Structural Basis for Immune Recognition of Lysozymes. I. Effect of Cyanogen Bromide on Hen Egg-White Lysozyme*

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ABSTRACT: The two methionine residues (positions 12 and 105) of hen egg-white lysozyme are cleaved by cyanogen bromide in 70% formic acid. Cleavage of the two peptide bonds was shown by the absence of methionine and the appearance of homoserine and homoserine lactone on amino acid analysis, detection of two new amino end groups, and demonstration of three polypeptide chains upon Sephadex chromatography after reduction of cyanogen bromide treated hen egg-white lysozyme. Under optimal conditions, cyanogen bromide treated hen egg-white lysozyme exhibits 10% of the enzymatic activity of native hen egg-white lysozyme, but is more sensitive to inhibition by certain salts than hen egg-white lysozyme. Cyanogen bromide treated hen egg-white lysozyme, unlike hen egg-white lysozyme, is susceptible to limited tryptic hydrolysis. One of the first peptides to be cleaved is a heptapeptide, immediately C terminal from the point of cleavage at residue 105, which contains two tryptophans. Conversion of hen egg-white lysozyme to cyanogen bromide treated hen egg-white lysozyme causes a red shift in the absorption spectrum. The difference spectrum shows a peak at 2980 Å which has been associated with a shift of tryptophan to a more hydrophobic environment. Immunological studies show that cyanogen bromide treated hen egg-white lysozyme can cause up to 70% inhibition of the binding of hen egg-white lysozyme by antihen egg-white lysozyme serum, suggesting considerable retention of native conformation. Gel diffusion tests show that cyanogen bromide treated hen egg-white lysozyme has lost some antigenic determinants, but gained new determinants not present on hen egg-white lysozyme. It is suggested that close to the points of cleavage, there is relative flexibility in cyanogen bromide treated hen egg-white lysozyme. This permits realignment of hen egg-white lysozyme residues which, in the parent molecule, are in an unfavorable environment, accounting for the altered spectral, enzymatic, and immunological behavior of cyanogen bromide treated hen egg-white lysozyme.

We are engaged in the study of the recognition of antigenic determinants [epitopes (Jerne, 1960)] on a native, well-characterized protein molecule, hen egg-white lysozyme (HEL[1]). Our interest is in establishing how much alteration must take place before one epitope loses its identity and may be recognized as another by the immune machinery.

Typically, epitopes on native protein molecules have been studied after complete or partial digestion of the antigens, by assay of the precipitating or inhibitory activity of the resultant peptides (Lapresle and Durieux, 1957; Benjamini *et al.*, 1964, 1965; Crumpton, 1965; Cebra, 1961). Another approach has been to grossly modify a specific residue type, such as iodinating the tyrosines (Pressman and Sternberger, 1950), or by acetylating all the lysines on bovine albumin (Maurer, 1963), or by adding polypeptides to a native protein (Landsteiner and van der Scheer, 1934; Sela, 1966), and comparing the product with the parent molecule. Sometimes one or several amino acids or peptides have been altered or removed from a protein (Harris and Knight, 1955; May and Brown, 1968; Atassi, 1967; Atassi and Caruso, 1968) and its subsequent antigenicity examined.

With a molecule such as hen lysozyme available, about which information exists describing its tertiary as well as primary structure, it seemed possible to approximate a physical description of conformational changes in molecules (a) with specific residues altered, as has already been attempted with myoglobin (Atassi and Caruso, 1968), or (b) with closely related molecules, as has been also done with the cytochromes (Reichlin *et al.*, 1966). The gallinaceous egg lysozymes are a convenient system for this study: they are antigenic in many laboratory animals; many related lysozymes are readily available; they are easily purified, owing to their basicity and small size; a further advantage is the possession of a readily measurable property, enzymatic activity.

