cross-immunoadsorbents, chymopapain, which has a larger molecular weight than papain, was shown to possess more antigenic determinants than papain. One of these determinants elicited the formation of antibodies which inhibit papain activity on a macromolecular substrate and also on a small substrate.

III. Synthetic Antigens and Antigenic Determinants

From the study of synthetic polypeptide antigens, several factors influencing antigenicity may be determined (Sela, 1966). Gill, Kunz and Papermaster (1967) carried out extensive studies on the immunogenic properties of synthetic polypeptide antigens in order to elucidate several factors influencing antigenicity. The quantitative control of antibody production was found to depend upon (i) the chemical composition of the antigen, where polymers containing aromatic amino acids generally elicited more antibody than those containing no aromatic residues; (ii) an excessively high charge on polypeptides depressed their antigenic potency; (iii) the optical configuration of the component amino acids, where D-amino acid polymers exhibited poorer immunogenicity than those composed of L-amino acids. In order to elicit antibody formation, however, it was essential to administer D-amino acid polymers in small doses in order to prevent immunological paralysis.

Jaton and Sela (1968) used multichain polyproline as the carrier macromolecule for attachment of D- or L-amino acids to test the role of optical configuration in immunogenicity. These multichain polyproline macromolecules of either optical configuration did not provoke antibody formation in rabbits and therefore could be used as inert or nonantigenic carrier macromolecules. Attachment of as little as 8.5% of L-amino acid residues on the outside of a macromolecule composed exclusively of D-amino acid residues converted it to a good immunogen. These workers also demonstrated that a macromolecule containing as much as 95.5% of L-amino acids, but having all its polymeric side chains terminating in D-amino acids, was just as poor an immunogen as a similar macromolecule composed exclusively of D-amino acids. These results of Jaton and Sela's work substantiated exactly the work of Gill et al.(1967).

IV. Determination of the Size of Antigenic Determinants

From work using synthetic peptides of known sequence attached to inert carrier molecules, the smallest antigenic fragments have been shown to be 6 to 7 amino acids in length. Arnon, Sela, Yaron and Sober (1965), who used polylysyl rabbit serum albumin with an average chain length of 5.5 lysines, showed that the inhibiting power of the peptides for the homologous antigen at the equivalence point of the antigen-antibody reaction increased sharply up to pentalysine and then

only slightly more up to nonalysine. The ultimate goal in determining the measurement of the size of the antibody combining site is to isolate a single antigenic determinant and know its structure and conformation in solution. Immunochemical studies on the poly-D-glutamyl capsule of Bacillus anthracis by Goodman, Nitecki and Stoltenberg (1968) have shown that the maximum size for the region on the polypeptide which combines with the antibody site is six residues of D-glutamic acid. This was tested by the quantitative precipitin inhibition reaction. The dimensions of hexaglutamic acid are 36 x 10 x 6 A. These dimensions are in close agreement with the dimensions found for the antigenic determinants on dextran which were 34 x 12 x 7 A (Kabat, 1960). It is interesting to note the close agreement in size reported above, in which instances two different antigens, a polypeptide and a carbohydrate, and two different species of animals, rabbits and human beings, were used respectively. The work on peptides derived from this homologous capsular polypeptide also demonstrated the heterogeneity of the combining sites of antibodies in the sera tested from three rabbits similarly immunized with whole capsular polypeptide. With two of the sera, increments of precipitin-inhibiting-efficiency were obtained up to the pentamer, which was equal in inhibiting capacity to the hexamer. However, the third antiserum could distinguish between the pentapeptide and hexapeptide, by the greater-inhibiting-efficiency of the hexapeptide, indicating that there may be individual animal differences in the response to antigenic stimulus.

A number of experiments demonstrated that when a single hapten is attached to a known residue of a pure protein, or to a polypeptide backbone (Kantor, Ojeda and Benacerraf, 1963; Eisen and Siskind, 1964) not a single antibody species, but whole families of antibodies were produced in response. This heterogeneity of most antibodies is partly due to the presence of different antibody classes such as IgG, IgM and IgA and their respective subclasses, and to genetic differences, as well as being dependent on the antigenic complexity of the immunogen. Because the dimensions of the antibody combining site are considerably larger than most small organic molecules used as haptens (Kabat, 1960; 1966; Sage, Deutsch, Fasman and Levine, 1964), the sites may encompass not only the hapten but also different sections of the protein to which the hapten is attached. Richards et al. (1969) studied the relationship between antigenic complexity and heterogeneity in the antibody response. In this respect, Richards and workers tried to elucidate the role of the immediate environment in its interaction with hapten. They synthesized amino acid copolymer with a defined sequence and an approximate molecular weight of 10,000 which had the 2,4-dinitrophenyl-hapten (2,4-DNP-hapten) at regular intervals of about 30 A along the backbone, when the polymer was in its extended form. The structure was $(\alpha\text{-DNP-L-lys-(D-ala-L-ala)}_5)_n$ where n = 10.2 on the average. The microenvironment around the hapten was relatively homogeneous and

nonantigenic, and in its extended form the hapten groups were separated by distances greater than the largest dimensions estimated for the antibody combining site, approximately 34 Å, as reported by Kabat (1960). In aqueous solution at pH 7.0, the polymer is a random coil with no evidence of β-conformation or α-helix because of the alternating D- and L-amino acid sequence. This defined sequence polymer with the DNP-hapten was studied along with the DNP-hapten in two other environments of increasing heterogeneity.

The second antigen was a statistical N-carboxyanhydride polymer with a residue ratio of DL-alanine to DNP-lysine of 10.6:1, and having the same amino acid composition as the defined sequence polymer. The third, most complex antigen was randomly dinitrophenylated bovine γ-globulin. Studies of the immune response produced in rabbits injected with these three antigens indicated that as increasing degrees of "order" are imposed on the haptenic environment, the antibody response becomes more homogenous. The experiments involved dispersion of molecular charge distribution, and variations in the magnitude of noncovalent interactions between the hapten and the antibody populations given as association constants.

These workers also produced evidence that antibodies to the defined-sequence polymer had early maturation properties showing no rise in the average binding energy later in the immune response, and were all of the same charge. The N-carboxyanhydride polymer having the same composition, but a less rigidly ordered environment of the

dinitrophenyl-amino alkyl residue, gave rise to antibodies having intermediate maturation properties and some difference in charge distribution. Antibodies produced to randomly dinitrophenylated bovine γ-globulin showed a marked rise in association constants with late response antibodies, and also showed a greater difference in charge distribution.

V. Antigenic Determinants on Protein Antigens

A major approach to the study of antigenic determinants and the dimensions of antibody combining sites must ultimately involve research on natural protein antigens. In instances where this approach has been undertaken, antigens have been degraded and the haptenic peptides isolated and characterized. It has been shown from these studies that proteins may contain many different antigenic determinants.

1. Tobacco mosaic virus protein subunit (TMVP)

The most extensive of these studies so far have been those on TMVP by Benjamini and his associates (Benjamini, Young, Shimizu and Leung, 1964; Benjamini et al.,1965). From the TMVP subunit, a single polypeptide chain of 158 amino acid residues, a twenty amino acid peptide was found to be the only haptenic one present after trypsin digestion. Further investigation of this peptide showed that the

C-terminal five amino acids (sequence Leu-Asp-Ala-Thr-Arg) were the critical ones for antigenicity (Young, Benjamini, Stewart and Leung, 1967). Subsequent work involving solid phase peptide synthesis demonstrated that the hydrophobicity of the N-terminal portion of this pentapeptide enhanced the binding of antibodies (Benjamini, Shimizu, Young and Leung, 1968). The N-(^{14}C) octanoyl derivatives of the C-terminal tetra-, tri-, and di-peptides of the haptenic pentapeptide were synthesized and tested for their capacity to bind specifically with anti-TMVP antibodies. The results showed that the octanoyl tripeptide and tetrapeptide exhibited specific binding, but not the dipeptide (Benjamini et al.,1968). By solid phase peptide synthesis, Benjamini's group showed that the shape of the aspartic acid residue was more important than its negative charge, since asparagine could be substituted for aspartic acid, but glutamic acid could not (Young, Benjamini and Leung, 1968). In another set of experiments, the C-terminal arginine residue was shown to be more important than the N-terminal leucine, in that removal of the arginine from the C-terminal of the peptide resulted in a pronounced decrease in haptenic activity, whereas removal of the N-terminal leucine or both the leucine and aspartic acid did not (Young, Benjamini, Shimizu and Leung, 1966).

2. Bradykinin

The work of Spragg et al. (1968) on antibody to bradykinin was

also an attempt to elucidate the structural and minimal requirements for the binding of antigen to antibody. In this study, antibodies were produced by rabbits injected with a branch-chain copolymer of bradykinin, a nonapeptide, and poly-L-lysine. Intrinsically labelled (^{14}C)-bradykinin and singly substituted alanine analogues of bradykinin and bradykinin fragments were tested in competition experiments for binding to the antibody. The amino acid sequence of bradykinin is Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. Alteration of charge by replacing arginine with nitroarginine or the hydrophobic character, by replacing phenylalanine with alanine, had relatively little effect on binding to antibody. However, substitution at proline, position 3, or glycine, position 4, which are obligatory for conformation, produced the most profound change in binding. From the experiments utilizing fragments of the sequence, it was shown that the antibody recognized the peptide in a preferred conformation which required the entire nonapeptide sequence.

3. Human serum albumin

Lapresle and Webb (1965), using prolonged peptic, chymotryptic and tryptic digestion of human serum albumin, showed that one fragment with a molecular weight of 6,600 possessed only one antigenic determinant which partially inhibited the precipitation reaction between serum albumin and its homologous antiserum. Using the

immunoadsorbent technique it was found that only about 1% of the antibody population to whole serum albumin was directed against this determinant.

4. Sperm whale myoglobin

In sperm whale myoglobin, Crumpton and Wilkinson (1965) found eight chymotryptic peptides which had the capacity to inhibit precipitation using both antisera to metmyoglobin and apomyoglobin, and their respective antigens. However, the greatest degree of hapten inhibition obtained was 15% which indicates: that there are many more antigenic determinants present in other portions of the molecule; that these peptides could not assume the correct conformation to show maximal inhibition; or that these peptides did not represent the complete sequence of any single determinant.

Atassi's work (1968) on nitrated apomyoglobin and metmyoglobin showed that one or both tyrosines 146 and 151 are present in a reactive region of the molecules, since the nitrated derivatives possessed lowered antigenic reactivities relative to apomyoglobin and metmyoglobin with their homologous antisera. A peptide fragment containing residues 56-131 and its derivative nitrated at tyrosine 103 (the third tyrosine in metmyoglobin) showed identical reactivity to the amount of antibody specifically adsorbed from antiserum to metmyoglobin, and therefore it was concluded that this tyrosine residue was not located in an antigenic region.

By similar methods, Atassi and Caruso (1968) showed that a derivative of apomyoglobin modified at tryptophan 7 was identical immunochemically to apomyoglobin pretreated with 8.0 M urea - 5% methanol at pH 3.0 (U-ApoMb) and with metmyoglobin prepared by complex formation between ferriheme and U-ApoMb respectively, and therefore it was concluded that tryptophan 7 was not an essential part of an antigenic site. If conditions were chosen so that both tryptophans 7 and 14 were modified, there was a drastic decrease in antigenic reactivity due probably to changes in conformation of the molecule by unfolding. Using mild periodate oxidation conditions, so that only methionine residues at positions 55 and 131 were oxidized, Atassi (1967) showed that these two residues were also not essential parts of the antigenic determinants of metmyoglobin.

An interesting observation made from studies on tryptic digests of sperm whale myoglobin following isolation of soluble peptides by chromatography was that the five immunochemically reactive tryptic peptides occupied mainly the four corners on the surface in the three-dimensional structure of this molecule proposed by Kendrew et al. (1961). The peptides only showed inhibitory activity by quantitative precipitation, and did not precipitate antibody. Therefore, each peptide was assumed to contain only one antigenic determinant.

Cleavage at the two methionine residues at positions 55 and 131 shown previously to be non-essential for antigenicity, produced three fragments which accounted for almost all the immunochemical reactivity of the intact molecule. The two largest fragments, 1 - 55 and 56 - 131

had the capacity to precipitate with antiserum against the whole protein, and therefore contain at least two antigenic determinants but fragment 132 - 153 acted as a true hapten possessing only the ability to inhibit precipitation between the whole molecule and its antiserum. In the fragment containing the residues 1 - 55, the tryptophan at position 7 can be cleaved to yield a small peptide, 1 - 7, and the remaining 8 - 55. The small N-terminal peptide was found to possess no immunological reactivity. However, there was some evidence that this sequence might be important for the proper orientation of the reactive regions on fragment 1 - 55, since the precipitation obtained with fragment 8 - 55, for a given serum, was considerably less than that from the complete fragment 1 - 55.

5. Ribonuclease

Oxidized ribonuclease was digested with trypsin, chymotrypsin, and pepsin, and various peptides were isolated. Brown (1962) found that peptides from two different regions of the protein specifically inhibited the immune reaction between rabbit antibody and oxidized ribonuclease. On a molar basis, the larger, 24-amino acid peptide caused 50% inhibition of the precipitin reaction between oxidized ribonuclease and its homologous antiserum. Further enzyme digestion of this peptide showed that the antigenic region lay within the first 15 amino acids of the sequence. The other peptide, 19 amino acids

in length, and near the C-terminal of the antigen, caused 22% inhibition of the precipitin reaction with the same system. Both these haptenic peptides are rich in non-polar amino acids which may be important in maintaining their stability and secondary structure in an aqueous environment.

It is interesting to note that performic acid oxidized, or reduced and carboxymethylated ribonuclease, lacking disulfide bonds and therefore much of its tertiary structure, does not precipitate antibody directed against native ribonuclease. This lack of cross-reactivity between antibodies directed to the native well-characterized three-dimensional form of the protein, and the reduced and carboxymethylated form of an antigen was also demonstrated with the lysozyme and CM-lysozyme system (Gerwing and Thompson, 1968). It should also be noted here that in both these instances, all haptenic peptides so far demonstrated, contain one or more cysteinyl residues.

6. Bacterial ferredoxins

In proteins, such as bacterial ferredoxins, in which the total tertiary structure has not been established, the immunological method may elucidate in part the significance of conformation in the antibody combining site. Work on clostridial ferredoxins, using the performic acid oxidized derivative and reacting it with antibodies to native ferredoxin, has shown that a high degree of cross-reactivity

exists in this instance (Nitz, Mitchell, Gerwing and Christensen, 1969). In fact, it was found that the immune reaction appeared to be greater between oxidized ferredoxin and antiserum against the native molecule than it was with the homologous system. This might indicate that in the animal body, the native ferredoxin undergoes alteration, possibly resulting from the removal of iron molecules which are bound to the cysteine residues by noncovalent intermolecular forces. The observation that a considerable degree of cross-reactivity also existed between performic acid oxidized and carboxymethylated ferredoxin and their respective antisera also indicated that the cysteine residues in this system, none of which are involved in disulfide bonds, do not play a direct role in the regions responsible for antigenicity.

7. Native lysozyme

Shinka et al. (1967) recently published evidence that in native lysozyme, four peptic peptides exhibited haptenic properties. All these peptides contained the sequence from Gln 57 to Ala 107 (peptide 7a), and differed only in their cleavage points in the loop between Cys 80 and Cys 94. Further work by this group (Fujio, Imanishi, Nishioka and Amano, 1968a) showed that another immunologically active peptide could be isolated by limited pepsin digestion, which contained both the N- and C-terminal peptides

linked by a single disulfide between Cys 6 and Cys 127. This peptide contained the sequence Lys 1 to Asn 27, and Ala 122 to Leu 129 and was designated peptide 17. Equilibrium dialysis experiments showed that 47% of the antibody in the 7 S anti-hen egg-white lysozyme was directed against this peptide. These two antigenically active peptides were independent of each other, as shown by the observation that there was no inhibition of binding of one of these peptides to antiserum in the presence of the other (Fujio, Imanishi, Nishioka and Amano, 1968b).

Arnon and Sela (1969) have also studied the antigenic properties of native lysozyme. They prepared a synthetic conjugate composed of a fragment of lysozyme (sequence Cys 64 to Leu 83) which they designated as the "loop" peptide, and branched poly-DL-alanine. This peptide comprises a portion of the longer haptenic peptide isolated by Shinka et al.(1967) which was designated peptide 7a. This conjugate was used to immunize rabbits to produce antibodies specifically against this unique region in the native lysozyme molecule, and the purification of the antibodies was carried out using a lysozyme-cellulose immunoadsorbent. By preparing an immunoadsorbent from this "loop" peptide, Arnon and Sela were able to isolate antibodies of similar specificity from anti-lysozyme serum. These anti-"loop" antibodies were able to distinguish between the "loop" peptide, containing a disulfide bond, and the open-chain carboxymethylated peptide derived from it, suggesting that they may be directed against a conformation-dependent determinant.

These workers felt that the availability of the two populations of antibodies, directed most likely to the same region within the native protein, will provide an opportunity to compare them and to learn the role of the antigenic carrier in the homogeneity of antibodies to a specific determinant. This proposed work will be interesting in view of the previously mentioned work of Richard et al.(1969) on the role of the environment around the hapten in the immune response.

The lysozyme molecule was chosen for study for several reasons. Firstly, the complete amino acid sequence is known; secondly, X-ray crystallography has been completed on the molecule and a three-dimensional structure has been proposed, so that the areas found to be haptenic can be correlated with their location in the crystalline structure; thirdly, the four disulfide bonds in the single polypeptide chain give the native molecule a fairly rigid tertiary structure; finally, lysozyme is a highly basic protein with a pI (isoelectric point) of pH 11.05, and a study of its antigenic determinants might be interesting in voew of the work of Sela and Mozes (1966) demonstrating that antibodies produced to basic protein antigens are found in the acidic fraction of immunoglobulins after chromatography on DEAE-Sephadex.

8. Reduced and carboxymethylated lysozyme (CM-lysozyme)

In this study, reduced and carboxymethylated lysozyme (CM-lysozyme) was used as the antigen. Results from initial experiments indicated that CM-lysozyme did not cross-react at the precipitin level with native lysozyme antiserum, and none of the tryptic peptides isolated from it showed any capacity to act as haptens with the native system.

Unpublished work by Young and coworkers from Benjamini's group has substantiated these observations showing the lack of inhibition of the lysozyme-anti-lysozyme reaction by small peptide fragments isolated from CM-lysozyme, when they tested them either by complement fixation or radio-immunoassay techniques. They found that CM-lysozyme would react with anti-lysozyme serum at antibody concentrations higher than those required for the binding of an equivalent amount of lysozyme. This CM-lysozyme-anti-lysozyme reaction could not be inhibited by chymotryptic digests, but could be inhibited by tryptic digests of CM-lysozyme, or by peptides containing the N- and C-terminal regions, Lys 1 to Met 12, and Asn 106 to Leu 129, isolated from cyanogen bromide (CNBr) cleavage of CM-lysozyme. Because this CM-lysozyme-anti-lysozyme reaction could not be inhibited by native lysozyme unless the lysozyme was in extremely high concentration (1,000 fold excess), these workers

suggested that the antibodies involved in binding the CM-lysozyme are, in fact, more specific to a denatured form of lysozyme which may be present in small amounts in preparations of this enzyme. The determinant areas existing in the tryptic peptides and the C-terminal CNBr peptide may not have the same conformation as in the native crystalline lysozyme, and this may explain their inhibitory activity.

From the work reported in this thesis on CM-lysozyme, it was shown that a single tryptic peptide encompassing the sequence Asn 74 - Lys 96 exhibited strong haptenic activity. It is interesting to note that this peptide falls within the fragment isolated by Shinka et al. (1967) from peptic digests of native lysozyme which showed haptenic activity, and overlaps the "loop" peptide studied by Arnon and Sela. (1969).

MATERIALS AND METHODS

1. Immunization Procedures

The hen egg-white lysozyme used in all experiments was purchased from Worthington Biochemical Corporation. Reduction and alkylation of the four disulfide bonds of the lysozyme molecule with iodoacetic acid was carried out according to the method reported by Canfield and Anfinsen (1963). The treated lysozyme, termed CM-lysozyme, was lyophilized and stored at 4 C as a powder until required. For immunization, 30 mg of CM-lysozyme was suspended in 1.5 ml sterile 0.9% saline and emulsified with 1.5 ml complete Freund's adjuvant (Bacto-Difco). Immunization of the three rabbits with the material was carried out as follows. Rabbits were anaesthetized with 1.0 ml of nembutal (Abbott Laboratories) administered intravenously. The emulsion (0.2 ml) was injected into a foot pad of each of the four extremities, and a further 0.2 ml was injected intramuscularly in the left hind leg. On the following day, 1.0 ml of alum-precipitated antigen containing 1.5 mg of CM-lysozyme was injected intravenously into each animal. The alum-precipitated antigen was prepared according to the method described by Kabat and Mayer (1961 - p. 309).

The antibody titer was elevated four weeks after the last injection, and remained unchanged in successive samples collected weekly for six

months. The serum was stored in 50.0 ml batches at 4 C. After it was determined that the titer of the serum from two of the rabbits was identical, the two sera (from rabbits 1 and 2) were pooled as they were collected. Merthiolate at a final concentration of 1:10,000 was added as a preservative.

After six months, the rabbits were given booster injections of 0.5 ml Freund's adjuvant emulsion containing 3.0 mg CM-lysozyme intramuscularly in the left hind leg. Additional serum samples were collected at weekly intervals starting two weeks after the booster injection, and stored as above.

II. Quantitative Precipitin Reaction

The immune precipitation reaction was used for initial studies on the antigenic properties of reduced and carboxymethylated lysozyme (CM-lysozyme). Optimal proportions were determined by incubating varying amounts of antigen, in a constant volume, with constant amounts of antiserum at 37 C for 1 hr followed by 18 hr at 4 C. The immune precipitates were centrifuged at 2000 g, washed twice with 0.9% NaCl, and dissolved in 1.0 ml of 0.1 N NaOH. Then the absorbance at 2800 A was read on a Beckman DB-G spectrophotometer. A stock solution of CM-lysozyme containing 100 µg/ml in 0.9% NaCl was used to titrate sera from all three rabbits. Sera 1 and 2 which had identical titers precipitated optimally with 25.0 µg of CM-lysozyme/ml of undiluted serum. Serum 3

precipitated optimally with 50 µg of CM-lysozyme under the same conditions. Because of its higher titer, serum 3 was selected for use in all further extensive precipitin tests.

Hapten inhibition studies with tryptic peptides were performed as follows. The peptide in 0.1 ml of 0.9% NaCl was mixed with 0.4 ml of undiluted serum and incubated at 37 C for 1 hr followed by 18 hr at 4 C. Antigen was then added at the calculated amount to yield optimal precipitation with 0.4 ml of antiserum, and the incubation at 37 C and 4 C was repeated. Control tubes using 0.1 ml of saline instead of peptide were treated identically. The resulting precipitates were then prepared as previously described and read for 2800 A. The percent inhibition was calculated as the percentage decrease in optical densities at 2800 A taking the saline control as 100%. These studies were always conducted in triplicate unless otherwise stated.

III. Complement Fixation Reaction

Freeze-dried guinea pig serum (3.0 ml quantities) obtained from Hyland Company was used as a source of complement. The serum was reconstituted for each test in the diluent provided and further dilutions were made in veronal buffered saline, between 1:25 and 1:250, between 1:50 and 1:500, or between 1:75 and 1:750 depending on the batch of guinea pig serum. The veronal buffered saline used for all dilutions of antigen, haptens, complement and antiserum was

prepared according to the method of Brooksby (1952). A pooled sample of antiserum 1 and 2, and a stock solution of CM-lysozyme containing 100 µg/ml were used throughout.

The complement fixation titer of the antiserum was determined by using ten dilutions of complement between the values given above, doubling dilutions of the antiserum starting at 1:10 up to 1:160, and one dilution of antigen, 0.4 µg/ml. The complement, antigen and antiserum dilutions were all used in 1.0 ml quantities per tube. A similar test to test for anticomplementary activity in the antiserum was set up simultaneously using 1.0 ml of veronal-buffered saline instead of the antigen. The tubes were mixed by shaking and incubated overnight at 4 C. A 2% suspension of twice-washed sheep erythrocytes in veronal-buffered saline was sensitized by mixing it with an equal volume of a 1:50 dilution of the recommended standard solution of hemolysin (Difco-Bacto Antisheep Hemolysin) for 10 min. This preparation was added in 1.0 ml aliquots to the test series and incubated at 37 C for 30 min in a water bath. The tubes were shaken once at 15 min. Following the 30 min incubation, unhemolyzed red blood cells were sedimented by centrifugation at 1800 g and the supernatants were read on a Klett colorimeter using the green filter. End points were calculated as 50% hemolysis on a probit plot of the actual percentages of the Klett readings according to the method described by Wright (1963). Titers of the pooled test antisera 1 and 2 were 1:80 in the presence of the standard amount of CM-lysozyme, 0.4 µg/ml.

The fresh pooled antiserum was heated at 55 C for 20 min to inactivate any complement. No anticomplementary activity was demonstrated by the pooled antiserum preparation (even at a 1:20 dilution) after heat inactivation.