Nonenzymatic, specific alteration of various amino acid residues has been described by Gross and Witkop (1961). Cyanogen bromide has been found to spe-

* From the Department of Bacteriology, University of California, Los Angeles, California. *Received September 16, 1968.* This work was supported in part by National Science Foundation Grant GB 5410 and U. S. Public Health Service Grant AI08098 and Predoctoral Traineeship GM-01297 from the U. S. Public Health Service. This paper is derived from a thesis submitted by Benjamin Bonavida in partial fulfillment of the requirement for the degree of Doctor of Philosophy at the University of California, Los Angeles, Calif.

[1] The following abbreviations are used: HEL, hen egg-white lysozyme; HEL(CB), cyanogen bromide treated hen egg-white lysozyme; HEL(NBS_{62}), *N*-bromosuccinimide-treated hen egg-white lysozyme.

cifically alter methionine residues (Gross and Witkop, 1961, 1963), and a small number (two to three) of methionines is characteristic of all known gallinaceous lysozymes (Imanishi *et al.*, 1966; Arnheim, 1968; R. Scibienski, A. Miller, B. Bonavida, and E. Sercarz, 1968, unpublished data). We chose to examine the cyanogen bromide cleavage product before reduction of its four disulfide bridges, for conformational changes which would be reflected in alteration of the enzymatic or immunological activity of hen egg-white lysozyme (see Figure 1). The conversion of two methionyl residues to homoserine or homoserine lactone with peptide chain cleavage might be associated with local distortions very close to the points of chain disruption, or might exert a more profound effect on the shape of the molecule. Disulfide-bound, multiple-chain molecules such as the cyanogen bromide cleaved, unreduced hen egg-white lysozyme [HEL(CB)] have not been examined from this point of view. Although rabbit immunoglobulin G, a native four-chain molecule, has been cyanogen bromide treated (Cahnman *et al.*, 1966), the Fab portions containing the antibody active sites have not been characterized antigenically.

In this report we studied the effect of cyanogen bromide on hen egg-white lysozyme. Some physical, enzymatic, and immunological characteristics of the product cyanogen bromide treated hen egg-white lysozyme were compared with the parent molecule.

Materials and Methods

Hen egg-white lysozyme was obtained from Worthington Biochemical Corp., twice crystallized and salt free. Cyanogen bromide, analytical grade, was obtained from Eastman Organic Chemicals Division, Rochester, N. Y.

Diethanol disulfide was obtained from Aldrich Chemical Co., Milwaukee, Wis.

Agarose was obtained from L'Industrie Biologique Française, Genevilliers, France.

Hen Egg-White Lysozyme Derivatives. Heptasuccinyl hen egg-white lysozyme was a gift from Dr. M. Raftery. Hen egg-white lysozyme modified at tryptophan 62 by treatment with *N*-bromosuccinimide was prepared by the method of Hayashi *et al.* (1965).

CNBr-Treated Hen Egg-White Lysozyme. The method used is essentially that of Gross and Witkop (1963) using the modification described by Steers *et al.* (1965).

Purified hen egg-white lysozyme was dissolved in 70% formic acid at a final concentration of 10 mg/ml, and CNBr was added (50 molar excess relative to methionine residues in hen egg-white lysozyme). The flask was stoppered tightly, stirred manually for about 2 min, and incubated at 25° for 24 hr. At the conclusion of the incubation period, the solution was diluted tenfold with water and lyophilized. The products of the cleavage remained as white fluffy material.

Mixed Disulfides of Cyanogen Bromide Treated Hen Egg-White Lysozyme and of Hen Egg-White Lysozyme. Exchange reactions with HEL(CB) or HEL were carried out as described by Smithies (1965) using diethanol disulfide in the presence of 8 M urea. The exchange was carried out at 37° for 4 hr. Following the incubation period, the reaction mixture was chromatographed.

The mixed disulfide of HEL was concentrated by lyophilization and treated with CNBr in the same way as HEL. The formic acid was then removed and the reaction products chromatographed on Sephadex G-25.

Trypsin Action on Cyanogen Bromide Treated Hen Egg-White Lysozyme. Trypsin was added to a solution of 10 mg/ml of HEL(CB) in 1% NH_4HCO_3 (pH 8.0) to a final ratio of 1 part of trypsin to 100 parts of HEL(CB). The solution was stirred at 37° for 1–12 hr. The same procedure was performed with HEL as a control. After incubation, the reaction mixture was chromatographed on a Sephadex G-25 column using 0.2 N acetic acid as eluent. The tubes were read at 2800 Å. Amino acid analyses were performed on some peaks.

Amino acid analyses were performed according to the method of Spackman *et al.* (1958) with the automatic analyzer commercially available through the Beckman Spinco Division, Palo Alto, Calif. Samples were hydrolyzed in sealed and evacuated tubes in 6 N HCl at 100° for 22 hr. Tryptophan was determined by a modification of the method of Bencze and Schmid (1957) in which a limited alkaline hydrolysis (2.5 N NaOH, 90°, for 2 hr) of the protein under study was included before analysis. With this modification, a known control sample of HEL gave the expected tryptophan value as determined by other methods (Canfield, 1963).

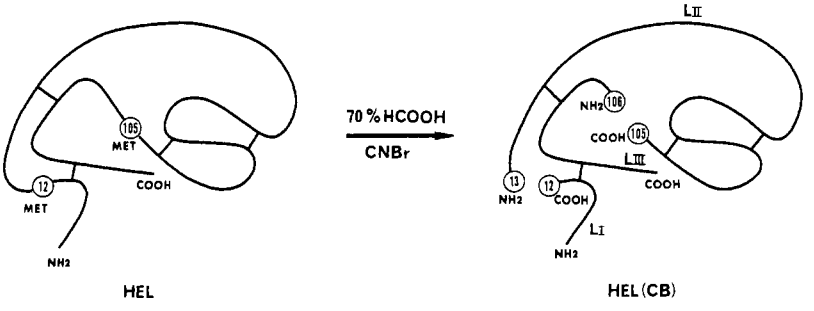

FIGURE 1: Linear representation of the lysozyme molecule and the product of CNBr treatment, HEL(CB). Notice the cleavage of the two methionyl peptide bonds at positions 12 and 105 in HEL(CB). HEL(CB) still retains the four disulfide bridges.

Amino-terminal analyses were performed on HEL, HEL(CB), and the polypeptides obtained after subjecting HEL(CB) to disulfide exchange. The method was that essentially described by Sanger (1945) using the solvent systems of Levy (1955). DNP-aspartic acid and di-DNP-lysine controls were chromatographed simultaneously with the test samples. For the quantitative analyses, after chromatography, the DNP-amino acid spots were cut out and eluted in a test tube with 1% $NaHCO_3$, and the solution was measured spectrophotometrically at 3600 Å. Correction factors to account for losses were determined with standard amounts of DNP-amino acid controls.

Column Chromatography. CNBr reaction products were dissolved in 0.2 N acetic acid and applied to a Sephadex G-25 column (3 × 100 cm) at room temperature. The effluent was analyzed by measuring the ultraviolet absorption at 2800 Å, and in some instances by ninhydrin assay after alkaline hydrolysis using the method of Hirs *et al.* (1956). The recovery of the small peptide (L_I) from 8 M urea (in the case of HEL(CB) disulfide exchange) was accomplished by chromatography of the 8 M urea-soluble fraction on Amberlite CG-50 according to the method of Dixon (1959).

Enzymatic Assays. Lysozyme activity was determined by measurement of the rate of decrease of optical density of a suspension of dried *M. lysodeikticus* cells (Worthington). A Gilford recording spectrophotometer was set to give a full-scale deflection of 0.200 optical density unit (with the sensitivity adjusted to give an initial reading of 0.900–0.950). Standard assays were run in a total volume of 2.0 ml containing 0.18 mg of cells, 0.1% bovine serum albumin, 0.067 M potassium phosphate (pH 6.24), and 0.1 M NaCl (Jollès, 1960). The equivalent of 1–10 μg of HEL in a volume of 1–20 μl was added at zero time. In general, a linear rate of decrease was observed for the full scale (0–100) over a period of 2–10 min. When the rate decreased with time, the initial slope was taken for rate determinations. An arbitrary unit of activity was defined as a change of 1 scale unit/min. Under standard conditions HEL gives 2.0 units/μg.