The antigen was titrated by repeating the tests using 1:80 antiserum throughout with doubling dilutions of antigen ranging from 6.4 to 0.0125 µg/ml. The data showing the calculation of the optimal antigen range are shown in Figure 1.

Hapten inhibition studies involved the use of standard amounts of antigen (0.8 µg/ml) and antiserum (1:80) and varying amounts of complement and peptide. The test peptides in 0.2 ml were all added initially at a concentration of 50 µg in 0.2 ml. Preliminary experiments with all the tryptic peptides from CM-lysozyme indicated inhibition in only two preparations, the pooled tryptic digest and one purified peptide. The positive tests were re-assayed over a range of 50.0 to 0.10 µg. The hapten inhibition tests were carried out as follows. The peptide-antiserum mixtures were incubated at 37 C for 1 hr and 18 hr at 4 C. Subsequently, 0.8 µg of antigen in 1.0 ml, and 1.0 ml of complement containing dilutions between 1:40 and 1:125 of guinea pig serum, were added and the mixtures were left overnight at 4 C. On the third day, the indicator hemolysin system was added as previously described, and end points were again taken as 50% hemolysis.

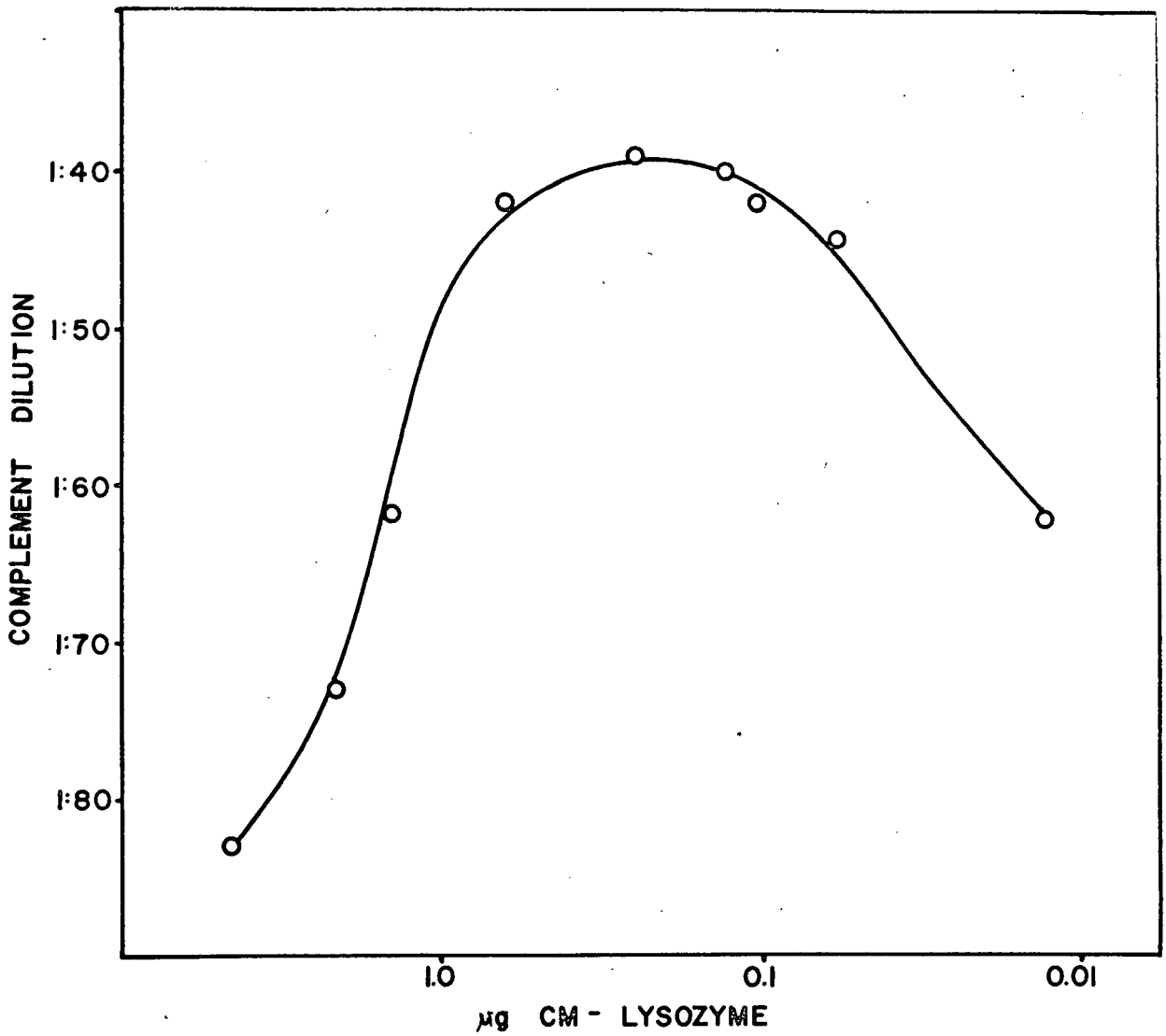


Figure 1. Complement fixation titration curve of CM-lysozyme.

IV. Preparation of Tryptic Peptides

The carboxymethylated lysozyme was digested with trypsin (Worthington, lyophilized containing 125 units/mg) as described by Canfield (1963a). The trypsin was assayed using the hydrolysis of p-toluenesulfonyl-L-arginine methyl ester (TAME) by trypsin and measuring an increase in absorbance at 2470 A (Hummel, 1959). One unit of trypsin activity is defined as the hydrolysis of 1 µmole of TAME/min at 25 C and pH 8.1 in the presence of 10 mM Ca^{++}. The assay for activity and the calculation of the units of activity is shown in Figure 2. The digestion of CM-lysozyme involved a 1% solution of CM-lysozyme in distilled water with the pH maintained at 8.0 with dilute NH_4OH, and trypsin in an amount equal to 2% of the CM-lysozyme by weight. The solution was stirred at 37 C for 2 hr for optimal trypsin digestion and the pH was periodically adjusted to 8.0. The insoluble core material was removed by centrifugation. Digests usually lacked the ability to precipitate with homologous antiserum by the ring test, but the precaution was taken of passing them through an ultrafilter designated to retain material of more than 10,000 molecular weight (Amicon ultrafilter, UM-1 filter pad) before purification of the peptides chromatographically. In the initial stages of the work, Canfield's method (1963a) for peptide separation, using a Dowex 50 x 4 (Bio-Rad) column of dimensions (0.7 x 150.0 cm) at 35 C, with an eight-chambered

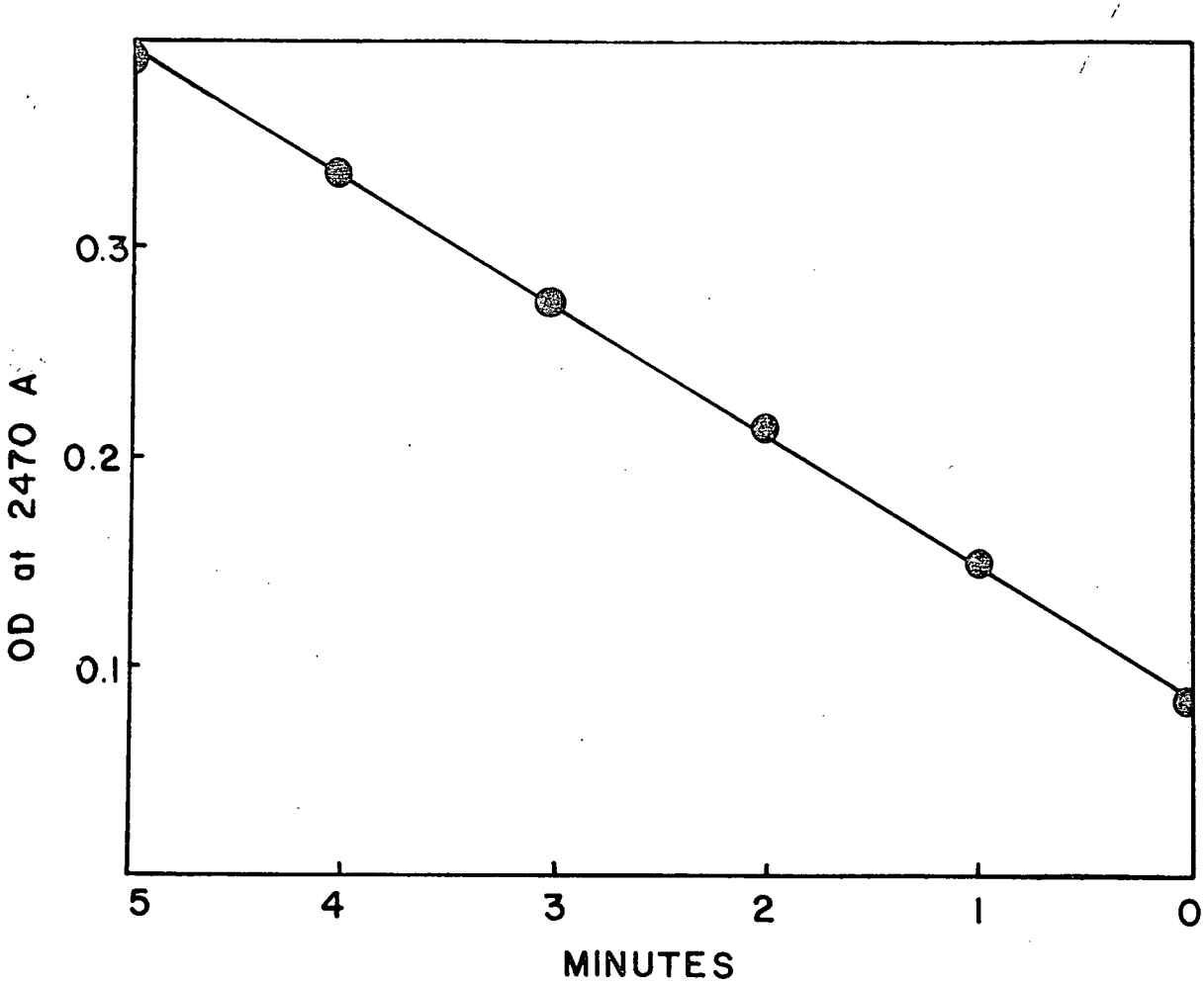


Figure 2. Assay of enzyme activity of the trypsin preparation by hydrolysis of the substrate p-toluenesulfonyl-L-arginine methyl ester.

gradient system, was employed, with each buffer at a volume of 500.0 ml. In the first column separation, 100 mg of tryptically digested CM-lysozyme was adjusted to pH 2.8 with formic acid, applied to the column, and washed with three 1.0 ml aliquots of the starting buffer. Gradient elution with an increasing pH and salt concentration was used with pyridine-acetate buffers ranging from 0.10 M acetic acid pH 3.8 up to 2.0 M acetic acid pH 5.18. Fractions of 5.0 ml were collected on an LKB fraction collector, and 0.3 ml aliquots of every other tube were taken and analyzed by the quantitative ninhydrin reaction after alkaline hydrolysis according to the method of Hirs et al. (1956). A representative elution profile with the eight-chambered gradient system is shown in Figure 3. Each peak was pooled and dried by flash evaporation at 45 C, washed with distilled water, freeze dried, and dissolved or resuspended (some peptides were only sparingly soluble) in 0.9% NaCl.

From initial immunological studies, strong haptenic activity was observed in the peak containing the T-11 peptide (Canfield's (1963b) nomenclature), and a concomitant lack of haptenic behaviour on the part of any other peptide fraction was noted. Therefore, in further peptide separations, the main purpose of which was to collect T-11 material, a simplified four-chambered gradient system was set up using the first four pyridine-acetate buffers recommended by Canfield, ranging from 0.1 M acetic acid pH 3.8 to 0.4 M acetic acid pH 4.47. In this modified system, 500 ml of each buffer were again used and the peptides

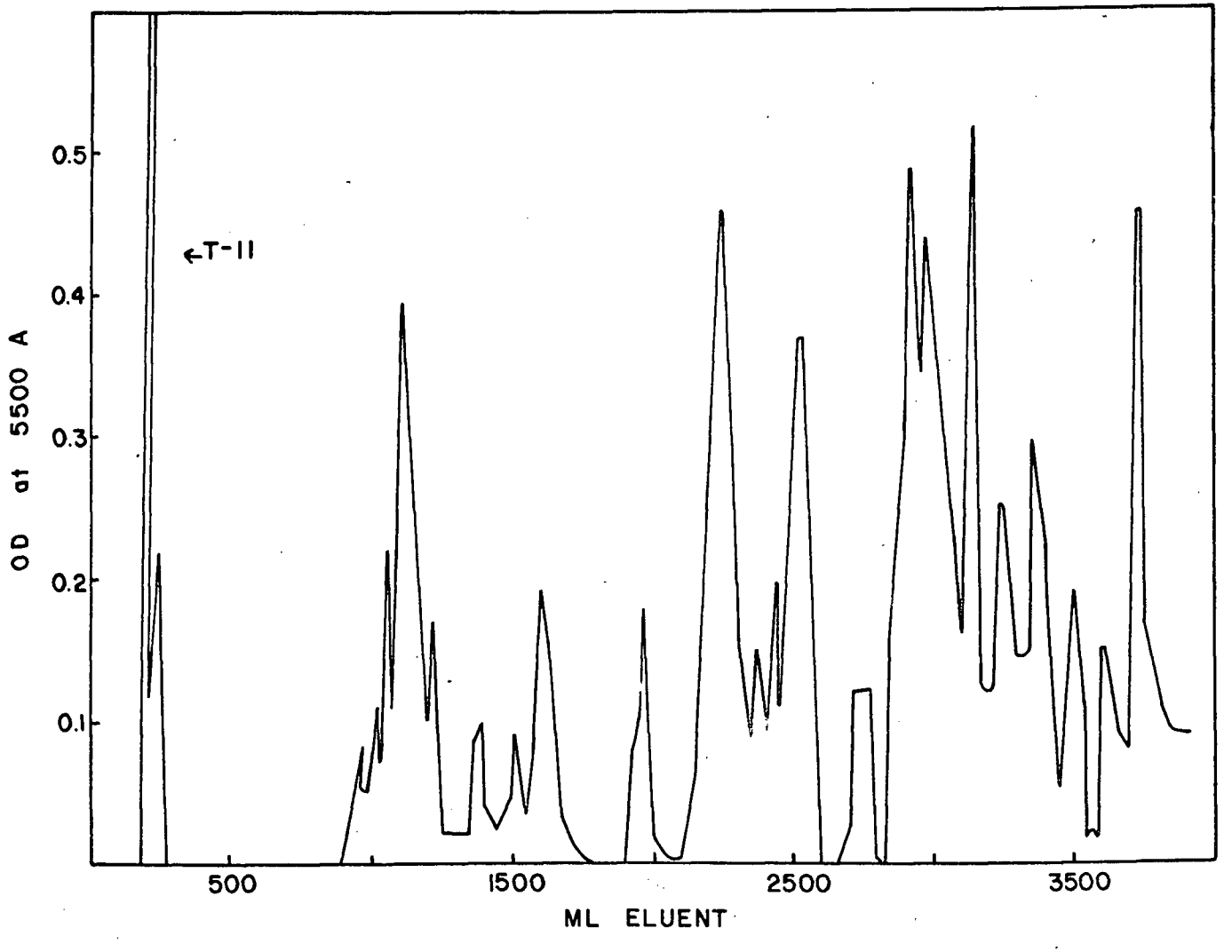


Figure 3. Elution profile of tryptic peptides of CM-lysozyme using the eight-chambered gradient system of Canfield (1963a).

were eluted through the Dowex 50 x 4 column at 35 C until 1.0 l had been collected. Then the remaining adsorbed peptides were eluted by running 2.0 M buffer at pH 5.18 through the column. Fractions of 10.0 ml were collected from which 0.3 ml aliquots were taken and analyzed by the quantitative ninhydrin reaction as described above. Figure 4 shows a representative elution profile of the modified system.

The T-11 peptide was eluted as the first sharply delineated peak, and was free from other peptides eluted later. Amino acid analysis of this material established its purity and identity with Canfield's T-11 peptide. Table II presents the results of the amino acid analysis of this material as well as the results reported by Canfield. The ninhydrin positive material eluting with the 2.0 M pyridine-acetate buffer at pH 5.18 from the column was pooled and tested for haptenic activity at a concentration of 200 µg, to confirm that no haptenic peptides were present in this mixture of tryptic peptides.

V. Protein and Peptide Analysis

Quantitative estimation of carboxymethylated lysozyme was calculated initially by the Lowry method (Lowry et al.,1951) for protein determination, and equated on a weight basis with its extinction at 2800 A. Subsequently, estimations were made on a spectrophotometric basis. The T-11 and other peptides were quantitated initially on a dry weight basis. Closer estimations of

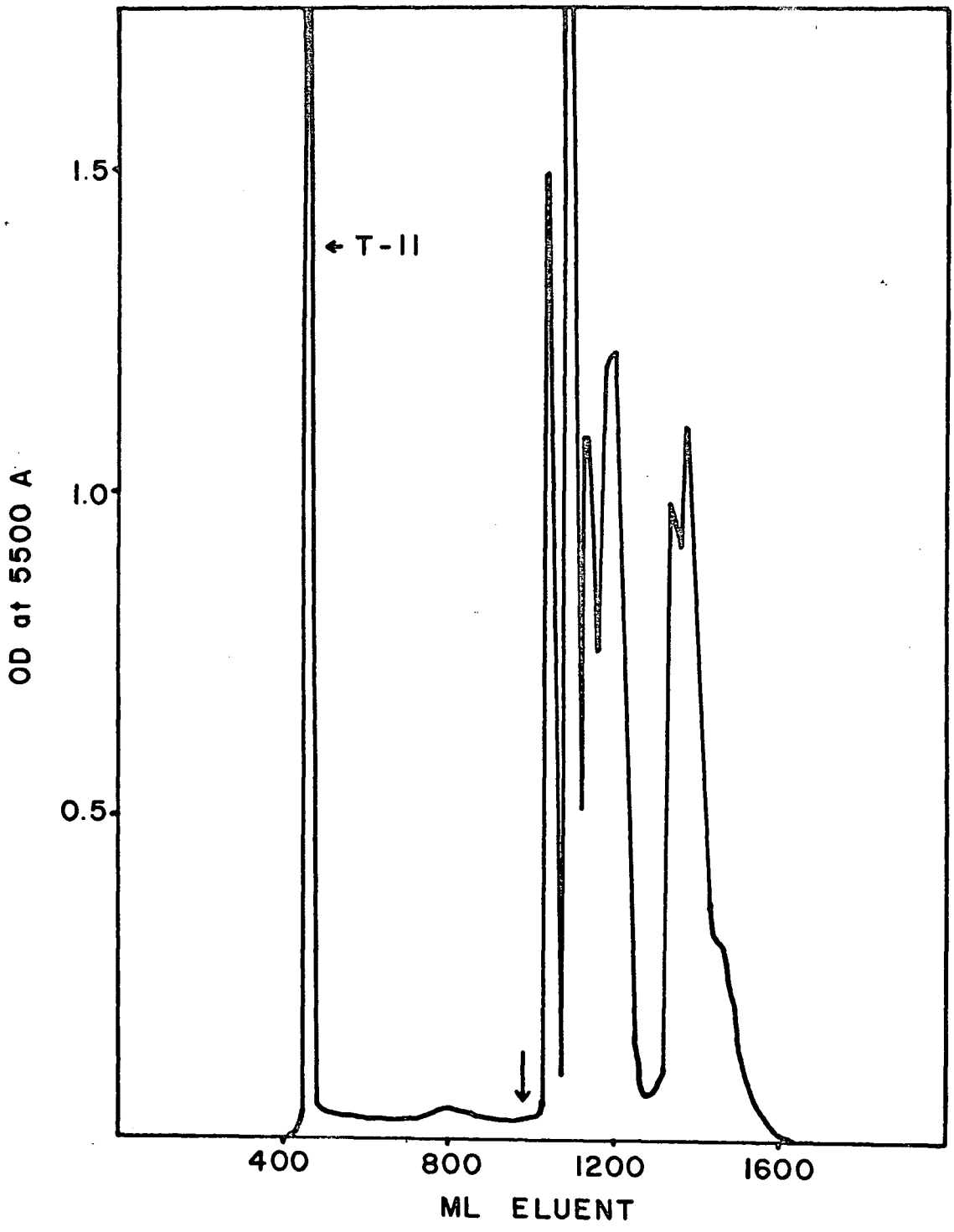


Figure 4. Elution profile of tryptic peptides of CM-lysozyme using the simplified four-chambered gradient system. Arrow designates change to 2.0 M buffer at pH 5.18.

the T-11 peptide were obtained from the calculation of micromoles of singly occurring amino acid residues present after quantitative amino acid analysis. Amino acid analyses were carried out according to the method of Spackman et al. (1958) on a Beckman model 120 Amino Acid Analyser.

The T-11 peptide could not be quantitated on the basis of its extinction spectrophotometrically since it contains no aromatic amino acids. However, its absorption spectrum at least indicated no contamination with tryptophan-containing peptides (this would not show up on amino acid analysis). A typical scan of this peptide from 3000 - 2200 Å is shown in Figure 5.

The main indication of the purity of the T-11 peptide was that finger print analysis showed the presence of only one ninhydrin positive spot. High voltage electrophoresis was carried out for 45 min at 3000 V on a Gilson High Voltage Electrophoretor in pyridine-acetate buffer (10%; 0.4%) pH 6.5 on Whatman #3 filter paper with 0.1 mg of the T-11 preparation. For the second dimension, the electrophoresed strip was sewn onto another piece of Whatman #3 filter paper and descending chromatography was carried out in butanol-pyridine-acetic acid-water solvent (10:15:3:12, v:v:v:v) at 25 C for 18 hr.

VI. Carboxypeptidase Digestions

Carboxypeptidase B (Worthington COB, not DFP-treated) was assayed for enzyme activity according to the rate of hydrolysis of

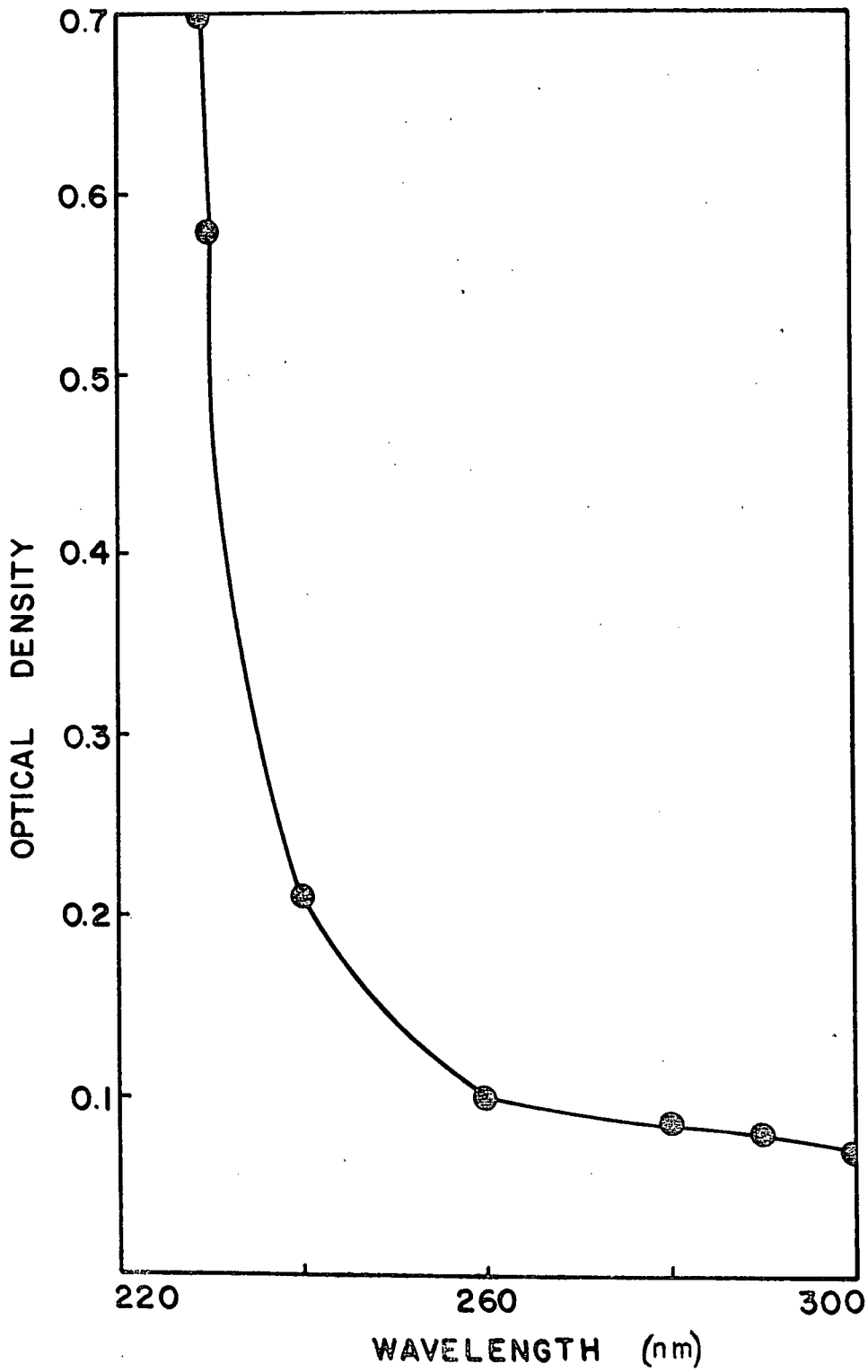


Figure 5. Typical scan of the T-11 peptide at a concentration of 0.10 mg/ml.

hippuryl-L-arginine (Folk et al., 1960). The rate of hydrolysis of the substrate was determined by recording the increase in absorbancy at 2540 A over one minute intervals. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1.0 µmole of substrate per min under the conditions specified. Once the activity of the carboxypeptidase B (COB) had been determined (shown in Figure 6), an experiment was set up using 1.0 ml of T-11 in saline (containing 1.875 mg or 0.75 µmole) and 0.4 ml of COB in 0.025 M Tris buffer (containing 120 µg which possessed 0.25 units of enzyme activity) at pH 8.5 for 4 hr at 37 C. A substrate control containing 0.5 ml of T-11 and 0.2 ml of 0.25 M Tris buffer at pH 8.5, and an enzyme control containing 1.0 ml saline and 0.4 ml of COB in Tris buffer at pH 8.5 were treated similarly. After 4 hr at 37 C, duplicate samples of 0.1 ml were taken from the T-11 digest and the control tubes, and the pH was adjusted to below 4.0 with glacial acetic acid to terminate the reaction and denature the enzyme. Duplicate hapten inhibition tests using 0.4 ml of antiserum 3 were set up. Digestion of the remaining material in the tubes was allowed to continue overnight at 37 C and then duplicate samples of 0.1 ml from all tubes were again adjusted to below pH 4.0 and tested for hapten inhibition. High voltage electrophoresis at pH 1.9 in formate-acetate buffer (2.5% formic acid; 7.8% acetic acid) was done on samples from all three tubes after overnight digestion.