Acrylamide Gel Electrophoresis. The methods of Reisfeld *et al.* (1962) and of Reisfeld and Small (1966) were used in the presence of 10 M urea. Staining was done with 1% Amido-Schwartz for 1 hr, and destaining was accomplished by electrophoresis in 7% acetic acid.

Preparation of Antisera. Rabbits were immunized to HEL and HEL(CB) by an initial injection of 1 mg in complete Freund's adjuvant, distributed in the four footpads and at several subcutaneous sites. Starting 3 weeks later, repeated injections of 5 mg of soluble protein were administered intravenously at weekly intervals for 3 weeks. Blood was collected 1 week after the last injection.

Immunological Methods. DOUBLE GEL DIFFUSION. Gel diffusion analyses were performed in 1% agarose–0.1 M sodium acetate (pH 5.6) in the presence of 0.05 M glycine.

FARR INHIBITION ANALYSES. ^{125}I-HEL was prepared according to the method of McConahey and Dixon (1966). For the inhibition test, a precipitation analysis was first performed with a standard anti-HEL serum. An amount of ^{125}I-HEL was chosen for addition to the standard serum, equivalent to the maximal amount precipitated in the preliminary test. (a) Increasing amounts of inhibitor(s) were incubated with the antiserum at 37° for 1 hr. (b) The prescribed amount of ^{125}I-HEL was added (0.3–1.0 μg) and incubated for 30 min at 37°, and 4 hr at room temperature. (c) Following this second incubation, 30% sodium sulfate was added to a final concentration of 15%, and the solution was mixed and left at room temperature for 4 hr. (d) The tubes were then centrifuged and the supernatant was discarded. The precipitate was washed with 15% sodium sulfate in 0.05 M phosphate buffer (pH 7.0), centrifuged, then resuspended in 0.1 N NaOH, and counted for radioactivity in a Baird-Atomic crystal scintillation detector.

INDUCTION OF TOLERANCE. Newborn mice were injected subcutaneously on three occasions with a total dose of 15 mg of soluble antigen during the first week of life; 6 weeks later the animals were challenged with 0.2 mg of protein in Freund's adjuvant intraperitoneally and subcutaneously. After 3 weeks, they were rechallenged with 1 mg of soluble protein intravenously and bled 5 days later. The sera were tested for their antigen binding capacity following the procedure of Farr (1958), with a modification described by Reichlin *et al.* (1966).

Results

Cleavage of Hen Egg-White Lysozyme with CNBr. Gross and Witkop (1961, 1963) have demonstrated that treatment of ribonuclease with CNBr in dilute acid results in conversion of methionine into homoserine lactone with concomitant cleavage of the methionyl peptide bond. HEL has two methionine residues at positions 12 and 105 (Jollès and Jollès, 1961; Canfield, 1963). Using the conditions of Gross and Witkop (0.1 N HCl for 25 hr at 25°), HEL was treated with CNBr. The reaction mixture was lyophilized, the residue was hydrolyzed in 6 N HCl, and an amino acid analysis was performed (Table II). No reaction of methionine or other residues was evident. However, when 70% formic acid was used as the solvent (Steers *et al.*, 1965), amino acid chromatography revealed the complete loss of the two methionines and the appearance of homoserine and its lactone (Table I). In the absence of CNBr, 70% formic acid did not alter the amino acid composition of HEL.

The action of CNBr on HEL under other solvent conditions was tested and the results are summarized in Table II. No reaction was observed in 2 N HCl nor in 10% formic acid. Partial reaction occurred, under the conditions used, with 15 and 20% formic acid and reaction was complete with 25% formic acid. Conditions have not yet been found which permit differential action of CNBr on a single methionine of HEL.

Amino-Terminal Residues of Cyanogen Bromide Treated Hen Egg-White Lysozyme. Cleavage of the two methionyl peptide bonds of HEL should result in the formation of two new free amino groups, at residue 13 (lysine) and residue 106 (asparagine) (see Figure 1).