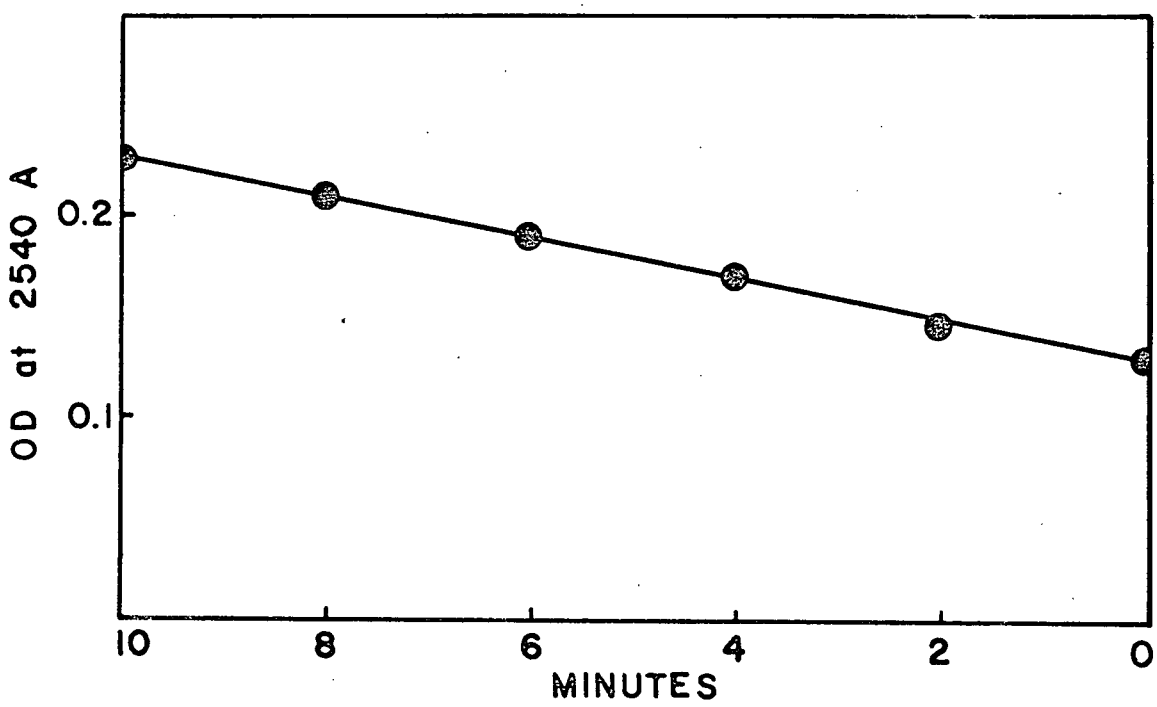


Figure 6. Assay of enzyme activity of the carboxypeptidase B preparation by the hydrolysis of the substrate hippuryl-L-arginine.

A second carboxypeptidase experiment was tried after the initial COB experiment indicated that the removal of lysine (and possibly alanine) from the C-terminal of the T-11 peptide had no effect on its haptenic activity. In the second experiment, both COA and COB were used to try to degrade the peptide from the C-terminal sequentially as far as possible, to test the limit of degradation before loss of haptenic activity occurred. The COA (Worthington, DFP-treated) was assayed for enzymatic activity and its rate of hydrolysis of hippuryl-L-phenylalanine measured by the increase in absorbancy at 2540 A at 25 C according to the method of Folk and Schirmer (1963). One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1.0 µmole of substrate per min under the conditions specified. Figure 7 shows the results of the assay of this enzyme. The methods used for the second experiment were basically those described by Young et al. (1966) in their work on an immunologically active tryptic peptide from tobacco mosaic virus protein. For this experiment 3.0 ml of T-11 in saline containing 0.75 µmole, and 3.0 ml of saline as a control were used. The pH of the test and the control were adjusted to 8.0 with 2% $NaHCO_3$, and 0.01 ml of COB (100 µg containing 0.4 units of activity) was added to each tube and the reaction was allowed to proceed for 60 min at 37 C. Then 0.01 ml of COA (250 µg containing 23 units of enzyme activity) and 0.1 ml of 10% LiCl were added to both tubes and the incubation at 37 C was continued. Because COA is known to autodegrade on lengthy digestion, an

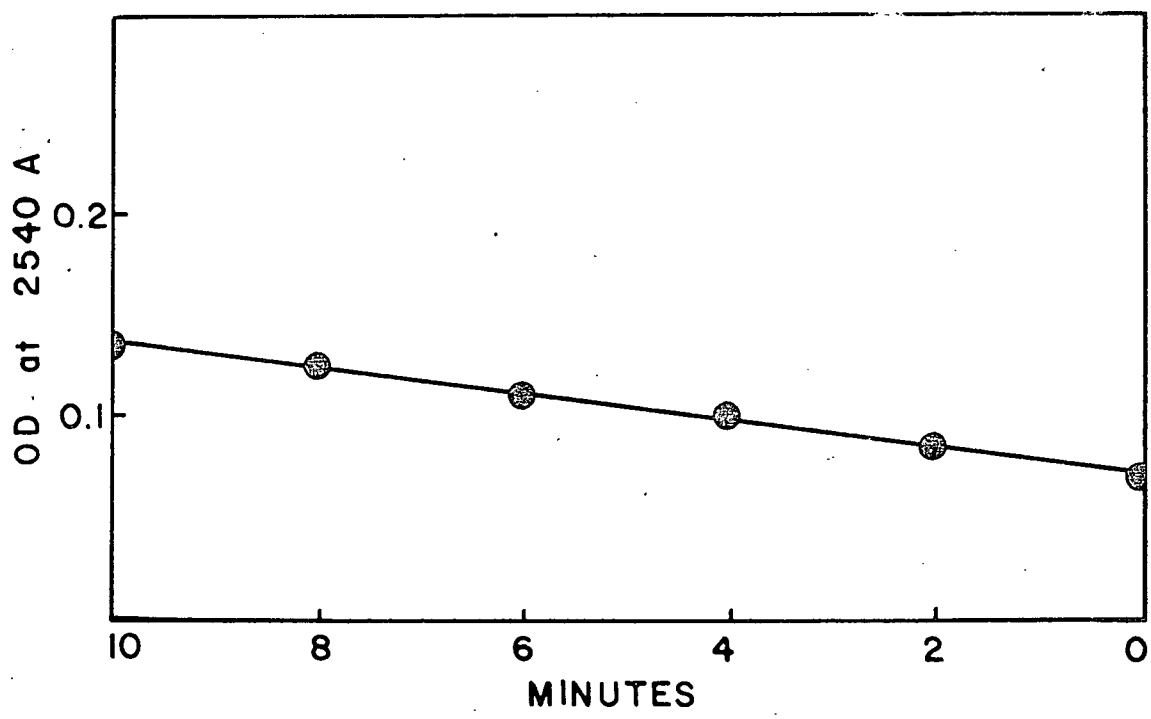


Figure 7. Assay of enzyme activity of the carboxypeptidase A preparation by the hydrolysis of the substrate hippuryl-L-phenylalanine.

additional 0.01 ml of the enzyme was added at 2 hr, 15 hr and 24 hr to both tubes. Samples of 0.3 ml were taken from both test and control tubes at 2, 6, 15, 24 and 40 hr. From the aliquots taken, 0.1 ml was dried and taken up in the starting buffer for amino acid analysis, and duplicate hapten inhibition tests were carried out on the remainder.

VII. Edman Degradation of T-11

Because the results from C-terminal degradation indicated that you could remove several amino acids from the C-terminal end of the T-11 peptide without loss of haptenic activity, the next experiments involved degrading the peptide from the N-terminal. Edman's degradation was tried first. A Sephadex G-25 (Pharmacia Fine Chemicals) column (2.5 x 40.0 cm) was equilibrated with distilled water and 2.0 ml of T-11 containing 1.5 µmoles in saline was desalted by running it through the column. The peptide, which came out near the void volume was flash evaporated at 45 C to dryness and used as the starting material for serial Edman's degradation. The method followed was described by Konigsberg and Hill (1962) with minor modifications.

The first stage in the Edman's reaction involves the coupling of the unprotonated amino group (therefore the reaction is carried out at an alkaline pH) of the peptide with the thiocarbonyl group of phenyl isothiocyanate (Eastman Organic). A suitable solvent

giving both the peptide and the reagent appreciable solubility was found to be 50% aqueous pyridine containing 2% trimethylamine. In this first coupling reaction, it is also important to exclude oxygen by flushing the reaction vessel well with nitrogen and stoppering the tube, since oxygen will replace sulfur in the thiocarbonyl group of the phenylthiocarbamyl peptide and will prevent the later cyclization and elimination of the phenylthiohydantoin of the terminal amino acid. The coupling stage was carried out for 2 hr at 45 C using a 1:50 molar ratio of peptide to phenyl isothiocyanate in a volume of 0.215 ml.

The second stage, after removal of excess reagents with two benzene extractions of 1.0 - 2.0 ml, was the cyclization of the phenylthiocarbamyl group on the amino group with the carbonyl carbon of the amino terminal amino acid, resulting in the formation of a thiazolinone derivative. It is necessary to perform the cyclization under anhydrous conditions with strong acid in order to prevent hydrolytic cleavage of acid-sensitive bonds. The relatively mild conditions permitted by anhydrous trifluoroacetic acid at 45 C for 30 min in a volume of 0.3 ml under nitrogen were used on the T-11 peptide.

Following cyclization, the trifluoroacetic acid was removed by vacuum evaporation at 25 C over P_2O_5, and the residue was dissolved in 0.2 ml of 0.2 M acetic acid and extracted twice with 1.0 - 2.0 ml amounts of benzene to remove the phenylthiocarbamyl amino acids and phenylthiohydantoins of most acidic and neutral

amino acids.

Peptide devoid of the N-terminal amino acid remained in the aqueous phase. The dried residue was resuspended in 0.1 ml of 0.2 N acetic acid and adsorbed onto a small Dowex 50 column (4 x 50 mm) in the pyridine form equilibrated with 0.2 N acetic acid. The column was washed by running approximately 10 ml of 0.2 N acetic acid through it, followed by 5 - 10 ml of 2.0 M pyridine-acetate buffer at pH 5.0. The acetic acid washings contained ninhydrin negative material which, after acid hydrolysis was shown by Konigsberg and Hill (1962) to contain all the amino acids present in the parent peptide, but this peptide derivative is devoid of free amino groups and therefore is not adsorbed under the stated chromatographic conditions. The true remaining peptide with free amino groups elutes with the pyridine-acetate buffer. In the present instance, this material was collected and dried down and resuspended in 50% aqueous pyridine which completed one cycle. This purification step cannot be used if the peptide to be degraded contains cysteic acid since it will not be adsorbed, or if it contains a high concentration of basic or aromatic residues, since it will be eluted only with difficulty with the 2.0 M pyridine-acetate buffer. However, the T-11 peptide contains only one lysine residue, no aromatic residues, and the cysteine residues are all carboxymethylated, so no problems of this type were encountered.

At the end of one cycle, which removed one amino acid, three samples of 10.0 µl (approximately 0.05 µmoles) were taken, one for amino acid analysis, and two for setting up duplicate hapten inhibition tests. Three cycles were repeated, the only difference being that the volumes of the aliquots taken after the second and third cycle were 20 and 30 µl respectively to compensate for losses in peptide resulting from handling and column chromatography. The concentration of peptides was determined by amino acid analysis and then hapten inhibition tests were performed on the three samples designated T-11-1, T-11-2 and T-11-3. Aliquots used for hapten inhibition tests were dried and redissolved in 0.9% NaCl. Each time a cycle was completed, the remaining peptide was taken up in the amount of 50% pyridine to give the same micromolar concentration present in the starting material. Control tests for the hapten inhibition were set up in duplicate using three concentrations of T-11, 0.04, 0.02 and 0.01 µmoles. Negative controls contained 0.9% saline in equivalent volumes, in place of peptide.

Since these experiments indicated the importance of the N-terminal portion of the T-11 peptide as an antigenic determinant, the complete experiment was repeated, which confirmed the observation that removal of three amino acids from the N-terminal caused loss of haptenic activity. Because Edman's degradation involves somewhat rigorous treatment of biologically active materials, the possibility was considered that the loss of haptenic activity could

be due to manipulation rather than to the importance of the three N-terminal residues. In order to test this, another series of experiments were set up using enzymatic cleavage at the N-terminal to ascertain whether or not the observations made here were valid.

VIII. Leucine Amino Peptidase Experiments

The leucine amino peptidase (LAP), diisopropyl phosphoro-fluoridate-treated, was obtained from Worthington Biochemical Company. Before use in any experiments, the enzyme preparation was assayed for activity by noting its rate of hydrolysis of the substrate L-leucine p-nitroanilide. Before this enzyme can be assayed, it is necessary to activate it in the presence of 2 mM $MgCl_2$ in 0.14 M triethylamine adjusted to pH 8.5 with acetate at 40 C for 2.5 hr. It has been found that magnesium ion is better than manganese ion for activation of this metal-requiring enzyme, as manganese ion slowly forms manganese dioxide under slightly alkaline conditions, and this will allow a reversible oxidation-reduction system to be formed capable of destroying tryptophan and sulfur-containing amino acids. The assay of the enzyme activity showing the determination of the units of activity per mg is shown in Figure 8.

When the activity of the activated LAP was determined, an experiment following the procedure of Light (1967) and using the following solutions was carried out.

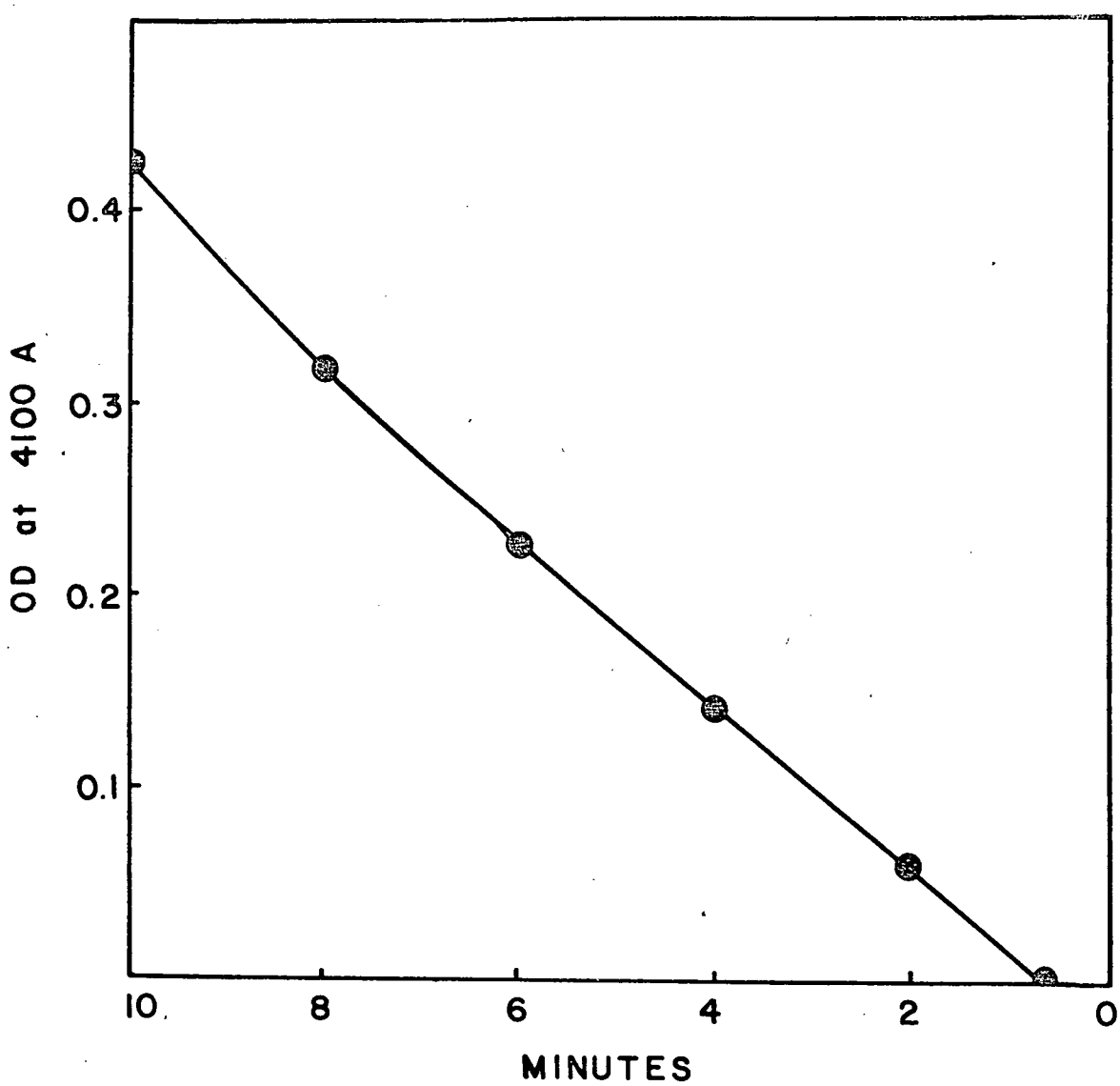


Figure 8. Assay of enzyme activity of the activated leucine amino peptidase preparation by the hydrolysis of the substrate L-leucyl-p-nitroanilide.

1. Experimental system

8.0 mg (3.2 µmoles) of T-11 peptide was dissolved in 1.0 ml of 0.14 M triethylamine acetate at pH 8.5 containing 0.002 M $MgCl_2$. At zero time, 0.25 mg (0.3 ml of the activated enzyme preparation containing 25.0 units of enzyme activity) of LAP was added.

2. Substrate controls

a. 1.0 mg (0.4 µmoles) of T-11 peptide dissolved in 0.1 ml of triethylamine acetate buffer containing $MgCl_2$ at pH 8.5.

b. 4.0 mg (1.6 µmoles) of T-11 peptide dissolved in 0.5 ml of triethylamine acetate buffer containing $MgCl_2$ at pH 8.5. At zero time, 0.125 mg (0.15 ml of the activated enzyme preparation containing 12.5 units of activity) of heat-inactivated (boiled) LAP was added.

3. Enzyme control

0.125 mg (12.5 units of activated enzyme preparation) of LAP was dissolved in 0.5 ml of triethylamine acetate buffer containing $MgCl_2$.

All the tubes were incubated at 37 C, and samples of 0.15 ml were taken from the test, the enzyme control and the substrate controls at 8, 16, 24, 40 and 48 hr. A sample of 0.15 ml was taken from the test solution at 32 hr also just before fresh

enzyme in the same amount as at the start was added. Heat-inactivated enzyme, at an equivalent amount to that added at the start, was added to the appropriate control, as well as 0.125 mg of enzyme to the enzyme control at 32 hr. As aliquots were taken, the pH was lowered in each one to below 3.5 with acetic acid (0.1 N) in order to terminate the action of the LAP.

From the samples removed at the times given above, aliquots of 0.03 ml were taken from the test series, dried down and taken up in 0.5 ml of starting buffer (pH 2.2), and an amino acid analysis was carried out on each one to determine the extent of digestion. A 0.03 ml aliquot of the sample taken at 48 hr from the enzyme control was also analysed for free amino acid content, to check for any autodegradation of the LAP. Under the above conditions, 0.004 µmoles of leucine only was detected in the enzyme control, a value which was about 10% of the amount of leucine released in the test series. Correction was made for this in calculating the amount of digestion in the experimental samples.

When quantitative amino analyses had been carried out on all the digest samples, and the extent of enzyme cleavage had been determined, hapten inhibition tests were done in triplicate on 0.01 ml (60 µg of the original T-11) aliquots of the test samples and controls, With the T-11 digest, all times were tested, but with the boiled LAP control, only 8, 16 and 48 hr samples were tested, and with the enzyme control tests were run on the 8, 24 and 48 hr samples. The T-11 control was tested only at 48 hr to see

if the prolonged incubation at 37 C had had any effect on its haptenic activity, since shorter times would have less effect. A control set in triplicate using an equivalent amount of triethylamine acetate buffer containing $MgCl_2$ rather than digest was done to obtain the maximum precipitation level.

IX. Purification of Specific Antibodies

Rabbit anti-CM-lysozyme antibody was specifically purified from pooled sera from rabbits 1 and 2 according to the method of Fujio et al. (1968a). One hundred ml of pooled sera was precipitated at the equivalence point with a calculated amount of the stock solution of CM-lysozyme, by incubating the mixture for 1 hr at 37 C and then for 2 days at 4 C. The specific immune precipitate was centrifuged at 20,000 g for 20 min and washed three times with 0.02 M sodium phosphate, 0.15 M NaCl, pH 6.0 buffer (PBS). The washed precipitate, containing about 25 mg of antibody, was dissolved in 5.0 ml of 0.2 N acetic acid and incubated at 37 C for 2 hr with stirring to dissociate the antigen-antibody complex. The dissociated mixture was passed through an ascending Sephadex G-200 column (2.5 x 90.0 cm) equilibrated with 0.2 N acetic acid. The gel filtration was carried out at room temperature and 5.0 ml fractions were collected. The flow rate was adjusted to 12.0 ml/hr. The absorbance of each fraction was read at 2800 A, and the elution profile is shown in Figure 9. Blue dextran was run through first to determine void volume. There was no 19S

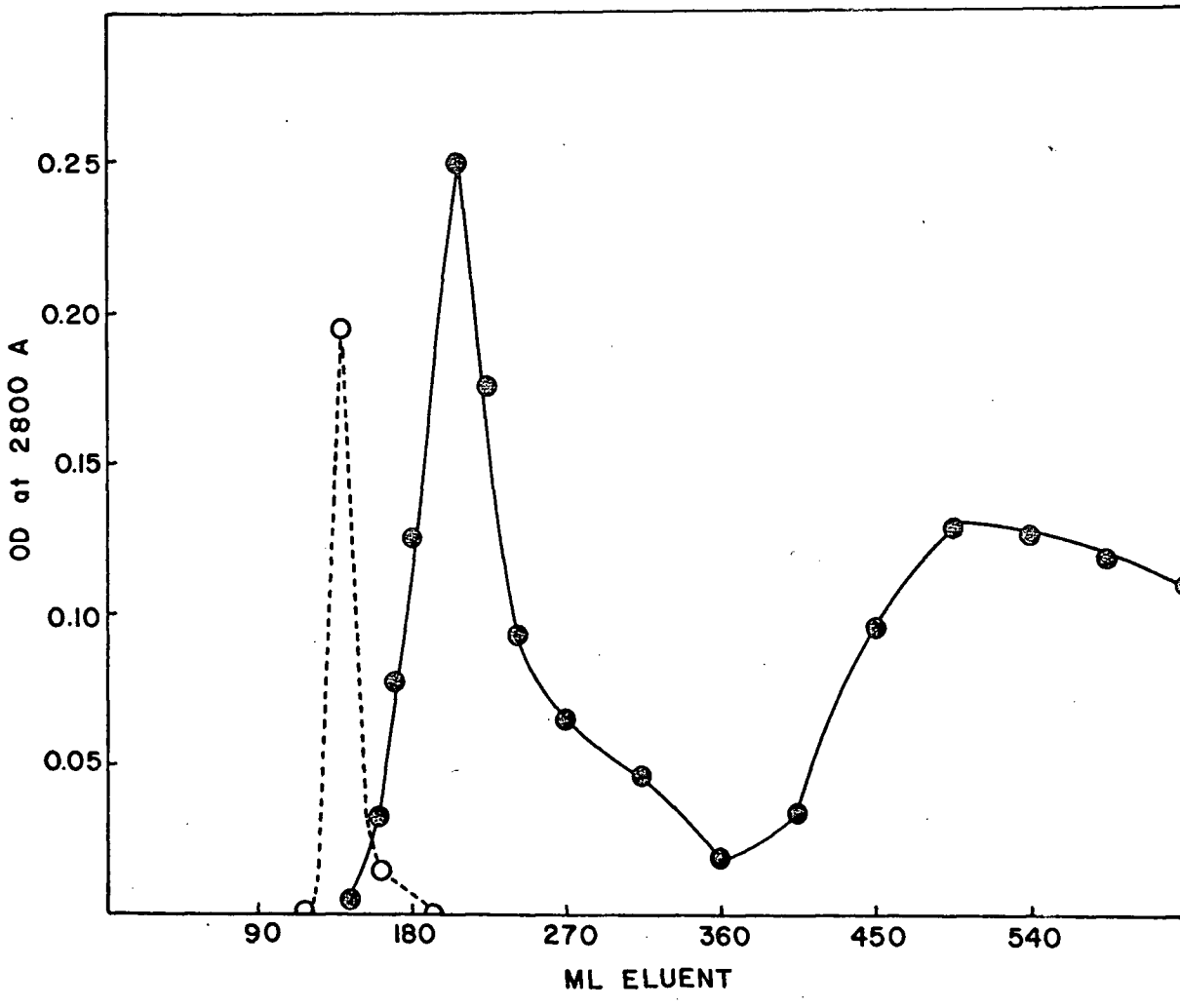


Figure 9. Elution profile of the purification of specific anti-CM-lysozyme antibodies on Sephadex G-200. The dotted line represents blue dextran and the sharp, major solid line peak represents the IgG (7S) specific antibodies.

gamma globulin peak which would have come out in the void volume so that only the retarded IgG peak is demonstrated. The fractions containing the IgG were pooled and dialysed against 100 volumes of 0.02 M PBS at pH 6.0 at 4 C for 3 days with stirring and changes in buffer twice daily. The stock solution of purified antibody was concentrated by ultrafiltration through an Amicon ultrafilter apparatus using a UM-1 filter pad, so that the final concentration was 1.10 mg protein/ml. This material was stored at -15 C until required.

Nonspecific rabbit gamma globulin which had been concentrated by precipitation with 50% $(NH_4)_2SO_4$ and purified by DEAE-cellulose (Calbiochem) column chromatography in 0.05 M phosphate buffer at pH 7.5, according to the method used by Fujio et al. (1968a), was used for the gamma globulin control in equilibrium dialysis experiments. The gamma globulin prepared in this way was stored as a lyophilized powder, and was dissolved in small amounts of 0.02 M PBS at pH 6.0 and dialysed against this buffer for 3 days at 4 C as required. The dialysed preparation contained 17 mg protein/ml.

Another method for the purification of specific antibody was also employed using a specific immunoadsorbent. The method used for the preparation of the immunoadsorbent and the purification of antibody was basically that of Robbins et al. (1967). The antigen, CM-lysozyme was reacted with the bromoacetyl cellulose (obtained by reaction of bromoacetic acid and bromoacetyl bromide with powdered Whatman cellulose) in a manner permitting covalent

bonding to occur between the antigen and the adsorbent. The conjugate of antigen and bromoacetyl cellulose was then used to adsorb specific antibody from pooled sera from rabbits 1 and 2 in 0.15 M phosphate buffer pH 7.4 at 4 C. Immunoadsorbent conjugates prepared in this way have a high capacity for extracting IgM and IgG classes of specific antibodies from antisera, and they are stable for months and may be used repeatedly without significant loss of antibody binding capacity. With large protein antigen-cellulose conjugates, most of the different types of antigenic determinants are still available for interaction with antibody.

The serum to be adsorbed was clarified first by centrifugation at 20,000 g for 1 hr at 4 C, and the floating lipid material and any sedimented material was removed. Then the immunoadsorbent was dispersed in the serum, and the suspension was stirred at 4 C for 2 hr. At the end of this time, the cellulose conjugate was centrifuged at 20,000 g for 20 min, and all the supernatant serum drained off (tests on this material indicated that most of the specific antibody had been removed in this step). After washing the adsorbent with 0.15 M NaCl and recentrifuging until the absorbance of the washes was less than 0.08 at 2800 Å, the antibody was eluted from the immunoadsorbent by stirring the complex in 0.1 M acetic acid (pH 2.8) for 1 hr at 37 C. The suspension was centrifuged for 30 min at 20,000 g to remove the cellulose-antigen conjugate, and the supernatant fluid containing the antibody, was dialysed against 350 - 700 volumes of 0.1 M NaCl - 0.01 M Tris HCl

at pH 7.0. This purified antibody stock solution was stored at -15C until required after mixing it with an equal volume of glycerine.

X. Solid Phase Peptide Synthesis

1. Peptide synthesis

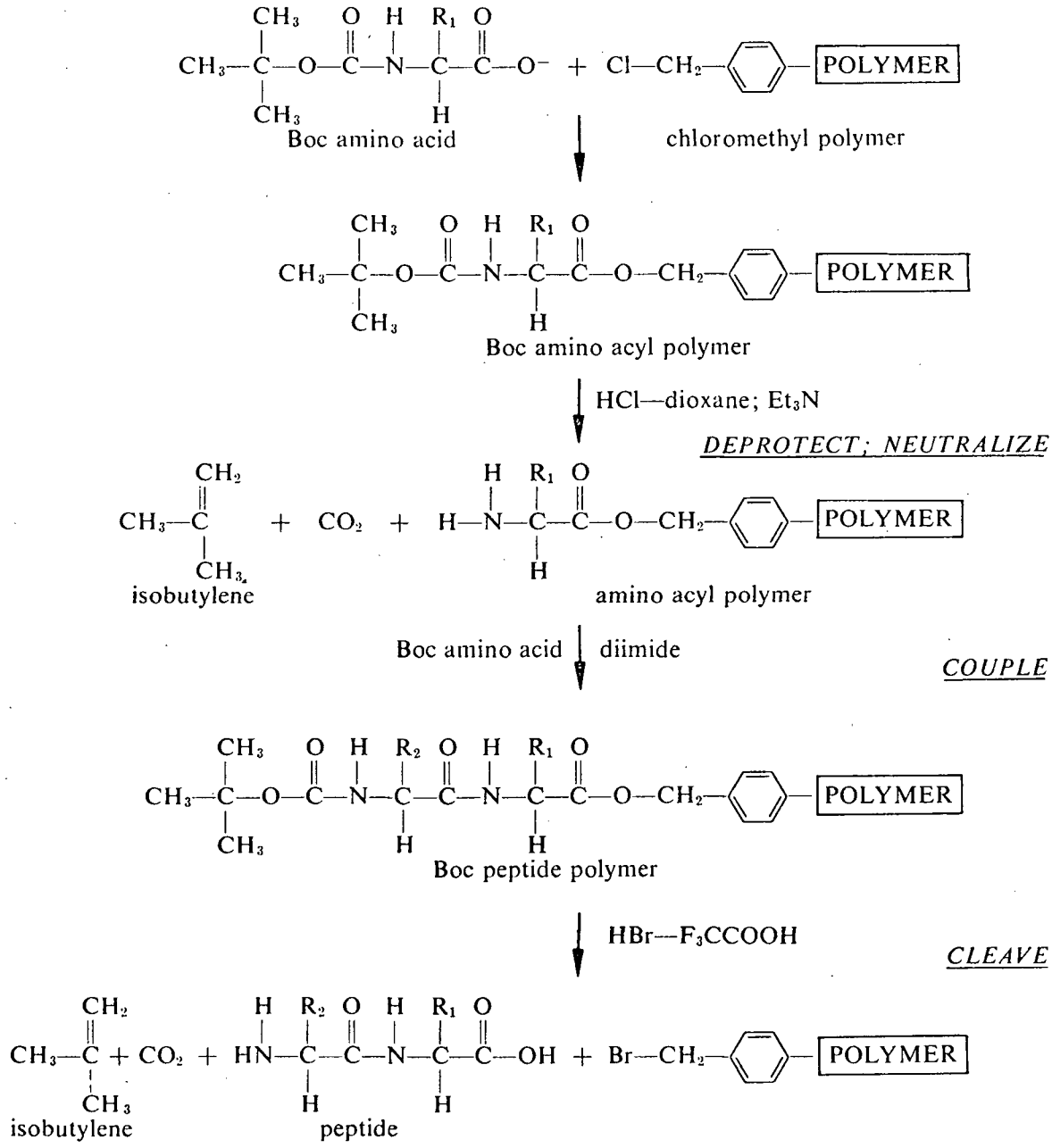
The Merrifield (1964) method of solid phase peptide synthesis was followed with the modifications as given in the recent book Solid Phase Peptide Synthesis (Stewart and Young, 1969). The method basically involves the growth of peptides by stepwise additions of the required amino acids, while the peptide is covalently bonded to an insoluble support. The insoluble support is chloromethylated copolystyrene-divinylbenzene resin (Bio-Rad). The reactive groups, the chloromethyl groups, react with the salt of a t-butyloxycarbonyl (t-Boc) amino acid (obtained from Mann Research Biochemicals and Sigma Chemicals) to form an ester bond between the COOH group of the amino acid and the resin. Thus the peptide is lengthened from the C-terminal amino acid toward the N-terminal. The t-Boc group blocks the free amino group of each incoming amino acid so that only the carboxyl group is free to react. The t-Boc group of the attached amino acid is removed by treatment with anhydrous HCl to allow this free amino acid group to react with the incoming protected

amino acid. The coupling reaction involving the formation of a peptide bond requires the presence of dicyclohexylcarbodiimide and neutral pH in most instances, but active esters of incoming t-Boc amino acids may be used with no coupling reagent. The excess amino acids and other reagents are easily washed out of the resin by appropriate solvents, thus eliminating laborious purification procedures at intermediate steps during the synthesis.

After the synthetic cycles are completed, the peptide is cleaved from the solid resin support by passing HBr gas (Baker, Matheson Co.) through a suspension of the resin and peptide in anhydrous trifluoroacetic acid. The terminal t-Boc group still present at this time is removed by an elimination reaction, and the peptide is cleaved from the resin by a nucleophilic displacement reaction. Certain other side chain protecting groups such as benzyl groups on the hydroxyls of serine and threonine are also removed by this procedure. However, the side chain protecting groups such as the benzyl groups on the sulfhydryl of cysteine must be removed by reduction with sodium in liquid ammonia.

After all the side chain protecting groups are removed, the cleaved peptide is purified by a suitable chromatographic technique. A flow chart for a synthetic cycle is shown in Table II. In this instance, the peptide synthesized, which constituted a portion of the T-11 peptide, was cleaved from the resin and treated with sodium in liquid ammonia (Baker, Matheson Co.) to remove the S-benzyl groups on the cysteine residues.

Table I. Flow chart of Solid Phase Peptide Synthesis.



*Reproduced from Solid Phase Peptide Synthesis by John Stewart and Janis Young, p. 3.

2. Reduction and alkylation of synthesized peptides

The method followed for reduction and alkylation of the synthesized peptide was a modification of the carboxymethylation procedure used for native lysozyme as reported by Canfield and Anfinsen (1963a). The peptide was flash evaporated at 45 C following reduction in liquid ammonia, washed with dilute acetic acid (0.1 N) and suspended in 5.0 ml of dilute acetic acid.

A 50-fold excess of 2-mercaptoethanol was added, and the pH of the solution was adjusted to 8.6 with dilute NH_4OH. The flask was flushed with nitrogen, stoppered, and incubated at 37 C for 4 hr to ensure the reduction of the cysteine residues in the peptide. Following the reduction step, the pH was lowered to 2.0 with dilute HCl to prevent reformation of any disulfide bonds, and the preparation was flash evaporated at 45 C to dryness and washed once with acidified distilled water. The reduced peptide was dissolved in 5.0 ml of distilled water, and 2.5 µCi of (^{14}C)-iodoacetic acid (New England Nuclear Co. containing 0.19 µmole) was added. The pH was raised to 8.6 and alkylation with the labelled iodoacetate was allowed to take place for 20 min before cold iodoacetic acid (purified by one crystallization from petroleum ether) was added in a 1:1 ratio on a weight basis with the amount of peptide present. This constituted a large molar excess, and the reaction was allowed

to continue for a further 5 min at pH 8.6 to ensure that all the cysteine residues had been alkylated. A 125-fold molar excess (to the moles of cysteine residues present in the peptide) of 2-mercaptoethanol was then added to the reaction mixture to reduce the excess iodoacetate. The pH was maintained at 8.6 for 1 min after the addition of 2-mercaptoethanol and then lowered to pH 2.0 with dilute HCl. The alkylated peptide was dried by flash evaporation at 45 C, washed once with distilled water, dried again, and suspended in 10.0 ml of distilled water with the pH adjusted to 2.8 with formic acid.

3. Purification of reduced and carboxymethylated peptide

The reduced and S-carboxymethylated peptide in dilute formic acid at pH 2.8 was applied to a Dowex 50 x 4, Bio-Rad column (2.0 x 25.0 cm) which had been equilibrated with 0.1 M pyridine-acetic acid buffer at pH 3.8. The peptide preparation contained some insoluble black matter, but this gradually dissolved on the column. The four-chambered gradient modified from the original Canfield procedure (1963a) was used as the initial purification step for the synthetic peptide. Fractions of 3.0 ml were collected, and the peptide was detected by counting 0.1 ml aliquots from every other tube in 8.0 ml of scintillation fluid. The liquid scintillation fluid was prepared with 42 ml of liquifluor (New England Nuclear) in 1 l of toluene-methanol (60:40, v:v). All

counting was carried out on a Nuclear Chicago liquid scintillation counter (Model 725).

Using these chromatographic procedures, the unreacted iodoacetic acid eluted at the front. The second major peak contained peptide material which, after amino acid analysis was found to consist of the desired synthetic peptide. The pooled peak material was carefully flash evaporated at 45 C using NaOH pellets in a trap in case any unreacted (^{14}C)-iodoacetic acid was present. The material was taken up in 4.0 ml of distilled water and 0.02 ml was analyzed after acid hydrolysis for its amino acid content.

In order to purify the peptide further and free it from low molecular weight contaminants which were sometimes present, the material from the Dowex column was run through a Sephadex G-15 column (2.5 x 35.0 cm) equilibrated with 0.1 N acetic acid. The column was run at 12 ml/hr and 4.5 ml fractions were collected. The fractions were read for 2300 Å absorbance on a DB-G spectrophotometer. The peptide material thus detected was concentrated by flash evaporation at 45 C and redissolved in 4.0 ml of distilled water. A sample of 0.01 ml was hydrolyzed and another amino acid analysis was performed on this. Some improvement in the amino acid ratios was usually observed after this step.

High voltage electrophoresis of the peptide material was carried out using a Gilson High Voltage Electrophoretor. The sample, at 0.1 mg

quantity was run at pH 6.5 in pyridine-acetic acid buffer (10% pyridine-0.4% acetic acid) at 3000 volts/cm for 30 min. Only one ninhydrin positive spot was present in these samples.

The haptenic activity of the synthesized peptides was tested by the precipitation inhibition technique previously described at concentrations of 50, 25 and 12.5, 6.25, 3.0, 1.5 and 0.4 µg and also by equilibrium dialysis studies described later.

XI. (^{14}C)-Acetylation of Peptides

The preparation of (1-^{14}C)-acetyl peptides was carried out according to the method described by Fujio et al. (1968a) with slight modifications. The (1-^{14}C)-acetic anhydride (5 mCi/mM) was obtained in 0.1 Cl breakseal tubes from New England Nuclear Co. The breakseal tube was frozen in a dry ice-acetone bath until the tube and the vacuum seal was broken. Once the vacuum was broken, the benzene was drawn down into the tube. After warming the benzene solution which now contained the labelled acetic anhydride, it was withdrawn with a syringe and transferred to a small tube.

For the acetylation reaction, the peptide to be so treated was dissolved in 1.0 M sodium acetate at pH 8.0, and the benzene solution of (1-^{14}C)-acetic anhydride was layered on top of the peptide solution which had been cooled to 0 C. The molar ratio

of peptide to acetic anhydride was 1:20. The reaction was allowed to continue for at least 24 hr at 4 C. The aqueous phase was passed through a Sephadex G-15 column (2.5 x 40.0 cm) equilibrated with 50% acetic acid at 25 C and eluted with the same solvent. Peptides treated in this way had molecular weights of approximately 1200, so that they were only slightly retarded on Sephadex G-15. However, the unreacted labelled acetic anhydride which under these conditions would be completely hydrolyzed to acetic acid, did not elute until total exchange in the column with cold acetic acid had occurred at approximately one column volume. Fractions of 4.5 ml were collected and 0.1 ml of every other fraction was mixed with 8.0 ml of scintillation fluid and the radioactivity was counted on a Nuclear Chicago scintillation counter (Model 725). The peptide peak was pooled and the acetic acid was removed by flash evaporation through a NaOH trap at 45 C. The peptide was washed with distilled water and lyophilized. After taking the peptide up in a known volume of 0.02 M PBS at pH 6.0, containing a small amount of sodium azide (0.02%), a small sample was taken to be hydrolyzed for amino acid analysis. Another sample of 0.01 ml was put into 8.0 ml of scintillation fluid and the radioactivity was counted, so that quantitative evaluation of counts per micromole of peptide could be estimated.

XII. Equilibrium Dialysis

The dialysis apparatus used in these studies was a small sample vial with a screw cap. In the outside compartment 4.0 ml of the peptide solution was placed (all peptides had previously been quantitated with respect to specific counts per micromole). The various antibody preparations (purified as described previously), anti-CM-lysozyme, non-specific gamma globulin, and PBS as a buffer control, all in 1.0 ml quantities, were placed in the inside (dialysis sac) compartment. The PBS, in which the peptides and antibody were dissolved was at pH 6.0 and 0.02 M and contained 0.15% NaCl and 0.02% sodium azide. The antibody concentrations were calculated from their extinctions at 2800 Å.

The semipermeable membrane separating the outside and inside compartments consisted of Visking dialysis tubing (Union Carbide). The dialysis apparatus was rocked gently during incubation on the apparatus designed for this purpose. Dialysis was continued at 4 C for a number of days, the control well containing only PBS being checked at 48 hr intervals until equilibrium was established. This was tested by removing aliquots of 0.05 ml at these times and counting the radioactivity in the outside and inside compartments. When the counts were comparable, it was assumed that equilibrium

had been reached. This usually took between 4 and 6 days. At this time, 0.05 ml aliquots were taken from all compartments, and counts were made in 10 ml of scintillation fluid to test for the presence of specific binding.

RESULTS AND DISCUSSION

I. Haptenic Activity in Tryptic Digests of CM-lysozyme

The antigenic properties of reduced and S-carboxymethylated lysozyme were studied in order to elucidate the regions of this molecule responsible for antigenic specificity. The lysozyme molecule was chosen as the antigen, since the analysis of the entire amino acid sequence (Canfield, 1963b), and X-ray crystallography of the molecule showing its tertiary structure had been completed (Blake et al.,1965). Information regarding antigenicity could be more meaningful in this light.

To approach the problem of isolating and characterizing the antigenic determinants on the CM-lysozyme molecules, enzymatic degradation was chosen. Trypsin was used to obtain large peptide fractions, since this is a specific endopeptidase cleaving protein molecules only at the C-terminal of lysine and arginine residues. The lysozyme molecule is a highly basic protein containing 6 lysine and 11 arginine residues which allows the carboxymethylated derivative to be cleaved into as many as fourteen smaller peptide fragments and some single amino acids. The native lysozyme with its disulfide bonds intact is not susceptible to proteolytic cleavage by trypsin.

This is characteristic of many native molecules which have a rigid folded tertiary structure held together by disulfide bonds. After denaturation by reduction and alkylation of disulfide bonds these protein molecules are susceptible to considerable enzyme cleavage.

A study on the antigenic properties of native lysozyme had been carried out by another group of workers (Shinka et al., 1967), and it was felt that a comparative study on the reduced and carboxymethylated derivative would be interesting and might throw some light on the interrelated role of primary and tertiary structure in proteins with respect to their antigenic properties.

The digestion of CM-lysozyme with trypsin, and the separation of the tryptic peptides have been described in the previous section on methods. Initial screening for haptenic activity by precipitin inhibition involved mixing of triplicate samples of 0.4 ml of antiserum of known titer with 0.1 ml quantities of the various peptide fractions (containing between 200 and 1000 µg/ml depending on their solubilities) dissolved in 0.9% NaCl, and incubating the mixtures at 37 C for 1 hr followed by 18 hr at 4 C. At the same time, saline controls were set up in triplicate. On the following day, antigen was added to a concentration equivalent to optimal proportions (this varied with the sera being used), and the incubation was repeated. The immune precipitates formed were centrifuged, washed in 0.9% NaCl, and then dissolved in 1.0 ml of 0.1 N NaOH to be read for 2800 Å absorbance.

These experiments were repeated on the sera from three individual animals. In each instance the only peptide fraction which showed evidence of haptenic activity was that which correlated with the T-11 peptide isolated from the Dowex column run as recommended by Canfield (1963a). An amino acid analysis on this peptide fraction established its identity with the T-11 peptide isolated by Canfield. The close agreement between our results and Canfield's data is shown in Table II. The preparation was virtually free of all other contaminating amino acids as evidenced by the trace amounts of Glu and Gly present (these are not present in the peptide) in analyses at concentrations of less than 2.0% of other residue amino acids in the peptide. Ring tests on this fraction were negative, indicating that no precipitable and undigested material was present. For most of the tests reported here, a stock solution of the peptide was used which was at a concentration of 500 µg/ml.

A quantitative hapten inhibition test was carried out on T-11 using between 50 and 0.1 µg of the peptide, and compared on a weight basis with a similar test on the total tryptic digest. Because serum from rabbit 3 had a higher precipitating titer than did the other two sera, it was used for these tests. Figure 10 shows the results of these tests which were run in triplicate. These figures represent mean values for each concentration and were found to be closely reproducible, since on retesting the variation was found to be between 1.5 and 2.0%. Maximum inhibition of 60% was observed

Table II. Amino acid analysis of peptide T-11. The micromolar ratios are in agreement with those determined by Canfield (1963a) and shown in column 1.

Amino acid	Micromoles 1	Micromoles 2	Residues
Lysine	0.27	0.0211	1
S-CM-cysteine	0.73	0.0597	3
Aspartic acid	1.11	0.0722	4
Threonine	0.27	0.0195	1
Serine	1.03	0.0618	4
Proline	0.28	0.0154	1
Alanine	0.78	0.0516	3
Valine	0.25	0.0173	1
Isoleucine	0.55	0.0341	2
Leucine	0.82	0.0559	3

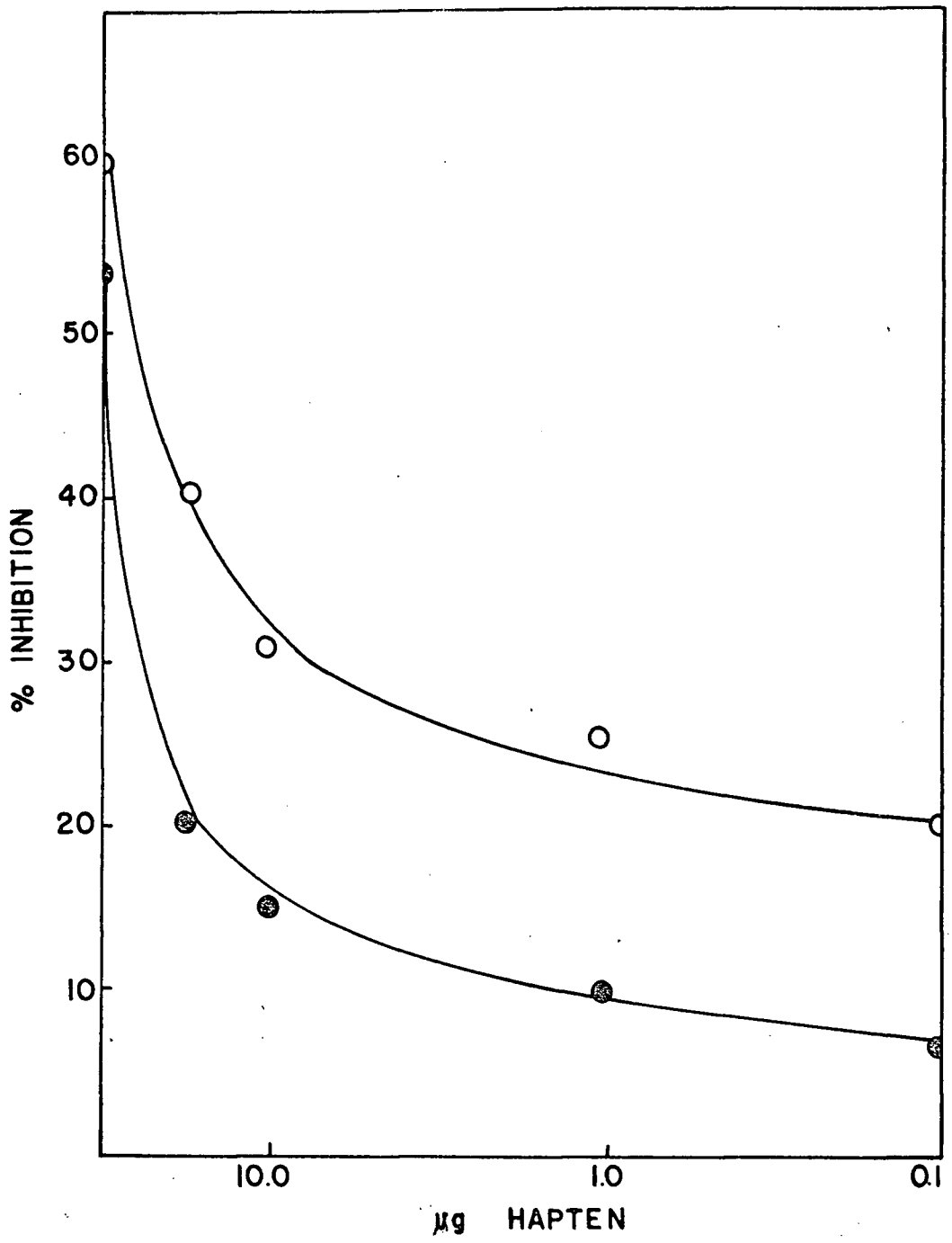


Figure 10. Inhibition of immune precipitation by peptide T-11 (o-o) and the total tryptic digest (●-●) at varying concentrations. Calculation of inhibition was based only on 50% hemolysis end point.

with 25 µg or larger quantities of T-11.