TABLE I: Amino Acid Composition of Hen Egg-White Lysozyme, Hen Egg-White Lysozyme (Treated with 70% Formic Acid), and Cyanogen Bromide Treated Hen Egg-White Lysozyme.

Amino Acid	Expected[a]	Found		
	Hen Egg-White Lysozyme	Hen Egg-White Lysozyme	Hen Egg-White Lysozyme (Formic Acid Treated)[b]	Cyanogen Bromide Treated Hen Egg-White Lysozyme
Lysine	6	6.2	5.8	6.1
Histidine	1	1.0	1.0	1.0
Arginine	11	10.8	10.3	10.9
Aspartic acid	21	21.2	21.5	20.5
Threonine	7	6.6	6.9	7.0
Serine	10	9.8	10.2	10.4
Glutamic acid	5	4.7	5.2	4.6
Proline	2	2.2	2.2	1.9
Glycine	12	12.0	12.1	12.4
Alanine	12	12.0	12.1	11.8
Half-cystine	8	8.7	8.0	8.4
Valine	6	5.9	6.0	6.3
Methionine	2	1.9	2.2	<0.01
Isoleucine	6	5.6	6.0	6.0
Leucine	8	7.8	8.0	8.2
Tyrosine	3	3.0	3.0	3.2
Phenylalanine	3	3.0	3.1	3.1
Homoserine and homoserine lactone	Nil	Nil	Nil	1.5
Tryptophan[c]	6	6		6

[a] From Canfield (1963). [b] Hen egg-white lysozyme treated with 70% formic acid without cyanogen bromide. [c] Tryptophan was determined by a modification of the Bencze and Schmid (1957) method.

TABLE II: Effect of Solvent on the Reaction of CNBr with Hen Egg-White Lysozyme.[a]

Solvent	Methionine Remaining[b] (%)
HCl (0.1 N)	100
HCl (0.5 N)	100
HCl (2 N)	100
Formic acid (70%)	0
Formic acid (25%)	0
Formic acid (20%)	60
Formic acid (15%)	85
Formic acid (10%)	100

[a] Time of incubation is 24 hr at 25°. [b] Fraction remaining was calculated from the methionine peak of the chromatogram obtained by amino acid analysis.

Since HEL has lysine at its amino terminus, HEL(CB) should have two lysines and one asparagine with free α-amino groups. Dinitrophenylation of HEL(CB) should yield twice as much di-DNP-lysine as DNP-aspartic acid. The results obtained are summarized in Table III and are consistent with the predicted positions of cleavage. No other α-amino DNP derivatives were detected and this fact taken together with the amino acid analysis of HEL(CB) (Table I) supports the contention that the reaction occurred solely as expected. Of particular importance are the identical tryptophan analyses of HEL(CB) and HEL (see Methods), which indicates that neither exposure to CNBr nor the acid reaction medium led to destruction of tryptophan.

Separation of Cyanogen Bromide Treated Hen Egg-White Lysozyme Peptides by Disulfide Exchange. An attempt was made to purify the three theoretical polypeptides obtainable by cleavage of the disulfide bonds of HEL(CB). To establish the chromatographic position of each polypeptide, we first reacted HEL with the Smithies' (1965) reagent, diethanol disulfide, plus a trace of mercaptoethanol, in the presence of 8 M urea. After incubation, the mixed disulfide of mercaptoethanol and HEL was readily separable from excess reagents by gel filtration on Sephadex G-25. The protein peak was concentrated by lyophilization and then treated with CNBr in the presence of 70% formic acid. After incubation, the material was again lyophilized to remove CNBr and formic acid. The product was dissolved in 0.2 N acetic acid and chromatographed on a Sephadex G-25 column. A typical chromatogram is shown in Figure 2. The eluates were read at 2800 Å and were analyzed with ninhydrin after alkaline hydrolysis. Three ninhydrin peaks were obtained. Amino acid

TABLE III: DNP Derivatives of Hen Egg-White Lysozyme and Cyanogen Bromide Treated Hen Egg-White Lysozyme.