In order to establish that the inhibition observed here was not unique for precipitation, inhibition tests using complement fixation with pooled sera from rabbits 1 and 2 were carried out. With serum titers at 1:80, the optimal antigen levels fell between 0.2 and 0.8 µg (Fig. 1). For hapten inhibition testing, 0.8 µg of CM-lysozyme was used standardly in order to work in a region of slight antigen excess. In the initial test on both T-11 and the total tryptic digest, only two dilutions of complement (1:42 and 1:50) were used, as these had previously been calculated to produce 50% hemolysis in the reaction of antigen and antibody. Under these conditions, the T-11 peptide caused total inhibition at concentrations between 50 and 10 µg, but the tryptic digest acted in the same way only at 50 and 25 µg. To determine the actual degree of inhibition, another test was set up expanding the range of complement and hapten levels. Tests were run concurrently on other tryptic peptides which had failed to cause inhibition of the precipitation reaction. These other tryptic peptides were tested at only one level varying between 50 and 200 µg depending on the maximum solubility of the particular peptide. As in the previous tests, the only fractions showing inhibition were the total digest and T-11. The results for these two preparations are documented in Figures 11 and 12, and are correlated in Figure 13, in which, on a weight basis, peptide T-11 showed considerably more inhibitory capacity than the tryptic digest. The T-11 demonstrated detectable

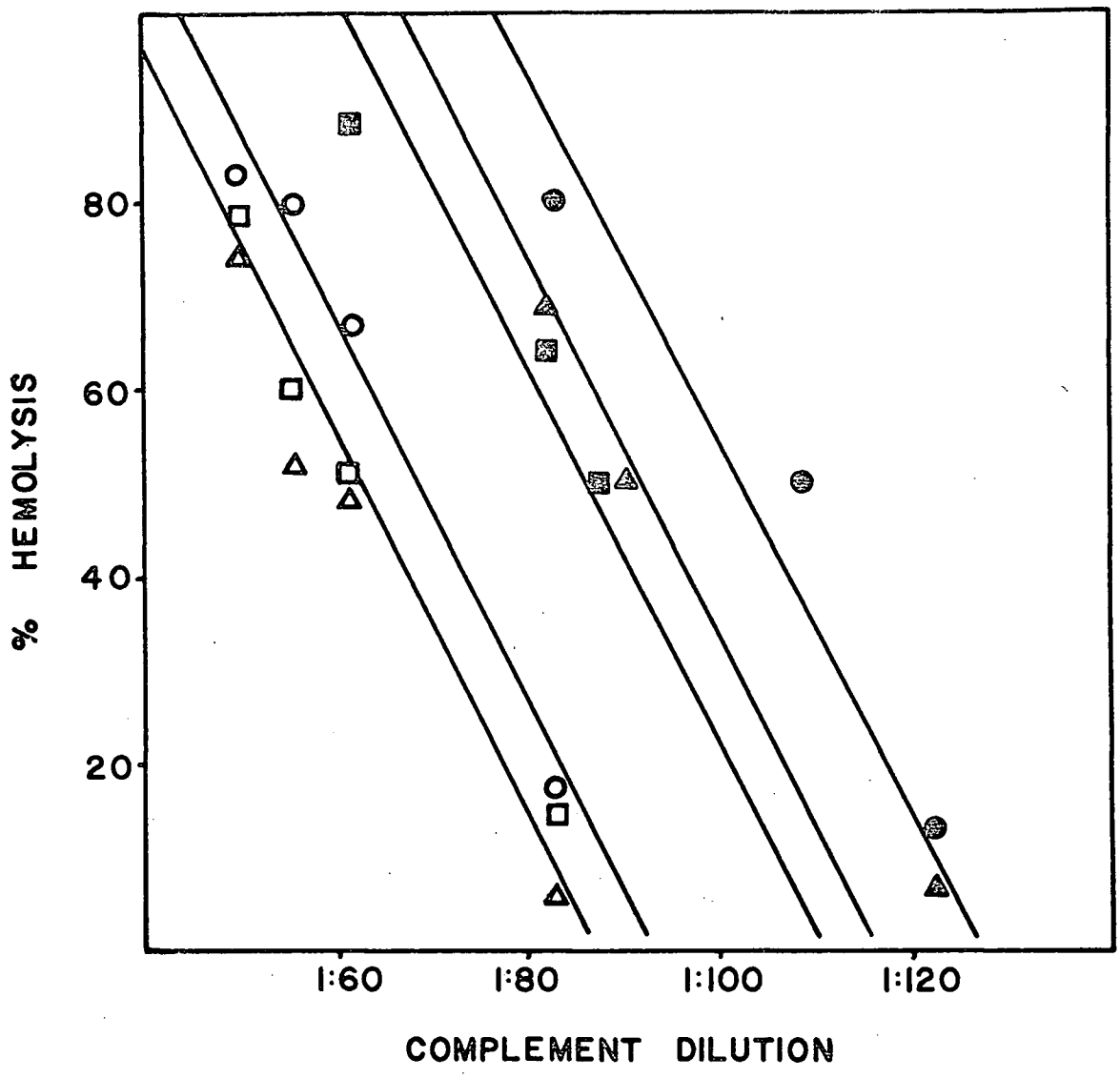


Figure 11. Inhibition of complement fixation with CM-lysozyme and homologous antiserum by peptide T-11 at varying concentrations; ●-●, 50 µg; ▲-▲, 25 µg; ■-■, 10 µg; o-o, 1.0 µg; Δ-Δ, 0.1 µg; and □-□ no hapten control. Calculation of inhibition was based only on 50% hemolysis end point.

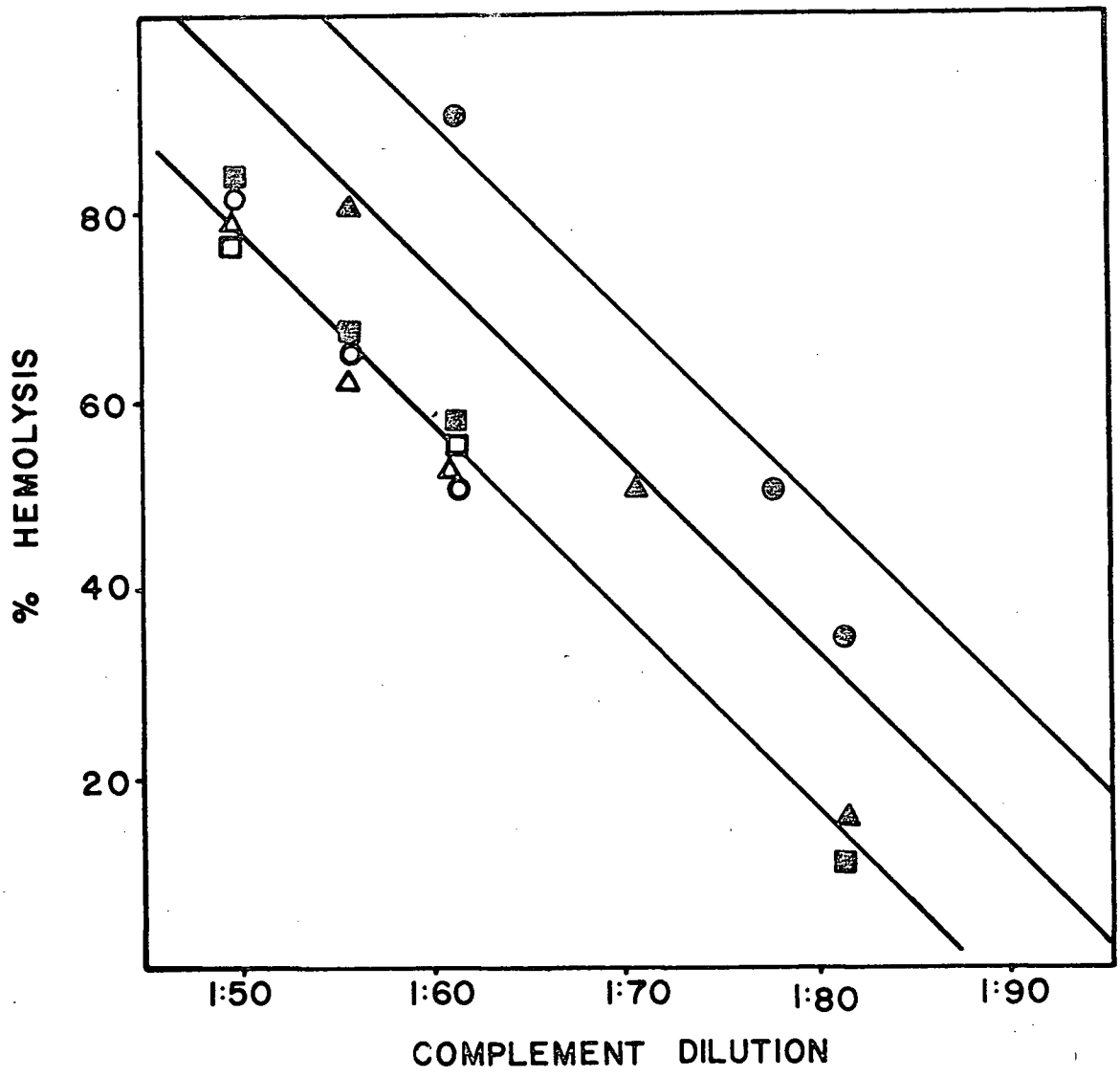


Figure 12. Inhibition of complement fixation with CM-lysozyme and homologous antiserum by the total tryptic digest at varying concentrations; ●-●, 50 µg; ▲-▲, 25 µg; ■-■, 10 µg; o-o, 1.0 µg; Δ-Δ, 0.1 µg; and □-□, no hapten control. Calculation of inhibition was based only on 50% hemolysis end point.

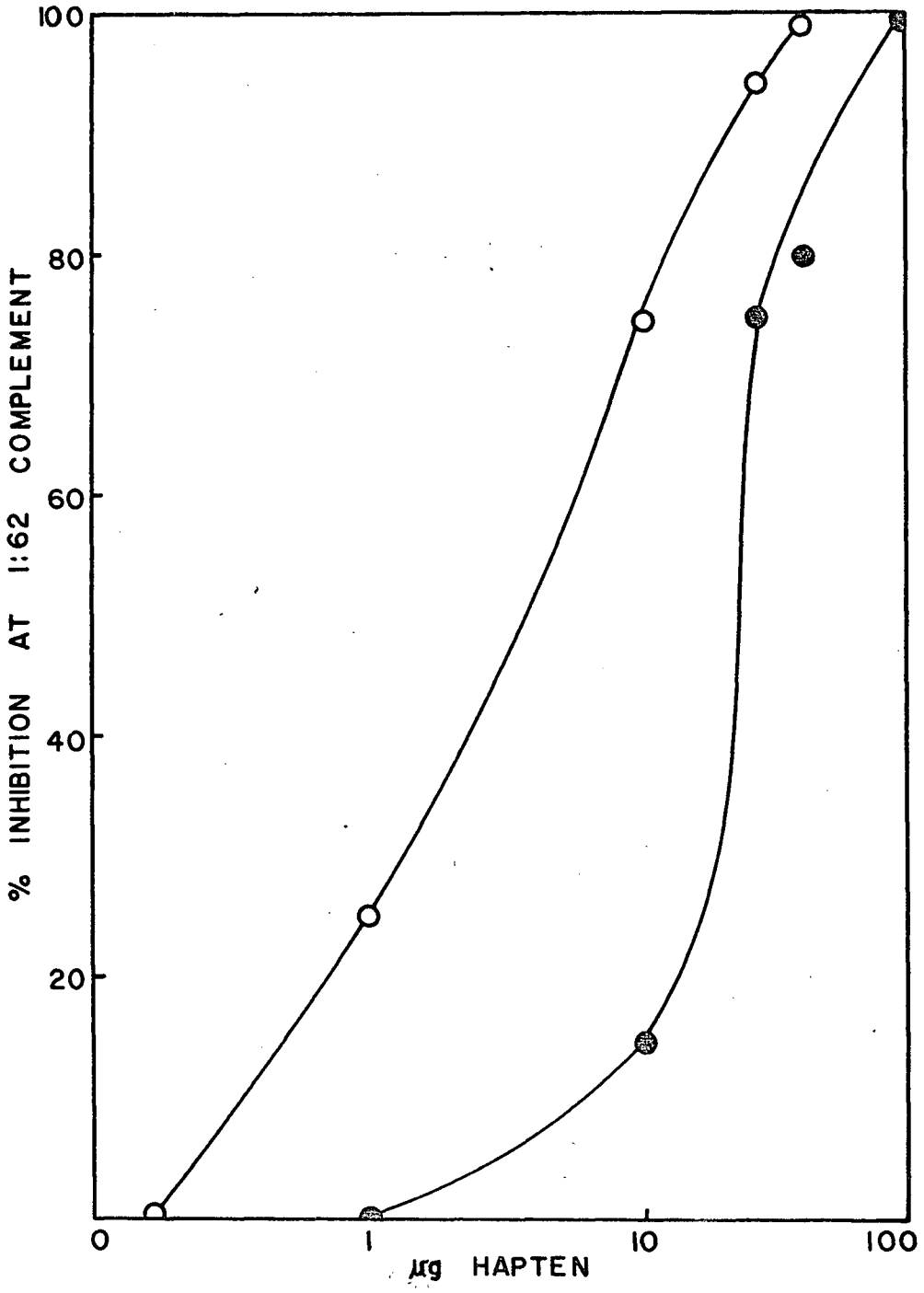


Figure 13. Correlation of inhibition of complement fixation at a 1:62 dilution of complement and varying concentrations of T-11 (o-o) and tryptic digest (●-●). Calculation of inhibition was based only on 50% hemolysis end point.

inhibition in concentrations as low as 1.0 µg, whereas the total digest showed only slight inhibition at 10 µg.

The testing of the specificity of the inhibition observed with the T-11 peptide was the final control measure taken in these experiments. For this purpose, a system of native lysozyme and its homologous antiserum was used, since it had previously been found that antiserum against native lysozyme did not cross-react at the precipitation level with CM-lysozyme. The antiserum had been prepared and titered in an identical manner to that described here for CM-lysozyme antisera. No detectable inhibition was produced by the T-11 peptide, indicating specificity for the CM-lysozyme system, and no affinity for the antiserum against the native antigen.

From these experiments, it would appear that the T-11 region of the CM-lysozyme molecule plays a significant role as an antigenic determinant, by its ability to inhibit the homologous immunological reaction, using both precipitation and complement fixation as test systems. The possibility that our inhibitory preparations of T-11, contained undigested protein, or a contaminating peptide which could be responsible for the observed reactions was discounted on several grounds: (i) the inability of peptide preparations to precipitate with antibody when tested either by ring test or by the testing of varying peptide concentrations with antiserum; (ii) the amino acid analysis which agrees closely with Canfield's (1963a) analysis of the peptide; (iii) the finger print analysis which demonstrated only one ninhydrin-positive spot; (iv) the absorption scan on T-11 which

showed no contamination with tryptophan-containing peptides; (v) the very low levels at which the peptide demonstrated haptenic activity; and (vi) the lack of complement fixing capacity on the part of the peptide fraction. Another important control was the proof that the inhibition by the T-11 peptide was specific for the homologous CM-lysozyme - anti-CM-lysozyme system. This effect was shown by the inability of T-11 to inhibit precipitation between native lysozyme and its homologous antiserum. Early experiments had shown that there was no cross-reactivity between CM-lysozyme and native lysozyme antiserum at the precipitin levels tested, and further, that none of the tryptic peptides or whole digest showed any capacity to act as haptens with the native system.

On a weight basis, it was found that the T-11 peptide was approximately two to three times as efficient as the tryptic digest as an inhibitor when tested either by precipitation or complement fixation. If this peptide represented the only determinant present in the tryptic digest, on a weight basis, one would expect about five times the specific activity for the peptide, since it comprises approximately 20% (23 amino acid residues to 129 residues in the total molecule) of the total molecule. However, in these experiments, this 5-fold activity was never observed, and at most a 3-fold greater specific activity of the peptide to the digest was noted. This result does not discount the probability that other regions of the CM-lysozyme molecule possess antigenic properties, but rather that the T-11 region is the only one retaining considerable activity after

trypsin digestion.

Because all the other peptides isolated from tryptic digests of CM-lysozyme on the eight-chambered gradient system recommended by Canfield (1963a) showed no haptenic activity, a simplified four-chambered system was set up. This used the first four buffers used in the original system, and permitted the elution of T-11 as the first sharply delineated peak. The purpose of these further peptide separations was mainly to collect large amounts of T-11 for further study.

The fact that the sera from three different rabbits all reacted identically in the systems tested, except with respect to antibody titer, indicated that the specificity of the system is not related to individual animals. It is interesting to note that this T-11 peptide is the largest tryptic peptide found in CM-lysozyme, containing 23 amino acid residues occupying between the 74th and 96th position in the molecule. The sequence of the peptide is as follows: NH_2-Asn-Leu - CMC - Asn - Ile - Pro - CMC - Ser - Ala - Leu - Leu - Ser - Ser - Asp - Ile - Thr - Ala - Ser - Val - Asn - CMC - Ala - Lys COOH (Canfield, 1963b).

II. Carboxypeptidase Digestions

Since T-11 is such a large peptide, the next series of experiments involved sequential degradation of the molecule in attempts to determine which portion of the whole peptide was responsible for its haptenic activity.

The first experiments employed the carboxypeptidase enzymes which, as the name indicates, sequentially cleave amino acids from the carboxy terminal of peptides and proteins. Initially, carboxypeptidase B was used to test whether or not the C-terminal residue (lysine) of T-11 was important to its haptenic activity. Carboxypeptidase B is an exopeptidase obtained from porcine pancreas which specifically cleaves basic amino acids (lysine, arginine or ornithine) from the C-terminal of peptides or proteins. The commercial preparations contain highly active homogeneous enzyme, essentially devoid of carboxypeptidase A activity which preferentially cleaves neutral and acidic amino acids sequentially from the C-terminal.

After the enzyme activity of the preparation had been determined, an experiment was set up for degradation of the T-11 peptide. Simultaneously, a substrate control, using only T-11 and no enzyme, and an enzyme control containing enzyme but no peptide, were set up and treated in a manner identical to the test digest solution as reported in the Methods. The substrate control tested whether the incubation conditions had any nonspecific effect on the haptenic activity of T-11. The enzyme control tested for any nonspecific effect the enzyme might have on the subsequent immunological testing of the digest, as well as for autodegradative release of amino acids from the enzyme itself rather than from the peptide.

Samples were removed for haptenic testing at 4 hr and at 18 hr, and the addition of glacial acetic acid terminated the reaction by denaturing and precipitating the enzyme. The substrate control was

used as the positive control in testing for haptenic activity. The enzyme control acted as the negative control and showed no nonspecific inhibition of the precipitation between the homologous CM-lysozyme - anti-CM-lysozyme system. Another negative control was also set up using an equivalent amount of saline-Tris buffer as used for the test solution to ensure that no nonspecific inhibition could be attributed to the enzyme control, due possibly to products of autodigestion. All the hapten tests were done in duplicate. The amount of T-11 present in aliquots taken for testing were equivalent to 65 µg, which from previous testing was known to yield maximum inhibition.

The results are presented in Table III. There was essentially no loss in the haptenic activity of the T-11 peptide after COB digestion for 18 hr. The enzyme control gave virtually no inhibition of precipitation and therefore the enzyme was assumed to have no nonspecific effect.

High voltage electrophoresis of test and control samples run with a lysine standard showed that lysine had been cleaved from the C-terminal of the peptide. There was also another ninhydrin-positive spot in addition to that for the remainder of the peptide. The substrate control containing only T-11 gave only one ninhydrin-positive spot, and the enzyme control similarly gave one major spot and a minor one which corresponded to the unknown one observed in the test sample. Thus, the second ninhydrin-positive spot in the digest and the enzyme control was thought to result from autodigestion

Table III. Hapten inhibition of specific immune precipitation by carboxypeptidase B digested T-11.

Digestion Time	O.D.* 2800 A	Percent Inhibition
4 hr		
Carboxypeptidase B digested T-11 (65 µg = 0.026 µmoles)	0.0805	24.8
Substrate (T-11) control (65 µg = 0.026 µmoles)	0.0835	22.0
Enzyme control	0.107	0.0 (100% precipitation level)
Saline control	0.107	0.0
18 hr		
Carboxypeptidase B digested T-11 (65 µg = 0.026 µmoles)	0.078	22.0
Substrate (T-11) control (65 µg = 0.026 µmoles)	0.079	21.0
Enzyme control	0.107	0.0 (100% precipitation level)
Saline control	0.106	0.0

*Represents the mean values of the duplicate samples.

of the enzyme.

In the second carboxypeptidase experiment, both COB and COA were used to degrade the peptide sequentially from the C-terminal in as far as possible, to test the limit of degradation before loss of haptenic activity occurred. COA is stopped in its cleavage by proline as the C-terminal amino acid. Commercial carboxypeptidase A is the bovine enzyme and still contains considerable activity against basic amino acids although it has been highly purified. The COA used here had been treated with diisopropylfluorophosphate (DFP) to inhibit any tryptic or chymotryptic endopeptidase activity. The COB was added first for 1 hr to ensure the removal of lysine from the C-terminal of T-11 before the COA was added. Additional aliquots of COA were added after 2, 15 and 24 hr since this enzyme is known to undergo autodegradation upon prolonged incubation.

Control tubes, both a substrate control and an enzyme control as described above, were set up and samples of 0.3 ml were removed from both the test and controls at 2, 6, 15, 24 and 40 hr. The reaction in the samples taken was terminated by denaturation of the enzyme by the addition of 20 µl of glacial acetic acid. Aliquots of 0.1 ml, containing 0.070 µmole of peptide in the case of the test samples, were removed from the 0.3 ml samples taken at various times, and dried and taken up in starting buffer for amino acid analysis. Analysis on the short column of the Beckman Analyzer showed that between 80 and 90% of the C-terminal lysine had been removed from T-11. However, the results of total amino acid analyses were not clear cut since an isoleucine peak and a carboxymethyl-

cysteine were present, but none of the other expected residues (Asp, Thr, Ser, Ala and Val respectively) were detected in equivalent amounts. Isoleucine is nine amino acids in from the C-terminal, and all the above mentioned amino acids would have to be cleaved before it would be present in digests of T-11. It is also known that S-carboxymethylcysteine is only released extremely slowly by COA, and since this is the third amino acid in from the C-terminal, all further degradation would be greatly reduced, so that one would expect only trace amounts of the other amino acids. Because of the high levels of enzyme used in this second experiment, and the fact that some free amino acids were detected in the enzyme control on amino acid analysis, it could not be conclusively determined how far in from the C-terminal the COA had degraded the peptide.

The digests, upon acidification to terminate the reaction, gave rise to considerable precipitates which interfered with the hapten inhibition tests. In fact, there appeared to be more precipitation observed between the homologous antigen-antibody system, which was used to yield the 100% precipitin level. Therefore, the results from the COA and COB experiment were not valid and did not give the sought-for answer. However, the original experiment indicated that removal of the C-terminal lysine had no effect on haptenic activity.