Protein	DNP Derivatives[a] (mμmoles)		% Recovery[b]		Ratio
	DNP-Asp	Di-DNP-Lys	DNP-Asp	Di-DNP-Lys	Di-DNP-Lys/ DNP-Asp
Hen Egg-White Lysozyme	Nil	12		70	
Cyanogen Bromide Treated Hen Egg-White Lysozyme	15	27	88	79	1.8

[a] The concentrations of the DNP derivatives were determined from standard controls run simultaneously with the test samples. [b] The amounts of DNP-hen egg-white lysozyme and DNP-cyanogen bromide treated hen egg-white lysozyme used for chromatography were 0.017 μmole.

analysis indicated that the first of these peaks was the core polypeptide, L_{II}, residues 13–105; the second was the carboxyl-terminal peptide, L_{III}, residues 106–129; and the third peak was the amino-terminal dodecapeptide, L_I (Table IV). L_I has no tryptophan or tyrosine residues and shows no absorption at 2800 Å, but is ninhydrin positive.

Upon exchange of HEL(CB) with diethanol disulfide one would expect HEL(CB) to dissociate into three polypeptide chains which could elute from Sephadex at positions comparable to L_{II}, L_{III}, and L_I above. The elution pattern of the reduced HEL(CB) mixture (reduced products, urea, and diethanol disulfide) from Sephadex G-25 is shown in Figure 3. By amino acid analysis it was shown that the first peak corresponds to the core polypeptide, L_{II}, and is followed by the middle-sized polypeptide, L_{III} (Table IV). Both of these peptides have three tryptophan residues each and thus should absorb at 2800 Å. Analysis of peak I indicated that it contained a mixture of urea and the diethanol disulfide reagent. By analogy with Figure 2, L_I should also be present in the tubes containing the excess reagents. The recovery of the peptide L_I from urea was attempted by chromatography on Amberlite CG-50 according to the method of Dixon (1959). Table IV summarizes the amino acid analyses of the peptides obtained from the Sephadex gel filtration. Dinitrophenylation of L_{II} and L_{III} produced di-DNP-lysine and DNP-aspartic acid as expected.

Enzymatic Activity of Cyanogen Bromide Treated Hen Egg-White Lysozyme. When the lytic activity of HEL(CB) on *M. lysodeikticus* cells was assayed under our standard conditions (see Methods), it was found that HEL(CB) was only 0.5% as active as HEL (Table V, line 1). However, it was also found that when HEL(CB) and HEL were present together (in a 100:1 ratio) in an assay, the activity was consistently only about 80% of that expected from separate assays

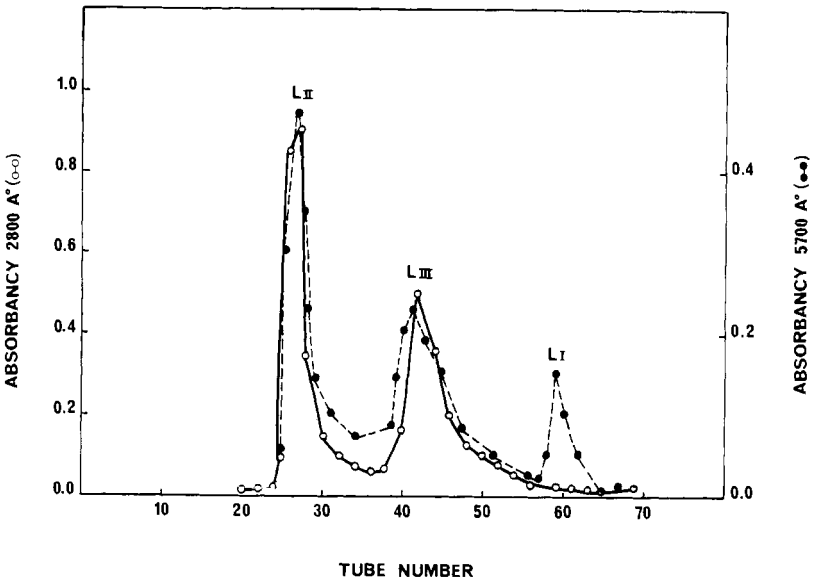

FIGURE 2: Gel filtration of the products obtained by the action of CNBr on the mixed disulfide of HEL and mercaptoethanol, fractionated on a 3 × 80 cm column of Sephadex G-25. Flow rate was 30 ml/hr. Absorbancy of the fractions was measured at 2800 Å (○—○) and aliquots tested for ninhydrin after alkaline hydrolysis at 5700 Å (●---●). The Roman numerals II, III, and I represent the three polypeptides obtained, numbered from the NH_2-terminal end of the hen egg-white lysozyme molecule.

TABLE IV: Amino Acid Composition of Peptides Separated on Sephadex G-25.

	Reduced Hen Egg-White Lysozyme Plus CNBr						Cyanogen Bromide Treated Hen Egg-White Lysozyme after Reduction					
	L_{II}		L_{III}		L_I		L_{II}		L_{III}		L_I	
Amino Acid	Theory	Found	Theory	Found	Theory	Found	Theory	Found	Theory	Found	Theory	Found
Lysine	4	3.8	1	1.0	1	1.0	4	3.9	1	0.8	1	0.9
Histidine	1	0.9					1	0.8				
Arginine	6	6.5	4	4.1	1	0.8	6	6.4	4	3.8	1	0.8
Aspartic acid	18	18.1	3	2.8			18	18.4	3	2.9		
Threonine	6	5.4	1	0.9			6	6.2	1	1.0		
Serine	10	8.6					10	9.4				
Glutamic acid	3	2.9	1	1.1	1	0.7	3	3.2	1	1.1	1	0.6
Proline	2	1.9					2	2.2				
Glycine	9	8.7	2	2.2	1	1	9	9.0	2	2.2	1	1.0
Alanine	6	6.1	3	3.1	3	2.9	6	6.8	3	3.1	3	2.7
Half-cystine	5	4.8	2	1.8	1	0.7	5	4.4	2	1.4	1	0.8
Valine	3	2.8	2	1.8	1	1.3	3	2.9	2	2.1	1	1.0
Methionine												
Isoleucine	5	4.4	1	0.9			5	4.5	1	0.8		
Leucine	6	5.6	1	1.1	1	1.3	6	6.1	1	1.1	1	1.2
Tyrosine	3	2.6					3	3.0				
Phenylalanine	2	2.1			1	0.9	2	2.4			1	0.9
Homoserine and homoserine lactone	1	0.8			1	0.7	1	0.9			1	0.6
Tryptophan	3	Nd[a]	3	Nd[a]			3	Nd[a]	3	Nd[a]		

[a] Not determined.

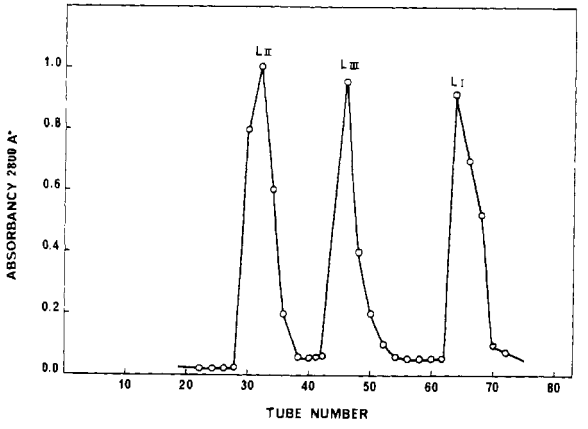

FIGURE 3: Chromatographic fractionation of the products obtained from the exchange reaction between HEL(CB) and diethanol disulfide, in the presence of a trace of mercaptoethanol and 8 M urea, on a 3 × 80 cm column of Sephadex G-25. Flow rate was 30 ml/hr. Absorbancy of the fractions was measured at 2800 Å. Peak I represents absorption of the mixture of urea and the diethanol disulfide reagents.