Because of the technical difficulties encountered using COA in these experiments, further work at this time involved investigation of the N-terminal of T-11. It was decided that if

the N-terminal region of T-11 was found to be uninvolved in antigenicity, then the carboxypeptidase experiments would be repeated, using more intricate techniques for purifying each digest aliquot before testing for hapten inhibition.

III. Edman Degradation of T-11

Degradation of the T-11 peptide sequentially from the N-terminal was carried out using the Edman's method. The subtractive Edman technique was used in which the N-terminal amino acid is removed and an aliquot of the remaining peptide is subjected to hydrolysis and amino acid analysis. Comparison of the remaining amino acids with the known analysis of the total peptide establishes the removal of the N-terminus.

The first stage in the reaction involves the attachment of the thiocarbamyl group of phenyl isothiocyanate to the unprotonated amino group of the N-terminal amino acid. After the coupling reaction has occurred and excess reagents have been removed, the phenylthiocarbamyl group on the amino group is coupled with the carbamyl carbon of the amino terminal amino acid, resulting in the formation of a thiazolinone derivative. The cyclization reaction and cleavage of the N-terminal amino acid from the peptide must be performed under anhydrous conditions to prevent hydrolytic cleavage of acid-sensitive bonds. After benzene extraction, to remove the phenylthiocarbamyl amino acids and phenylhydantoins of most acidic

and neutral amino acids, the remaining peptide was purified on a small Dowex 50 column equilibrated with 0.2 N acetic acid. Acetic acid was used to elute ninhydrin-negative material, and the peptide was then eluted with 2.0 N pyridine - acetic acid buffer at pH 5.0. The T-11 peptide contains only one basic residue, lysine, no aromatic residues, and the three cysteine residues are all carboxymethylated, and therefore not strongly acidic. Thus, no difficulties were encountered when using this purification technique. The eluted, purified remaining peptide was dried and resuspended in 50% aqueous pyridine from which 3 samples were taken. One sample was used for amino acid analysis after it had been dried and hydrolyzed for 18 hr. The other two samples were dried and redissolved in 0.9% NaCl for hapten inhibition tests. The amino acid analysis yielded quantitation of the amounts of peptide present in the samples used for hapten inhibition testing. Each time a cycle was repeated, there were losses in material resulting from handling and the column purification step, and in order to compensate for this, larger samples were removed each time, as stated in the methods. Duplicate samples of T-11 were set up as controls at three concentrations of 0.04, 0.02 and 0.01 μmoles, which were within the range of amounts of the T-11-1, T-11-2 and T-11-3 samples tested. Negative controls using equivalent volumes of 0.9% NaCl in place of peptide were also set up.

The results of amino acid analyses and the hapten inhibition tests are shown in Tables IV and V respectively, and demonstrate

Table IV. Amino acid analyses data of subtractive Edman's degradation of T-11.

Amino acid	Micromoles T-11-1	Residues	Micromoles T-11-2	Residues	Micromoles T-11-3	Residues	Residues of T-11 peptide
S-CM-cysteine	0.212	3.0	0.036	3.3	0.020	1.8*	3
Aspartic acid	0.217	3.1*	0.033	3.0*	0.031	2.8*	4
Threonine	0.068	0.97	0.013	1.2	0.012	1.1	1
Serine	0.261	3.7	0.042	3.8	0.040	3.6	4
Proline	0.097	1.4	0.016	1.5	0.015	1.4	1
Alanine	0.242	3.5	0.035	3.2	0.037	3.4	3
Valine	0.077	1.1	0.012	1.1	0.014	1.3	1
Isoleucine	0.151	2.2	0.022	2.0	0.022	2.0	2
Leucine	0.222	3.2	0.025	2.3*	0.024	2.2*	3
mcM in sample analyzed	0.070		0.011		0.011		

*Amino acids removed.

Sequence of N-terminal region of T-11 showing amino acids removed at each Edman's degradation cycle:

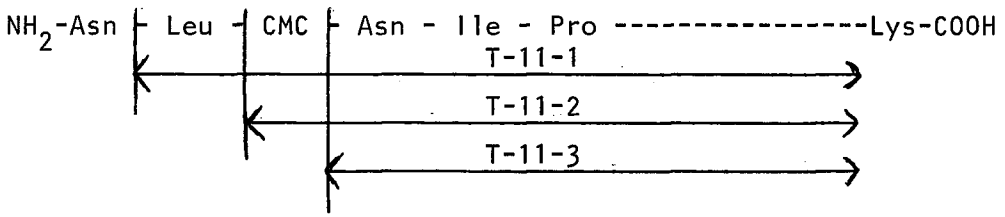


Table V. Hapten inhibition of specific immune precipitation by Edman's degradation of T-11.

Test	O.D.* 2800 A	Percent Inhibition
T-11-1 (0.07 µmoles)	0.118	18.6
T-11-2 (0.011 µmoles)	0.143	0.0
T-11-3 (0.011 µmoles)	0.167	0.0
Controls		
A. Positive hapten inhibition		
T-11 (0.04 µmoles)	0.063	57.3
T-11 (0.02 µmoles)	0.067	53.8
T-11 (0.01 µmoles)	0.073	49.7
B. Negative hapten inhibition (maximum precipitation level)	0.145	0.0

*Represents the mean values of duplicate samples.

that the removal of the asparagine from the N-terminal of T-11 decreased the haptenic activity by 67.5%, and removal of the next amino acid, leucine, caused essentially 100% loss of haptenic activity. The T-11-3, when mixed with antiserum, became insoluble immediately, which accounted for the higher readings of precipitate after completion of the test. This was possibly caused by loss of solubility of the peptide after the removal of the 3 N-terminal amino acids, or by the rigorous manipulations involved in the technique.

Because these results indicated the importance of the N-terminal region of T-11 in its haptenic activity, the complete experiment was repeated and the results were completely comparable, even to the loss of solubility of the T-11-3. However, because Edman's degradation involves fairly rigorous treatment of biologically active materials, the possibility was considered that the loss of haptenic activity was due to manipulation of the peptide rather than to the removal of the N-terminal amino acids. To test this possibility, another series of experiments was carried out to ascertain whether or not these observations were valid. These involved a second method of sequential degradation of peptides from the N-terminal, namely enzymatic cleavage with leucine amino peptidase.

IV. Leucine Amino Peptidase Experiments

Leucine amino peptidase is an exopeptidase obtained from swine kidney, which removes amino acids sequentially from the N-terminus of a protein or peptide. This exopeptidase is stopped in its cleavage reaction when proline is at the N-terminus, and is slow when aspartic acid is in this position. Leucine is removed faster than all other amino acids as the name implies. The commercial preparation of this enzyme has been treated with DFP to inhibit serine protease activity such as trypsin and chymotrypsin. The enzyme preparation must be activated in the presence of 0.002 M $MgCl_2$ at pH 8.5 at 40 C for 2 to 3 hr before use.

After activation and assay of the enzyme preparation, the experiment was set up as described in the methods. Amino acid analysis data on the samples taken at the various times from the LAP digest are presented in Table VI. Calculation of the percentage cleavage at the various times was determined by comparing the micromolar amounts of a single free amino acid released with the micromolar amount of the peptide present in the sample, which would represent the micromolar value for 100% cleavage.

The results of the hapten inhibition tests by precipitation are given in Table VII. In order to conserve antiserum, only three times (8, 16 and 48 hr) were tested with the boiled LAP - T-11 control, and (8, 24 and 48 hr) with the enzyme control.

Table VI. Amino acid analyses data of leucine amino peptidase digestion of T-11 peptide showing the amino acids removed.

Amino acid	8 hr	% cleavage	16 hr	% cleavage	24 hr	% cleavage	32 hr	% cleavage	40 hr	% cleavage	48 hr	% cleavage	48 hr boiled LAP + T-11 control
S-CM-cysteine	0.0124	16.8	0.028	37.8	0.047	63.5	0.052	70.3	0.094	75.8	0.302	202.7	0.0
Asparagine	0.016	22.0	0.028	37.8	0.043	58.1	0.054	73.0	0.165	133.0	0.360	241.6	0.0
Threonine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Proline	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.032	21.5	0.0
Isoleucine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.051	34.2	0.0
Leucine	0.022	24.3	0.037	44.6	0.057	71.6	0.058	73.0	0.102	79.0	0.163	109.4	0.004
Percent cleavage	$\left(\frac{0.016}{0.074} \times 100 = 22\right)$												

Sequence of N-terminal region of T-11 showing amino acids removed by the enzyme:

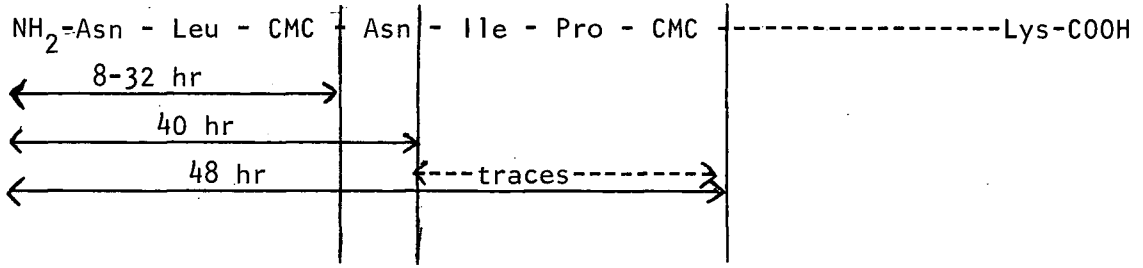


Table VII. Hapten inhibition of specific immune precipitation by leucine amino peptidase digested T-11.

	Sampling times (hr)	O.D.* 2800 A	Percent inhibition
A. Leucine amino peptidase digested T-11 (60 µg = 0.024 µmoles)	8	0.175	37.7
	16	0.165	41.3
	24	0.247	12.1
	32	0.275	2.0
	40	0.271	3.8
	48	0.280	0.0
B. Boiled leucine amino peptidase + T-11 (positive hapten control, 60 µg = 0.024 µmoles)	8	0.147	47.7
	16	0.162	42.2
	48	0.150	46.5
C. Enzyme (LAP) control (no inhibition, maximum precipitation)	8	0.276	1.7
	24	0.278	1.0
	48	0.281	0.0
D. Positive hapten (T-11) control (60 µg = 0.024 µmoles)	0	0.146	48.0
E. Negative control (no inhibition, maximum precipitation)	0	0.281	0.0

*Represents the mean values of the triplicate samples.

These inhibition tests confirmed the results of Edman degradation, in demonstrating the importance of the N-terminal region of T-11 in its haptenic activity. The aliquots tested for hapten activity in triplicate were estimated to contain 60 µg of the original T-11 preparation, which should yield maximum inhibition if enzyme cleavage at the N-terminal had no effect. The boiled LAP - T-11 control acted as a positive hapten control, but to rule out the possibility of a nonspecific effect on precipitation by boiled enzyme, a T-11 control at the same micromolar concentration was set up. The negative controls were represented by the enzyme control samples, and to discount nonspecific enzyme effect, a negative control was set up using the same buffer as in the digest. This latter negative control gave the maximum precipitation level which was used as the 100% figure. The results show that at 8 and 16 hr, virtually 100% of the haptenic activity remained. This correlated with the amino acid analysis data, which indicated that even at 16 hr, about 60% of the intact peptide was still present. This lowered the amount of hapten in these samples to 36 µg which still fell within the range of maximum haptenic activity (25 µg or greater). The boiled LAP - T-11 controls at comparable times showed essentially maximum inhibition. At 24 hr, there was only slight inhibition, and none was observed from 32 hr on. At 24 hr, there was 58% cleavage of the N-terminus, and the amount increased until at 48 hr, essentially 100% of the peptide had been degraded with the total release of at least the first three N-terminal amino

acids. At 48 hr, the boiled LAP - T-11 control still exhibited maximum inhibition, as did the positive control of T-11 alone.

V. Purification of Specific Antibodies

In order to obtain highly specific antibody for the CM-lysozyme molecule, the pooled antiserum 1 and 2 was purified by two different techniques. Once purified specific antibodies have been obtained, the detection of the specific immune reaction is made more sensitive. This was especially important in the CM-lysozyme system, since the antibody titers of the whole serum were low. Aside from this, immunospecific purification of antibodies is an essential step in understanding immunological phenomena at the molecular level.

In the first method (Fujio et al., 1968a), the specific antibodies in the pooled antiserum were precipitated by the homologous antigen at optimal proportions. The precipitate thus formed was centrifuged and washed well with PBS buffer. The antigen-antibody complex was dissociated under acid conditions, and the dissociated mixture was passed through an ascending Sephadex G-200 column. The major antibody component has a M.W. of about 150,000, and thus is slightly retarded under these conditions. However, any 19 S (IgM) immunoglobins would come out in the void volume. In our preparations, no 19 S antibody was detected. This was expected, since the immunization schedule used here selected for late response or

IgG (7S) antibody. IgM antibody may be obtained with very low antigen doses and the serum should be collected within two weeks of antigenic stimulus. In the present instance, collection of antisera was not started until four weeks after immunization, and a large antigenic stimulus was given. The CM-lysozyme antigen, represented by the third peak in figure 9, was extremely retarded on the Sephadex G-200 column. In fact it was more retarded than expected, possibly due to the high number of aromatic and basic amino acid residues it contains, which are known to adsorb weakly to Sephadex under some conditions. The IgG thus collected was dialyzed exhaustively against PBS and then concentrated to a final concentration of 1.10 mg protein/ml.

Nonspecific rabbit γ-globulin was also purified from nonspecific serum by precipitation with $(NH_4)_2SO_4$ and subsequent chromatography on DEAE-cellulose (Humphrey and Porter, 1957). This was used as control nonspecific γ-globulin in equilibrium dialysis experiments. The γ-globulin prepared in this way was lyophilized and stored as a dry powder until it was required. It was then dissolved in PBS and dialyzed exhaustively against this buffer at 4 C immediately before use.

The second method used for purification of antibodies involved the use of a specific immunoabsorbent. There are several criteria for an ideal immunoadsorbent: (i) it should be highly soluble; (ii) it should bind all the specific antibodies under conditions at which it will not retain other proteins; (iii) it should release

the adsorbed antibodies quantitatively without significant change in their activity; (iv) it should be stable with respect to time. The synthesis of bromoacetyl cellulose (BAC) met the above requirements and permitted the preparation of highly substituted protein-cellulose conjugates which retained much of the biological activity of the protein. This method can be used to purify anti-hapten antibodies which cannot be obtained by the first method given. Also, there are several examples of antibodies that do not form insoluble complexes after they interact with antigen, and therefore could not be purified by the immune precipitation method. However, in the case of the anti-CM-lysozyme serum, whole antigen was conjugated with the bromoacetyl cellulose immunoadsorbent. The preparation of bromoacetyl cellulose followed the modified method given by Robbins et al.(1967). The reaction involved mixing bromoacetic acid and powdered Whatman cellulose in absolute dioxane for 20 hr, and then adding bromoacetyl bromide, and allowing the reaction to continue for 10 to 12 hr. The Whatman cellulose had been exhaustively washed with acetone and dioxane, and dried to a constant weight before being used. The bromoacetyl cellulose (BAC-cellulose) was then precipitated by adding the reaction mixture to a large excess of deionized water which had previously been cooled to 4 C. The BAC-cellulose was then washed with deionized water and 0.1 M $NaHCO_3$ over a sintered glass funnel to get rid of excess unreacted reagents.

It was found that the BAC-cellulose changed in physical properties

if it was dried out completely, and the binding capacity with antigens was reduced, so the prepared immunoadsorbent was stored in a moist state at 4 C. The average degree of substitution obtained by this procedure was 1.3 meq Br/g cellulose (the range was 1.0 - 1.4) and the BAC-cellulose was stable for up to three months. The amount of antibody bound to BAC-protein conjugates was found to be low when compared with reports by Robbins et al. (1967) on several different antigens tested in which recovery of specific antibody ranged between 20 and 40% as opposed to 80 - 90% reported by Robbins et al.

The antibody adsorbed to the immunoadsorbent was eluted with 0.1 N acetic acid (pH 2.8) for 1 hr at 37 C, and then the cellulose-antigen conjugate was centrifuged out, and the supernatant fluid containing the antibody was dialyzed exhaustively against 0.1 M NaCl - 0.01 M Tris HCl at pH 7.0. The purified stock antibody preparation was stored at 4 C after merthiolate to a final concentration of 1:10,000 was added. The complement fixation titer of the purified antibody preparation was 1:300 when the protein concentration was 0.8 mg/ml which represented only 40% recovery but a marked increase in specific activity when compared with whole serum which had a titer of 1:80 and contained about 30 mg protein/ml.

VI. Solid Phase Peptide Synthesis

1. Synthesis

Since the results from sequential degradation of the T-11 peptide indicated the importance of the N-terminal portion in its haptenic activity, solid phase peptide synthesis was chosen to confirm these results and possibly pinpoint the antigenic region. The ultimate test for identification of an antigenic determinant is the synthesis of the amino acid sequence thought to be haptenic and show the demonstration of its ability to react with antibodies directed against the natural antigen.

The Merrifield method for peptide synthesis was a significant advance in peptide synthesis, because it allowed the peptide being synthesized to be attached to an insoluble support, the chloromethylated copolystyrene-divinylbenzene resin, and therefore excess reagents and amino acids of the soluble phase could be washed out of the resin. This eliminates the necessity for extensive purification procedures at intermediate steps in the synthesis. The reactive chloromethyl groups on the resin react with the salt of a t-butyloxycarbonyl (t-BOC) amino acid to form an ester link between the carboxyl group of the amino acid and the resin. The growth of the peptide occurs from the C-terminal amino acid toward the N-terminal. The free amino group of each incoming amino acid

is blocked by the t-BOC leaving only the carboxyl group free to react. Anhydrous HCl is used to remove the t-BOC group from the attached amino acid so that the amino group is free to react with the incoming protected amino acid. For the coupling reaction, which involves the formation of a peptide bond, dicyclohexylcarbodiimide (DCC) and a neutral pH are required in most instances, but active esters of incoming amino acids may be used with no coupling reagent. The t-BOC residues of Asn and Gln must be added as active p-nitrophenyl esters since the DCC may dehydrate the ω-amide functions of Asn and Gln to nitriles during the coupling reaction, resulting in the permanent incorporation of a number of these ω-cyano derivatives into the peptide chain.

After completion of the synthetic cycles, HBr gas is used to cleave the peptide from the solid resin support. Anhydrous trifluoroacetic acid is used for suspension of the peptide - resin for cleavage. The terminal t-BOC group still present at this time is removed by an elimination reaction, as well as the cleavage of the peptide from the resin by a nucleophilic displacement reaction. The side chain hydroxyls of serine and threonine and the sulfhydryl of cysteine must be protected with benzyl groups during the synthetic cycles. These must be removed from the peptide after cleavage from the resin before it can be tested for biological activity. The benzyl group on serine and threonine hydroxyls are removed by the HBr cleavage step, but the benzyl group on the sulfhydryl of cysteine requires a more rigorous reduction involving Na in liquid

ammonia. When cysteine, methionine or tyrosine are present in synthetic peptides, ethyl methyl sulfide must be used to protect these reduced side chains from oxidation and destruction during this step.

After all the side chain protecting groups have been removed, the cleaved peptide is purified by a suitable chromatographic technique. Table I[illegible] is a flow chart for a synthetic cycle.

The peptides synthesized in this study, which constituted the N-terminal decapeptide of T-11, and the decapeptide plus Arg at the N-terminal (this is the next amino acid along in the sequence of lysozyme), required the sodium in liquid ammonia reduction, since they contained 2 cysteine residues. These synthetic peptides required the active ester coupling reaction for the 2 Asn residues. The sequence of the two solid phase peptides was: NH_2 - (Arg) - Asn - Leu - Cys - Asn - Ile - Pro - Cys - Ser - Ala - Leu - COOH.

2. Reduction and alkylation

Once the benzyl groups had been removed from the cysteine residues of the cleaved peptide, the synthetic peptides were immediately reduced and carboxymethylated to prevent disulfide bond formation between the two cysteine residues. The reduction step with sodium in liquid ammonia also converts the nitro-arginine residue to arginine. Although the cysteine residues should be in

the reduced sulfhydryl form, 2-mercaptoethanol was added prior to the alkylation to ensure that they were reduced. For the alkylation, (^{14}C)-iodoacetic acid was used, so that later testing for the binding of peptides with specific antibodies could be detected by the radioactive labelling. However, it was later found that the degree of labelling in cpm/µmole was far too low for binding studies. The large excess of iodoacetic acid required for alkylation made it impractical to use only radioactive iodoacetic acid. Therefore, a small amount of (^{14}C)-iodoacetic acid was added to react with the cysteine residues in the peptides for 20 min, followed by a large amount of cold iodoacetic acid, and the reaction was continued for another 5 min. After the excess iodoacetic acid had been reduced by addition of 2-mercaptoethanol, the reduced and alkylated peptides were dried by flash evaporation at 45 C, washed with distilled water, and suspended in 10.0 ml water. The pH of the peptide mixtures was adjusted to 2.8 with formic acid. An amino acid analysis was done at this time on an aliquot of the peptide preparations (Tables VIII and IX).

3. Purification of reduced and carboxymethylated peptides

The purification procedure used for the reduced and alkylated peptides was similar to that used for whole T-11 peptide, namely Dowex 50 cation exchange resin with the modified four-chambered gradient system (described in the methods). Because of the presence

Table VIII. Amino acid analyses of NH_2-Asn synthetic decapeptide from the N-terminal region of T-11.

Amino acid	A. After cleavage from resin		B. After Dowex 50 purification		C. After Sephadex G-15 purification		D. Expected residues
	µmoles	Residues	µmoles	Residues	µmoles	Residues	
S-CM-cysteine	0.075	0.45	0.160	1.7	0.140	2.2	2
Aspartic acid	0.512	3.0	0.250	2.7	0.146	2.2	2
Serine	0.194	1.2	0.084	0.90	0.081	1.2	1
Proline	0.167	1.0	0.093	1.0	0.065	1.0	1
Alanine	0.376	2.3	0.155	1.7	0.083	1.3	1
Isoleucine	0.161	0.96	0.087	0.94	0.071	1.1	1
Leucine	0.335	2.0	0.187	2.0	0.143	2.2	2

Expected sequence of correct synthetic decapeptide:

position in CM-lysozyme: NH_2 - Asn - Leu - CMC - Asn - Ile - Pro - CMC - Ser - Ala - Leu
74 ---------- 83

Table IX. Amino acid analyses of NH_2-Arg synthetic peptide comprising arginine plus the N-terminal region of T-11.

Amino acid	A. After cleavage from resin		B. After Dowex 50 purification		C. After Sephadex G-15 purification		D. Expected residues
	µmoles	Residues	µmoles	Residues	µmoles	Residues	
S-CM-cysteine	0.051	2.4	0.037	2.5	0.064	2.3	2
Aspartic acid	0.048	2.3	0.036	2.4	0.054	1.9	2
Serine	0.019	0.90	0.012	0.80	0.026	0.93	1
Proline	0.030	1.4	0.021	1.4	0.034	1.2	1
Alanine	0.021	1.0	0.013	0.87	0.030	1.1	1
Isoleucine	0.022	1.0	0.016	1.1	0.031	1.1	1
Leucine	0.048	2.3	0.033	2.2	0.051	1.8	2
Arginine	0.027	1.3	0.018	1.2	0.032	1.2	1

Expected sequence of above correct 11 amino acid synthetic peptide representing N-terminal decapeptide of T-11 plus arginine on N-terminus:

position in CM-lysozyme: NH_2 - Arg - Asn - Leu - CMC - Asn - Ile - Pro - CMC - Ser - Ala - Leu
73 ---------- 83

of the ^{14}C-carboxymethyl group on some of the cysteine residues, the synthetic peptides were detected by scintillation counting rather than by alkaline hydrolysis and quantitative ninhydrin reactions on aliquots of fractions. The unreacted iodoacetic acid eluted in the void volume of the column, and the second major radioactive peak, coming out well separated from the unreacted iodoacetic acid, was shown to contain peptide material which, after amino acid analysis (Tables VIII and IX) was shown to contain the desired synthetic peptide. Figure 14 represents the elution profiles of the two synthetic peptides. The synthetic peptides were eluted from two separate Dowex columns but the results are graphed together. The material from this peak was pooled and dried over a NaOH trap in case any unreacted ^{14}C-iodoacetic acid was present. The evaporated and washed peptide material was then taken up in a known volume of distilled water, and a sample was taken for hydrolysis and amino acid analysis.

In order to purify further the peptides, and free them from low molecular weight contaminants which were sometimes present, the material from the Dowex column separation was passed through a Sephadex G-15 column equilibrated with 0.1 N acetic acid. After reading the fractions for 2300 Å absorbing material (the peptides in these instances have molecular weights of about 1300 and only slightly retarded on G-15), the eluted peptide was concentrated by flash evaporation, washed and redissolved in distilled water. Then a sample was taken, hydrolyzed, and another amino acid analysis was

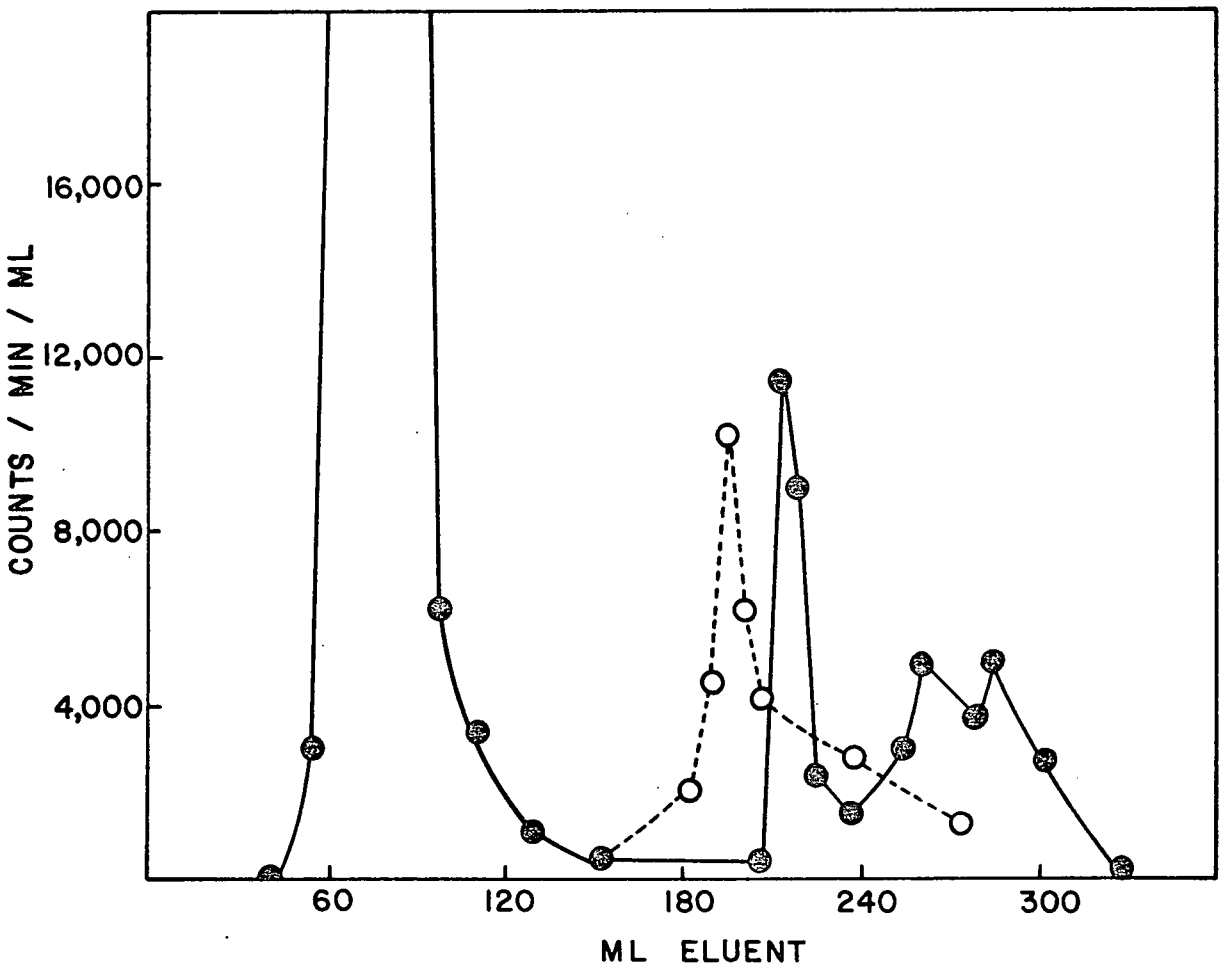


Figure 14. Elution profile of synthetic decapeptide (o---o) and synthetic decapeptide plus arginine (o-o). The first large peak represents the unreacted (^{14}C)-iodoacetic acid.

Table X. Hapten inhibition of specific immune precipitation by varying concentrations of the following peptides.

	Percent Inhibition			
µg Hapten	(^{14}C)-T-11	Arg N-terminal synthetic peptide	Asn N-terminal synthetic peptide	T-11
50.0	51.9	40.6	42.1	60.1
25.0	46.5	34.5	31.4	59.4
12.5	37.2	28.6	26.4	56.1
6.25	30.7	30.7	19.3	58.6
3.0	27.5	12.1	19.3	47.1
1.5	23.6	5.0	10.7	22.0
0.4	15.0	0	0	3.6

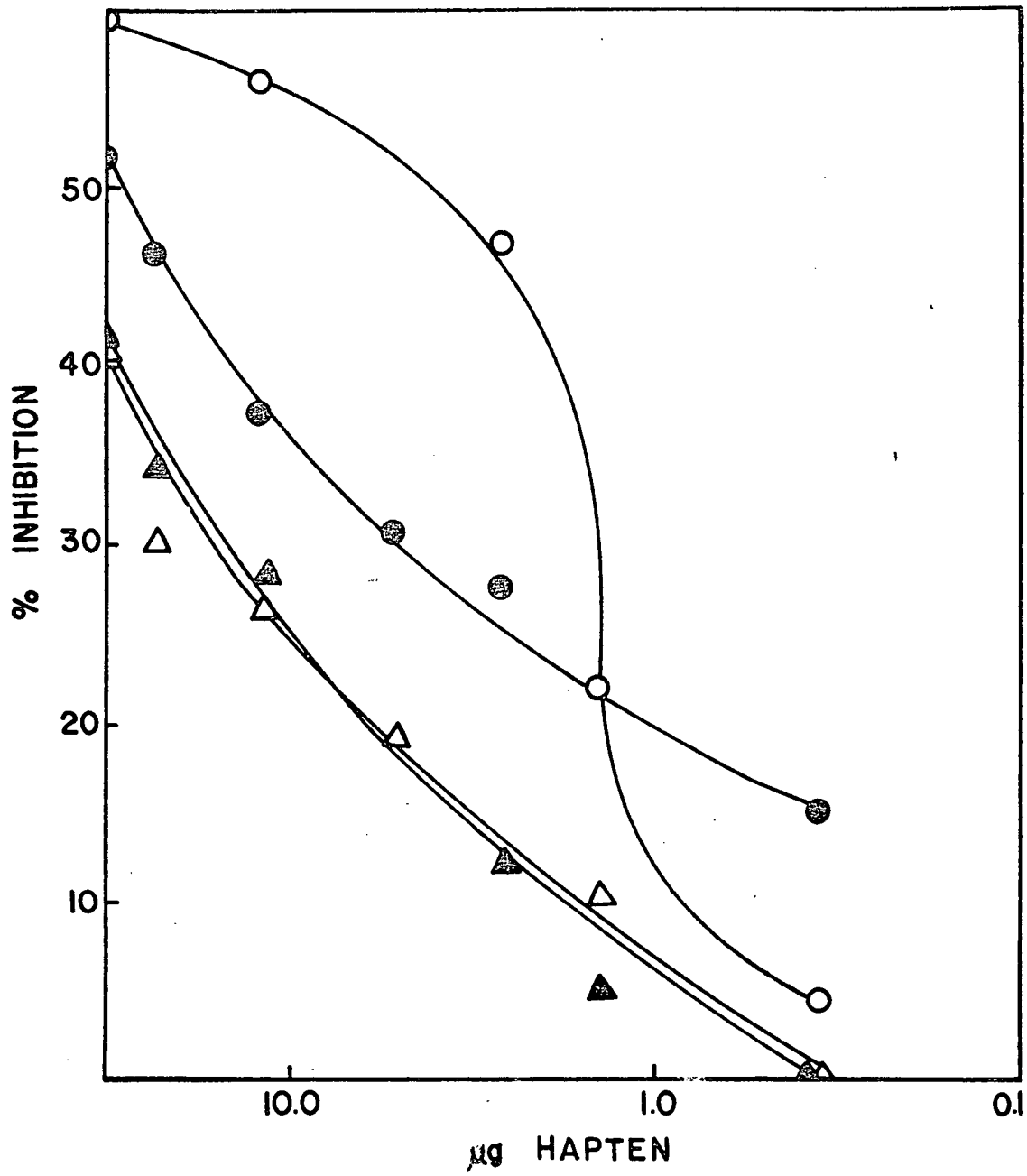


Figure 15. Inhibition of specific immune precipitation between CM-lysozyme and its homologous antiserum by: ●-●, (^{14}C)-T-11; ▲-▲ , NH_2-Arg synthetic peptide; o-o, T-11; Δ-Δ , NH_2-Asn synthetic peptide.

carried out (Table VIII and IX). There was invariably some improvement in the amino acid ratios.

High voltage electrophoresis at pH 6.5 confirmed that in both cases, only one ninhydrin positive component was present after purification of the peptides.

The haptenic activity of the synthesized peptides was tested by the precipitation inhibition technique, using serum 3 as previously described. The peptides were tested over a range between 50 and 0.4 mμg simultaneously with T-11 and (N-^{14}C)-acetylated T-11 (see the following section). The results are presented in Table X and in Figure 15. Tests were run simultaneously with the various peptides using native lysozyme and its homologous antiserum. No non-specific precipitin inhibition was noted in these instances, so it was assumed that the inhibition observed with the CM-lysozyme system was the result of specific haptenic activity. It can be seen from these data that both synthetic peptides cause inhibition of precipitation, although on a quantitative basis, they do not act as efficiently as the T-11 peptide.

VII. (^{14}C)-Acetylation of Peptides

Because the labelling of the synthetic peptides with radioactive iodoacetic acid did not give high enough specific activity for using these preparations in equilibrium dialysis studies, another radioactive label, (^{14}C)-acetic anhydride, was used. This

label adds an additional (^{14}C)acetyl group onto the N-terminal residue of the peptide, and also some secondary groups such as the ε-amino of lysine. However, under the conditions chosen, and considering the amino acid sequence of the peptides acetylated, the N-terminal acetylation reaction is highly favoured. The (^{14}C)-acetic anhydride was dissolved in benzene as stated in the methods, and then layered onto the particular peptide material in 1.0 M sodium acetate adjusted to pH 8.0 which is the optimum pH for N-terminal acetylation.

Following acetylation, the peptides were separated from unbound (^{14}C)-acetate by passing the aqueous phase through a Sephadex G-15 column equilibrated with 50% acetic acid. The elution was also carried out with 50% acetic acid using a very slow flow rate. Figure 16 shows the elution profile for the acetylated synthetic peptide (the decapeptide plus Arg) and also for acetylated T-11. The synthetic peptide had a molecular weight of about 1300 and was slightly retarded on Sephadex G-15. The larger T-11 peptide, containing 23 amino acid residues and having a molecular weight of about 2700 came out with the void and eluted just ahead of the synthetic peptide. The unreacted labelled acetic anhydride, which under these conditions would be completely hydrolyzed to acetic acid, came out much later after total exchange in the column with cold acetic acid had occurred at one column volume. The peptide peaks were detected by scintillation counting on 0.01 ml samples taken from every other fraction collected. The peptide material was pooled and flash evaporated to remove

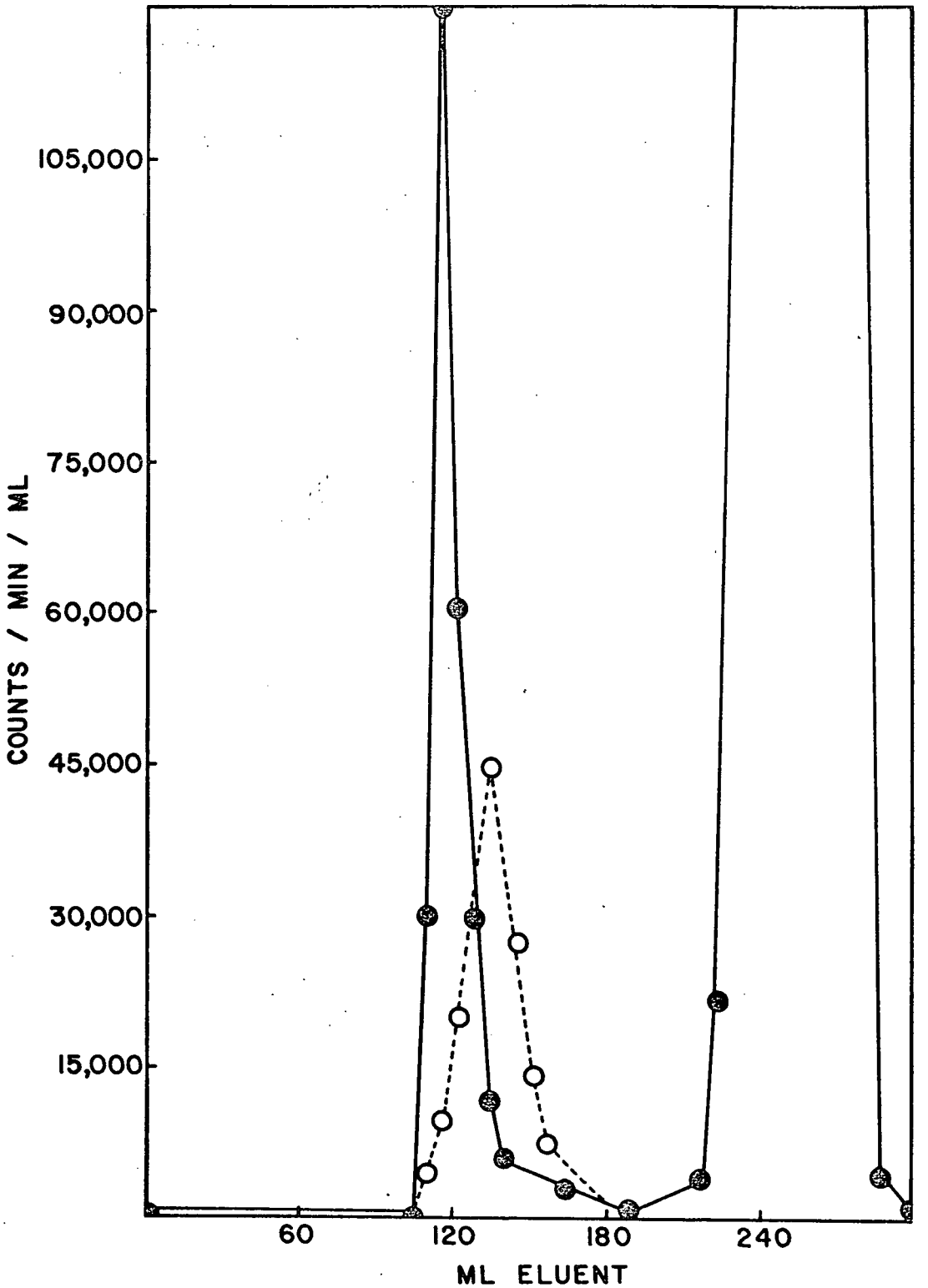


Figure 16. Elution profile for the acetylated NH_2-Arg synthetic peptide (⊙---⊙), and acetylated T-11 (o-o). The last large peak represents the unreacted (^{14}C)-acetic acid.

acetic acid, washed with distilled water and lyophilized. A sample of whole T-11 peptide was acetylated to test its haptenic activity by equilibrium dialysis and hapten inhibition simultaneously with the synthetic acetylated peptide. The lyophilized peptides were taken up in a known volume of PBS at pH 6.0 (containing 0.02% sodium azide) and two small samples from each were taken, one for hydrolysis and amino acid analysis, and one for radioactivity counting, so that quantitative counts per micromole of peptide could be calculated. These data showed that the synthetic peptide had a specific activity of 362,426 cpm/ μmole, and T-11 had 808,691 cpm/μmole.

Hapten inhibition tests by the precipitin method were set up on acetylated T-11 peptide. The results are reported in Table X and Figure 15, and show that acetylation does not inhibit the haptenic activity of T-11.

VIII. Equilibrium Dialysis

The equilibrium dialysis method for precise measurement of hapten-antibody interaction is the best method available in immunochemistry because it yields a direct measurement of the binding of antibody with the hapten. The other methods of hapten testing by precipitin inhibition and complement fixation inhibition both measure a negative reaction in which there is more room for nonspecific effects. The equilibrium dialysis method is also a

thermodynamically sound way to obtain measurements of the association constants for the interaction of hapten with an antibody combining site, since the measurements are made at equilibrium. The following equation expresses the interaction of a low molecular weight hapten with an antibody combining site, and the equilibrium or association constant.

$$H + B \rightarrow HB$$

$$\frac{(HB)}{(H)\ (B)} = K$$

The molar quantities of antibody and of bound and free hapten must be determined in order to evaluate K. In setting up the equilibrium dialysis experiments, known amounts of antibody and of hapten are used, and therefore the equilibrium dialysis essentially determines the amount of bound and free hapten. The antibody preparation is placed on one side of a dialysis membrane and hapten on the other, and the apparatus is rocked at constant temperature (4 C) for establishment of equilibrium. The dialysis membrane is such that hapten can pass through but antibody molecules cannot.

When equilibrium has been established, the free hapten concentration is measured by counting the radioactivity in samples from the hapten side of the dialysis system. At infinite hapten concentration, if the volumes on both sides of the membrane are equal, and also the free hapten concentration on both sides of the membrane are equal at

equilibrium, then the amount of bound hapten equals the total hapten added minus twice the free hapten. Expressed on a molar basis, it is possible to calculate the ratio of the number of moles of hapten bound per mole of antibody at the concentration of free hapten measured.

Controls using normal (nonspecific) γ-globulin and buffer must be set up at the same time as the test preparations containing specific antibody. When nonspecific γ-globulin is used, the hapten should equilibrate and distribute equally between the two sides. If any hapten is bound it is due to a nonspecific reaction. The buffer control is important also, since a small amount of adsorption of the hapten to the membrane occurs which may be measured by the difference between the amounts of hapten added and the amount recovered. Again, the hapten should distribute equally between the two sides in the buffer control.

This method of equilibrium dialysis was used to test haptenic activity of the acetylated 11 residue synthetic peptide and the acetylated T-11 peptide. Unfortunately, the levels of specific labelling in the acetylated peptides were not particularly good. This, coupled with the fact that the specific antibody concentration in the test was low (0.005 μmole/ml of specific antibody to CM-lysozyme) made this a poor experiment. However, the following preparations were set up for equilibrium dialysis. Both the acetylated 11 residue synthetic peptide and the acetylated T-11 peptide were tested at 0.0075 μmoles/ml and 0.0038 μmoles/ml. The

acetylated peptides were used in 4 ml quantities of the above concentrations, and 1 ml of the antibody preparation at 0.005 µmoles was placed in dialysis tubing and suspended in the peptide solutions. Controls were set up using the same two concentrations of peptide with nonspecific γ-globulin and also with PBS. After equilibrium had been established in 4 days, as shown by essentially equal counts inside and outside dialysis sac in PBS control tubes, the peptide solutions, specific γ-globulin and nonspecific globulin tubes were counted. The results were inconclusive, with only 1-3% specific binding with anti-CM-lysozyme serum which is within the limits of variability. Because the amount of specific radioactive labelling was low in the two peptide preparations, it was felt that possibly only 10% of the peptide material was radioactively labelled. This would mean that 90% cold, unlabelled peptide would compete with the 10% labelled peptide for the specific antibody molecules. At the low concentration of peptide used here, the counts would not be significant to detect specific binding to anti-CM-lysozyme antibodies.

GENERAL DISCUSSION

In this thesis, the antigenic properties of reduced and S-carboxymethylated lysozyme were studied, in order to determine the regions in the denatured molecule responsible for antigenic specificity. The results obtained from haptenic testing of tryptic peptides of CM-lysozyme demonstrated the participation of the T-11 peptide, on the basis of its ability to inhibit the homologous immunological reaction by precipitin and complement fixation inhibition. As discussed in the previous section, the possibility that the T-11 peptide preparations contained undigested protein or a contaminating peptide, was ruled out. This haptenic effect was also shown to be specific for the homologous CM-lysozyme - anti-CM-lysozyme system since the peptide caused no inhibition of the immune reaction of native lysozyme and its homologous antiserum. All other tryptic peptides, even when tested at high concentrations lacked haptenic activity. This does not mean that other regions of the CM-lysozyme molecule are not involved in antigenicity, but rather that other antigenic sites may be split by trypsin. The specificity of this system does not appear to be related to individual animals, since the sera from three different rabbits all reacted identically, except with

respect to antibody titer.

This haptenic peptide, T-11, is the largest tryptic peptide found in CM-lysozyme. It contains 23 residues and encompasses the sequence from Asn^{74} to Lys^{96}. The amino acid sequence is NH_2-Asn-Leu-CMC-Asn-Ile-Pro-CMC-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-CMC-Ala-Lys-COOH (Canfield, 1963b). This peptide shows similarities to the peptide T-8 from tobacco mosaic virus protein demonstrated to have haptenic activity by Benjamini et al. (1964), in that it contains no aromatic amino acids, it is rich in nonpolar residues and is thus sparingly soluble in aqueous solutions.

The most interesting aspect of these observations is that, although T-11 does not inhibit the precipitation reaction between native lysozyme and its homologous antiserum under the conditions used here, the sequence Asn^{74} to Lys^{96} falls within the longer peptide Gln^{57} to Ala^{107} demonstrated by Shinka et al. (1967) to exhibit haptenic activity in the native system (Figure 17). These workers used pepsin digestion of the native lysozyme molecule since it is relatively resistant to enzymatic cleavage by more specific enzymes such as trypsin. They were able to isolate four peptic peptides from the region Gln^{57} to Ala^{107} which all possessed the capacity to inhibit precipitation by 25%, and all had closely similar amino acid compositions. In fact, they differed only in their points of cleavage within the loop between Cys^{80} and Cys^{94}, which occurred at

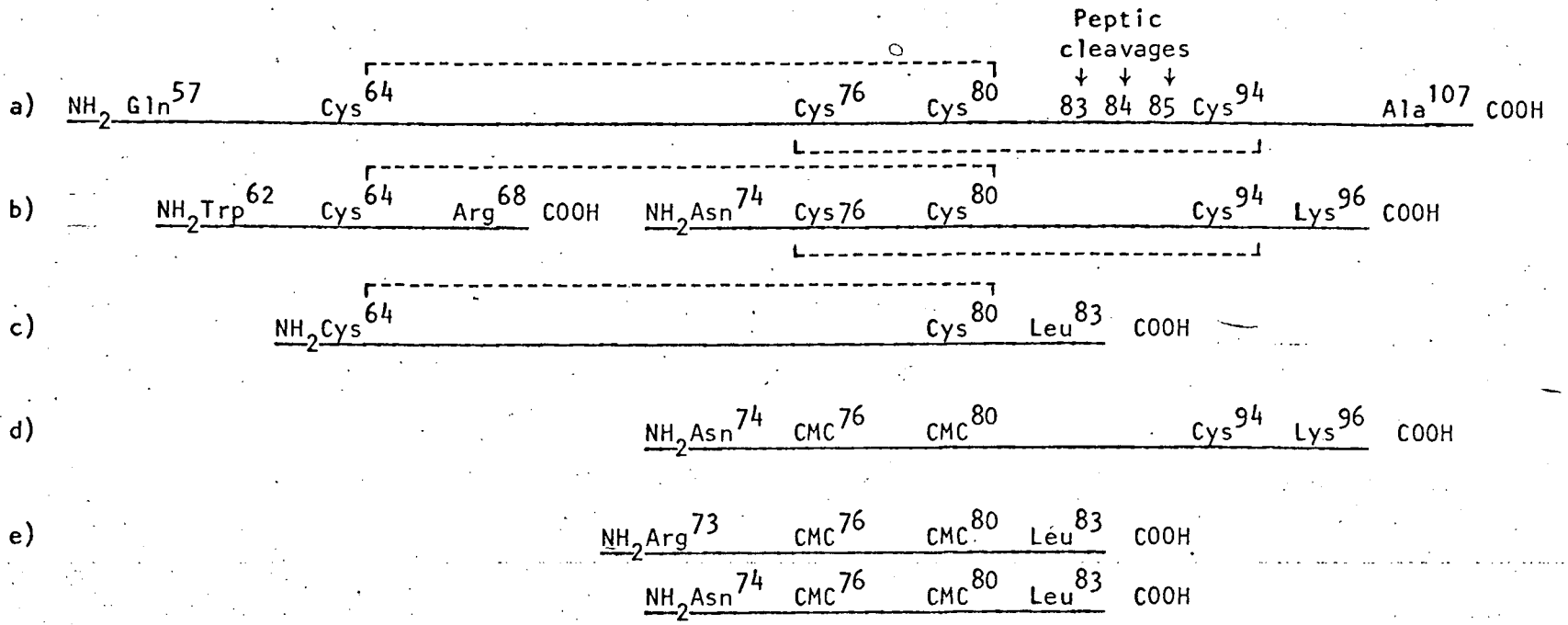


Figure 17. Correlation of the various peptide sequences exhibiting haptenic activity.
a) Haptenic peptic peptide isolated by Shinka et al.(1967) from native lysozyme;
b) haptenic tryptic peptide isolated from peptic peptide (a); Shinka et al.(1967);
c) haptenic "loop" peptide of Arnon and Sela (1969);
d) haptenic T-11 peptide isolated from CM-lysozyme in this thesis;
e) two haptenic solid phase peptides synthesized in this thesis.

Leu^{83}, Leu^{84} and Ser^{85}. It should be mentioned that this sequence contains two disulfide bridges between Cys^{64} and Cys^{80}, and between Cys^{76} and Cys^{94}, which would give this area considerable tertiary structure.

When Shinka's group digested their larger peptide with trypsin, they found one fragment capable of delaying precipitation in the homologous system, but no overall inhibition was observed. From their amino acid analysis data, it appeared that this fragment represented the sequences Asn^{74} to Ala^{95}, and Tyr^{62} to Arg^{68} (Fig. 17), joined at the Cys^{64} and Cys^{80} positions. Their conclusions are in agreement with our findings with CM-lysozyme although, with our system, inhibition of immunological reactivity by T-11 was far more pronounced.

Therefore it is possible that the determinants in this area may be the same for native lysozyme and its reduced and alkylated derivative, even though the two antisera do not appear to cross-react. It is possible that radical differences in conformation between the two antigenic preparations may be responsible for this. However, further work on these systems is necessary before this can be established.

In view of this latter proposal, it is interesting to note the work of Arnon and Sela (1969) on the native lysozyme molecule. They prepared a synthetic conjugate by covalent bonding of a lysozyme fragment (sequence Cys^{64} to Leu^{83}, denoted "loop" peptide) to a branched poly-DL-alanine peptide, and used this to elicit in rabbits, the formation of antibodies specifically directed

against this region of the lysozyme molecule. This loop peptide is part of the longer peptide shown by Shinka's group to be haptenic (Fig. 17). These antibodies were isolated immunospecifically on a lysozyme-cellulose immunoadsorbent, so no antibodies to the DL-alanine polypeptide portion were present. In addition, they obtained antibodies with a similar specificity from anti-native lysozyme serum, with an immunoadsorbent prepared from the same loop peptide. This loop peptide contains a disulfide bridge between Cys^{64} and Cys^{80}, giving it a rigid conformation. These workers demonstrated that the anti-loop antibodies were able to distinguish between the loop peptide containing a disulfide bond, and the open chain peptide derived from it, suggesting that the antibodies may be directed to a conformation-dependent determinant. However, the anti-loop antibodies obtained from immunization with the synthetic conjugate containing the loop peptide, were able to bind haptenically with both the loop peptide and its reduced and carboxymethylated derivative, although to a somewhat lesser degree in the latter case. In contrast, the anti-loop antibodies derived from antiserum prepared against native lysozyme, were able to bind haptenically only with loop peptide containing the disulfide bridge, thus discriminating to a greater extent between the "loop" and "open-chain" form of the peptide, than antibodies obtained from the synthetic polypeptide conjugate. Therefore, these two populations of antibodies, directed most likely to the same region of the native protein, will

provide an opportunity to compare them in order to learn more on the role of the antigenic carrier in the homogeneity of antibodies to a specific determinant.

Following the isolation and characterization of the haptenic T-11 peptide, the next series of experiments were aimed at pinpointing the critical antigenic region of this large 23 amino acid peptide. As reported in the literature review, with both synthetic and natural protein antigens, such as the TMVP, a sequence of five amino acids is frequently able to act as an antigenic determinant. From the results obtained from sequential degradation of T-11 from the C- and N-terminus, it appeared that the N-terminal region was important in haptenic activity. This is interesting, since the loop peptide prepared by Arnon and Sela (1969) overlaps the N-terminal region of T-11 by 10 amino acid residues (Fig. 17). This implied the importance of the N-terminal region, if the stated hypothesis of a common antigenic determinant in native and CM-lysozyme is correct. Figure 17 illustrates the various peptide sequences found that exhibit haptenic activity, and shows that the only sequence common to all of these is the decapeptide comprising the N-terminal region of T-11.

Because of these implications, the N-terminal region of T-11 was synthesized by the solid phase method, in order to determine if this smaller peptide would exhibit haptenic activity. The peptide comprised the sequence Asn^{74} to Leu^{83}, and another peptide comprising

this peptide plus Arg^{73}, were synthesized. The latter was prepared to determine whether or not this Arg residue would improve haptenic activity. It was synthesized because Sela and Mozes (1966) had shown that a high percentage of the antibodies directed against native lysozyme, a highly basic protein, were present in the acidic γ-globulin fraction isolated by DEAE-Sephadex chromatography, and an overall positive charge might enhance the haptenic activity of antigenic determinants. However, from the results of precipitin inhibition testing obtained with the two synthetic peptides, it appeared that they were both equally haptenic within the limits of variability of the test. When these two peptides were tested simultaneously with native lysozyme and its homologous antiserum, no inhibition of precipitation was detected. Therefore, the inhibition of the CM-lysozyme system was assumed to be the result of specific haptenic activity. On a quantitative basis, neither of the synthetic peptides were as efficient as the T-11 peptide in causing specific inhibition of precipitation. However, the calculation of the micromolar quantities of the synthetic peptides from amino acid analysis data may not be as accurate as with the T-11 peptide. Small amounts of biologically inactive peptide material may be present.

Another possible explanation for the greater haptenic efficiency of T-11 might involve the greater degree of hydrophobicity in the total sequence. In this respect, Benjamini (Benjamini,

et al., 1968a; 1968b) has shown that, with TMVP, increasing hydrophobicity by adding the (^{14}C)-acetyl group to the N-terminus of the haptenic pentapeptide, significantly increased its haptenic binding to specific antibodies compared to the non-acetylated pentapeptide. In fact, if the N-octanoyl derivatives of the C-terminal tetra- and tripeptides were used, specific binding with anti-TMVP serum was exhibited, whereas the non-octanoylated C-terminal tetra- and tripeptides did not. Thus, by increasing hydrophobicity on the end of the peptides containing the amino acids not essential for antigenicity, increased binding with specific antiserum was observed.

From the work of Metzger et al. (1963) on hapten-antibody interaction, it is known that in the binding of an antigenic area with specific antibody, a hydrophobic interaction is important. The enhancement of haptenic binding may be due to the orientation of the peptide away from water and into the antibody site, or due to a stabilizing effect of the fatty acid (octanoic acid in the case of the work mentioned above) on a given conformation exhibiting the best fit to the combining site of the antibody.

CONCLUDING COMMENTS

The following is a summary of the findings reported here on the antigenic properties of CM-lysozyme:

1) A tryptic peptide (T-11) was isolated which exhibited haptenic activity by causing inhibition of the immune reaction between CM-lysozyme and its homologous antiserum when tested by precipitation and complement fixation.

2) The N-terminal region of T-11 was shown to be important for the haptenic activity of the peptide. This was demonstrated by studies on sequentially degraded samples of T-11, using both enzymatic and chemical methods.

3) Ultimate proof of the importance of the N-terminal decapeptide of T-11 for haptenic activity was established by its synthesis using the solid phase method. The synthetic peptide demonstrated haptenic activity by inhibition of precipitation between CM-lysozyme and its homologous antiserum.

4) The observation was made that CM-lysozyme did not cross-react with antiserum prepared against native lysozyme. It was further noted that tryptic peptides isolated from digests of CM-lysozyme did not inhibit precipitation between native lysozyme and its homologous antiserum.

APPENDIX I

Chemicals and Materials	Place Purchased
Acetic acid	Fisher Scientific
Acetone	Fisher Scientific
(^{14}C)-Acetic anhydride	New England Nuclear
Aluminum potassium sulfate (alum)	Fisher Scientific
Ammonium chloride	Fisher Scientific
Ammonium sulfate	Fisher Scientific
Antisheep Hemolysin	Difco-Bacto
Barbituric acid	British Drug House
Benzene	Fisher Scientific
t-BOC-amino acids	Mann Research Biochemicals and Sigma Chemicals
Bromoacetic acid	Eastman Organic Chemicals
Bromoacetyl bromide	Eastman Organic Chemicals
Butanol	Fisher Scientific
Cadmium acetate	Fisher Scientific
Calcium chloride	Fisher Scientific
Carboxypeptidase A, DFP-treated	Worthington Biochemicals
Carboxypeptidase B, not DFP-treated	Worthington Biochemicals
Chloromethylated copolystyrene divinylbenzene resin	Bio-Rad
Citric acid	Fisher Scientific

Complete Freund's Adjuvant	Bacto-Difco
DEAE-cellulose	Calbiochem
Dicyclohexycarbodiimide	Eastman Organic Chemicals
N,N-Dimethylformamide	Fisher Scientific
Dioxane	Fisher Scientific
Dowex 50 x 4 cation exchange resin	Bio-Rad
Ethyl Methyl Sulfide	Eastman Organic Chemicals
Filter paper	Whatman Co.
Formic acid	Fisher Scientific
Glycerine	Fisher Scientific
Guinea pig serum (complement)	Hyland Co.
Hippuryl-L-arginine	Mann Research Biochemicals
Hippuryl-L-phenylalanine	Mann Research Biochemicals
Hydrochloric acid	Fisher Scientific
Hydrogen Bromide	Baker, Matheson
Iodoacetic acid	Eastman Organic Chemicals
(^{14}C)-iodoacetic acid	New England Nuclear
L-leucine-p-nitroanilide	Sigma Chemicals
Leucine amino peptidase	Worthington Biochemicals
Liquid ammonia	Baker, Matheson
Liquifluor	New England Nuclear
Lithium chloride	Fisher Scientific
Lysozyme	Worthington Biochemicals
Magnesium chloride	Fisher Scientific

Merthiolate	British Drug House
2-Mercaptoethanol	Eastman Organic Chemicals
Methanol	Fisher Scientific
Methyl cellulose	Fisher Scientific
Methylene chloride	Fisher Scientific
Nembutal	Abbott Laboratories
Ninhydrin	Pierce Chemicals
Nitrogen	Canadian Liquid Air
Petroleum	Fisher Scientific
Phosphorous pentoxide	Fisher Scientific
Phenyl isothiocyanate	Eastman Organic Chemicals
Pyridine	Fisher Scientific
Sephadex	Pharmacia Fine Chemicals
Sodium acetate	Fisher Scientific
Sodium azide	Fisher Scientific
Sodium barbitone	Fisher Scientific
Sodium bicarbonate	Fisher Scientific
Sodium chloride	Fisher Scientific
Sodium hydroxide	Fisher Scientific
Sodium metal	Fisher Scientific
Sodium phosphate, dibasic	Fisher Scientific
Sodium phosphate, monobasic	Fisher Scientific
Stannous chloride	Fisher Scientific
Toluene	Fisher Scientific

p-toluenesulfonyl-L-arginine methyl ester	Sigma Chemicals
Trifluoroacetic acid	Fisher Scientific
Trimethylamine	Fisher Scientific
Tris(hydroxymethyl)amino methane	Sigma Chemicals
Trypsin lyophilized	Worthington Biochemicals
Visking dialysis tubing	Union Carbide
Cellulose powder	Whatman Co.

LITERATURE CITED

1. Ambler, R. 1967. Carboxypeptidases A and B. p. 436-445; p. 650-655. In S.P. Colowick and N.O. Kaplan (ed.), Methods in Enzymology, vol. XI. Academic Press, Inc., New York.

2. Arnon, R., and M. Sela. 1969. Antibodies to a unique region in lysozyme provoked by a synthetic antigen conjugate. Proc. Nat. Acad. Sci. 62: 163-170.

3. Arnon, R., and E. Shapira. 1968. Comparison between the antigenic structure of mutually related enzymes. A study of papain and chymopapain. Biochem. 7: 4196-4202.

4. Arnon, R., M. Sela, A. Yaron, and H.A. Sober. 1965. Polylysine-specific antibodies and their reaction with oligolysines. Biochem. 4: 948-953.

5. Atassi, M.Z. 1967. Periodate oxidation of sperm whale myoglobin and the role of the methionine residues in the antigen-antibody reaction. Biochem. J. 102: 478-487.

6. Atassi, M.Z. 1967. Specific cleavage of tryptophyl peptide bonds with periodate in sperm whale myoglobin. Arch. Biochem. Biophys. 120: 56-59.

7. Atassi, M.Z. 1968. Immunochemistry of sperm whale myoglobin. III. Modification of the three tyrosine residues and their role in the conformation and differentiation of their roles in the antigenic reactivity. Biochem. 7: 3078-3084.

8. Atassi, M.Z., and D.R. Caruso. 1968. Immunochemistry of sperm whale myoglobin. II. Modification of the two tryptophan residues and their role in the conformation and antigen-antibody reaction. Biochem. 7: 699-705.

9. Atassi, M.Z., and A.F.S.A. Habeeb. 1969. Enzymic and immunochemical properties of lysozyme. I. Derivatives modified at tyrosine. Influence of nature of modification on activity. Biochem. 8: 1385-1393.

10. Atassi, M.Z., and B.J. Saplin. 1968. Immunochemistry of sperm whale myoglobin. I. The specific interaction of some tryptic peptides and of peptides containing all the reactive regions of the antigen. Biochem. 7: 688-698.

11. Benjamini, E., M. Shimizu, J.D. Young and C.Y. Leung. 1968. Immunochemical studies on the tobacco mosaic virus protein. VII. The binding of octanoylated peptides of the tobacco mosaic virus protein with antibodies to the whole protein. Biochem. 7: 1261-1264.

12. Benjamini, E., J.D. Young, W.J. Peterson, C.Y. Leung, and M. Shimizu. 1965. Immunochemical studies on the tobacco mosaic virus protein. II. The specific binding of a tryptic peptide of the protein with antibodies to the whole protein. Biochem. 4: 2081-2085.

13. Benjamini, E., J.D. Young, M. Shimizu, and C.Y. Leung. 1964. Immunochemical studies on the tobacco mosaic virus protein. I. The immunological relationship of the tryptic peptides of tobacco mosaic virus protein to the whole protein. Biochem. 3: 1115-1119.

14. Beychok, S., and E.A. Kabat. 1965. Optical activity and conformation of carbohydrates. I. Optical rotary dispersion studies on immunochemically reactive amino sugars and their glycosides, milk oligosaccharides, oligosaccharides of glucose, and blood group substances. Biochem. 4: 2565-2574.

15. Blake, C.C.F., D.F. Koenig, G.A. Mair, A.C.T. North, D.C. Phillips, and V.R. Sarma. 1965. Structure of hen egg-white lysozyme: A three-dimensional fourier synthesis at 2 Å resolution. Nature 206: 757-763.

16. Bonavida, B., A. Miller., and E.E. Sercarz. 1969. Structural basis for immune recognition of lysozyme. I. Effect of cyanogen bromide on hen egg-white lysozyme. Biochem. 8: 968-979.

17. Brooksby, J.B. 1952. A.R.C. Report, Series No. 12, A.R.C.

18. Brown, R.K. 1962. Studies on the antigenic structure of ribonuclease. III. Inhibition by peptides of antibody to performic acid oxidized-ribonuclease. J. Biol. Chem. 237: 1162-1167.

19. Canfield, R.E. 1963a. Peptides derived from tryptic digestion of egg-white lysozyme. J. Biol. Chem. 238: 2691-2697.

20. Canfield, R.E. 1963b. The amino acid sequence of egg-white lysozyme. J. Biol. Chem. 238: 2698-2707.

21. Canfield, R.E., and C.B. Anfinsen. 1963. Chromatography of pepsin and chymotrypsin digests of egg-white lysozyme on phosphocellulose. J. Biol. Chem. 238: 2684-1690.

22. Crumpton, M.J., and J.M. Wilkinson. 1965. The immunological activity of some of the chymotryptic peptides of sperm whale myoglobin. Biochem. J. 94: 545-556.

23. Crumpton, M.J., and P.A. Small. 1967. Conformation of immunologically-active fragments of sperm whale myoglobin in aqueous solution. J. Mol. Biol. 26: 143-146.

24a. Eisen, H.N., and G.W. Siskind. 1964. Variations in affinities of antibodies during the immune response. Biochem. 3: 996-1008.

24b. Folk, J.E., K.A. Piez, W.R. Carroll, and J. Gladner. 1960. Carboxypeptidase B. IV. Purification and characterization of the porcine enzyme. J. Biol. Chem. 235: 2272-2278.

24c. Folk, J.E., and E.H. Schirmer. 1963. The porcine pancreatic carboxypeptidase A system. J. Biol. Chem. 238: 3884-3888.

25. Fujio, H., M. Imanishi, K. Nishioka, and T. Amano. 1968a. Antigenic structures of hen egg-white lysozyme. II. Significance of the N- and C-terminal region as an antigenic site. Biken J. 11: 207-218.

26. Fujio, H., M. Imanishi, K. Nishioka, and T. Amano. 1968b. Proof of independency of two antigenic sites in egg-white lysozyme. Biken J. 11: 219-223.

27. Gill, T.J. III, H.W. Kunz, E. Friedman, and P. Doty. 1963. Studies on synthetic polypeptide antigens. VIII. The inhibition of antibody-synthetic polypeptide reaction by amino acids, amines, alcohols and dicarboxylic acids. J. Biol. Chem. 238: 108-123.

28. Gill, T.J., III, H.W. Kunz, and D.S. Papermaster. 1967. Studies on synthetic polypeptide antigens. XVIII. The role of composition, charge and optical isomerism in the immunogenicity of synthetic polypeptides. J. Biol. Chem. 242: 3308-3318.

29. Gerwing, J., and K. Thompson. 1968. Studies on the antigenic properties of egg-white lysozyme. I. Isolation and characterization of a tryptic peptide from reduced and alkylated lysozyme exhibiting haptenic activity. Biochem. 7: 3888-3892.

30. Goodman, J.W., D.E. Nitecki, and I.M. Stoltenberg. 1968. Immunochemical studies on the poly-γ-D-glutamyl capsule of Bacillus anthracis. III. The activity with rabbit antisera of peptides derived from the homologous polypeptide. Biochem. 7: 706-710.

31. Haber, E., and F.F. Richards. 1966. The specificity of antigenic recognition of antibody heavy chain, p. 176-187. In Proceedings of the Royal Society, Series B, vol. 166.The Royal Society, Bullington House, Piccadilly, London.

32a. Hirs, C.M.W., S. Moore, W.H. Stein. 1956. Peptides obtained by tryptic hydrolysis of performic acid-oxidized ribonuclease. J. Biol. Chem. 219: 623-642.

32b. Hummel, B.C.W. 1959. A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. Can. J. Biochem. Physiol. 37: 1393-1398.

33. Humphrey, J.H., and R.R. Porter. 1957. Reagin content of chromatographic fractions of human gamma-globulin. Lancet 1: 196-197.

34. Jaton, J.C., and M. Sela. 1968. Role of optical configuration in the immunogenicity and specificity of synthetic antigens derived from multichain polyproline. J. Biol. Chem. 243: 5616-5626.

35. Kabat, E.A. 1960. The upper limit for the size of the human antidextran combining site. J. Immunol. 84: 82-85.

36. Kabat, E.A. 1966. The nature of an antigenic determinant. J. Immunol. 97: 1-11.

37. Kabat, E.A. 1968. Structural concept in immunology and immunochemistry, p. 82-111. Holt, Rinehart and Winston, Publisher, New York.

38. Kabat, E.A., K.O. Lloyd, S. Beychok. 1969. Optical activity and conformation of carbohydrates. II. Optical rotary dispersion and circular dichoism studies on immunochemically reactive oligo- and polysaccharide containing amino sugars and their derivatives. Biochem. 8: 747-756.

39. Kabat, E.A., and M.M. Mayer. 1961. Experimental immunochemistry, 2nd ed., p. 309- , Charles C. Thomas, Publisher, Springfield, Ill.

40. Kantor, R.S., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. I. Antigenicity of DNP-polylysine and DNP copolymer of lysine and glutamic acid in guinea pigs. J. Exptl. Med. 117: 55-69.

41. Kendrew, J.C., H.C. Watson, B.E. Strandberg, R.E. Dickerson, D.C. Phillips, and V.C. Shore. 1961. A partial determination by X-ray methods, and its correlation with chemical data. Nature 190: 666-672.

42. Konigsberg, W. 1967. Subtractive Edman degradation, p. 461-469. In S.P. Colowick and N.O. Kaplan (ed.), Methods in Enzymology, vol. XI, Academic Press, Inc., New York.

43. Konigsberg, W., and R.J. Hill. 1962. The structure of human hemoglobin. III. The sequence of amino acids in the tryptic peptides of the α-chain. J. Biol. Chem. 237: 2547-2561.

44. Lapresle, C., and T. Webb. 1965. Isolation and study of a fragment of human serum albumin containing one of the antigenic sites of the whole molecule. Biochem. J. 95: 245-251.

45. Light, A. 1967. Leucine amino peptidase, p. 426-436; p. 655-660. In S.P. Colowick and N.O. Kaplan (ed,), Methods in Enzymology; vol. XI. Academic Press, Inc., New York.

46. Lowry, O.H., M.J. Roseborough, L. Fair, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

47. Merrifield, R.B. 1964. Solid-phase peptide synthesis. III. An improved synthesis of Bradykinin. Biochem. 3: 1385-1390.

48. Metzger, H., L. Holfsy, S.J. Singer. 1963. A specific antibody hapten reaction with novel spectral properties. Arch. Biochem. Biophys. 103: 206-215.

49. Nitz, R.M., B. Mitchell, J. Gerwing, and J. Christensen. 1969. Studies on the antigenicity of bacterial ferredoxins. I. The effect of modification of cysteinyl residues on the antigenicity of C. pasteurianum Ferredoxin. J. Immunol. (In press).

50. Richards, F.F., J.H. Pincus, K.J. Bloch, W.T. Barnes, and E. Haber. 1969. The relationship between antigenic complexity and heterogeneity in the antibody response. Biochem. 8: 1377-1384.

51. Robbins, J.B., J. Haimovich, and M. Sela. 1967. Purification of antibodies with immunoadsorbents prepared using bromoacetyl cellulose. Immunochem. 4: 11-22.

52. Sage, H.J., G.F. Deutch, G. Fisman, and L. Levine. 1964. Specificity of the poly-alanine immuno system. Immunochem. 1: 133-144.

53. Schlossman, S.F., H. Levine, and A. Yaron. 1968. Studies on the specificity of antibody to 2,4-dinitrophenyl-poly-L-lysines. Biochem. 7: 1-7.

54. Sela, M. 1966. Immunological studies with synthetic polypeptides. Adv. Immunol. 5: 29-129.

55. Sela, M. 1966. Chemical studies of the combining sites of antibodies, p. 188-206. In Proceedings of The Royal Society, Series B, vol. 166. The Royal Society, Bullington Press, Piccadilly, London.

56. Sela, M., and E. Mozes. 1966. Dependence of the chemical nature of antibodies on the net electrical charge of antigens. Proc. Nat. Acad. Sci. 55: 445-452.

57. Spackman, D.H., W.H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190-1205.

58. Shinka, S., M. Imanishi, M. Miyagawa, and T. Amano, M. Inouye, and A. Isugita. 1967. Chemical studies on antigenic determinants of hen egg-white lysozyme. Biken J. 10: 89-107.

59. Spragg, J., R.C. Talamo, K. Suzuki, D.M. Appelbaum, K.F. Austen, and E. Haber. 1968. Structural requirements for binding of bradykinin to antibody. II. Studies with bradykinin fragments. Biochem. 4086-4089.

60. Stewart, J.M., and J.D. Young. 1969. Solid phase peptide synthesis. W.H. Freeman and Company, Publisher, San Francisco, Cal.

61. Torii, M., E.A. Kabat, and A.E. Bezer. 1964. Separation of teichoic acid of Staphylococcus aureus into two immunologically distinct specific polysaccharides with α- and β-N-acetylglucosaminyl linkages respectively. J. Exptl. Med. 120: 13-29.

62. Webb, T., and C. Lapresle. 1964. Isolation and study of rabbit antibodies specific for certain of the antigenic groups of human serum albumin. Biochem. J. 91: 24-31.

63. Wright, N.S. 1963. Detection of strain-specific serological activity in antisera of tobacco mosaic virus clover yellow mosaic virus and potato virus X, by complement fixation. Virology 20: 131-136.

64. Young, J.D., E. Benjamini, and C.Y. Leung. 1968. Immunochemical studies on the tobacco mosaic virus protein. VIII. Solid-phase synthesis and immunological activity of peptides related to an antigenic area of tobacco mosaic virus protein. Biochem. 7: 3113-3119.

65. Young, J.D., E. Benjamini, M. Shimizu, and C.Y. Leung. 1966. Immunochemical studies on the tobacco mosaic virus protein. III. The degradation of an immunological active tryptic peptide of tobacco mosaic virus protein and the reactivity of the degradation products with antibodies to the whole protein. Biochem. 5: 1482-1488.

66. Young, J.D., E. Benjamini, J. Stewart, and C.Y. Leung. 1967. Immunochemical studies on the tobacco mosaic virus protein. V. The solid-phase synthesis of peptides of an antigenically active decapeptide of tobacco mosaic virus protein and the reaction of these peptides with antibodies to the whole protein. Biochem. 6: 1455-1459.