(Table V, lines 1, 2, and 4). This result suggests that HEL(CB) is able to compete with HEL for substrate sites on the cell wall. The availability of these sites appears to be rate-limiting under the conditions of assay.[2]

When assays were carried out in 0.02 M sodium acetate (pH 6.2), it was found that HEL(CB) had approximately 10% of the lytic activity of HEL (Figure 4). About the same ratio of activity was found when the assays were carried out in 0.02 M acetate (pH 5.5 or 5.0) or in 0.02 M cacodylate (pH 7.0).

In order to explain the low activity of HEL(CB) found in the standard assay system, each component was investigated separately. Bovine serum albumin was not inhibitory, and 0.067 M potassium phosphate (pH 6.2) was only slightly so. Sodium chloride caused some inhibition (Figure 4) which, however, is of insufficient magnitude to explain the results. Only when the phosphate and sodium chloride were both present was the marked reduction of HEL(CB) activity found which indicates that these salts act synergistically. A

[2] Under the standard assay conditions, about 10^{14} molecules of HEL and/or 10^{16} molecules of HEL(CB) are present and 0.18 mg of dry cells (about 10^8 cells). Assuming the cell wall contributes about 20% of the dry weight, the maximum number of susceptible bonds, based on the cell wall content of muramic acid and *N*-acetylglucosamine (Czerkawski *et al.*, 1963), would be about 2×10^{17}.

TABLE V: Inhibition of Hen Egg-White Lysozyme by Cyanogen Bromide Treated Hen Egg-White Lysozyme.

Addition	Units of Act.[a]
1. 206 μg of CNBr treated hen egg-white lysozyme	2.08
2. 2.0 μg of hen egg-white lysozyme	3.77
3. 1 plus 2 (expected)	5.85
4. 1 plus 2 (experimental)	4.85 (83%)

[a] Activity determined under standard conditions as described in Methods. The concentrations of hen egg-white lysozyme and cyanogen bromide treated hen egg-white lysozyme were calculated assuming that both molecules have equivalent absorption at 2800 Å.

similar but less severe effect was found on HEL activity (compare Table V, lines 1 and 2 with Figure 4).

Acrylamide Gel Electrophoresis. In glycine–acetate buffer at pH 4.5, under the conditions used by Reisfeld *et al.* (1962) in the presence of 8 M urea, HEL(CB) gave a single band on acrylamide gel electrophoresis, at the same position as HEL (Figure 5A). Under these conditions, L_{II} also gave a single, more anionic band, while L_{III} gave three bands. The multiple banding in L_{III} might be due to aggregation or alternatively to partial deamidation of asparagine and glutamine during the CNBr treatment in acid.

Using the conditions of Reisfeld and Small (1966) at pH 8.0, HEL(CB) again migrated as one band, at a position quite different from that of HEL (Figure 5B). No trace of native HEL in the HEL(CB) preparation was detected. Both L_{II} and L_{III}, at pH 8.0, gave multiple band patterns.

Ultraviolet Spectra. In an attempt to rationalize the reactivity of HEL with CNBr in 20% formic acid but not in HCl at a concentration as high as 2 N, the ultraviolet spectra of HEL under these two conditions were compared. The spectra showed no difference. However, conversion of HEL into HEL(CB) gives rise to a pronounced red shift in the spectrum. When the spectra of HEL and HEL(CB) are compared in 0.1 M sodium acetate (pH 4.5), the difference spectrum, with its peak at 2980 Å (Figure 6), closely resembles that obtained upon treatment of HEL with cationic detergents at this pH (Hayashi *et al.*, 1968).

Action of Trypsin on Cyanogen Bromide Treated Hen Egg-White Lysozyme. HEL(CB) undergoes limited enzymatic digestion with trypsin, whereas HEL is not attacked (Jollès, 1964). HEL(CB) becomes completely soluble after incubation at 37° for 1 hr, although there is still evidence of high molecular weight material left after digestion for 12 hr (Figure 7).

The nature of the digestion products is under study. Amino acid analysis of a peptide which is liberated within the first 4-hr digestion (Figure 7, peak G) suggests that it is the heptapeptide 106–112 immediately adjacent to methionine 105 (Asn-Ala-Trp-Val-Ala-Trp-Arg).

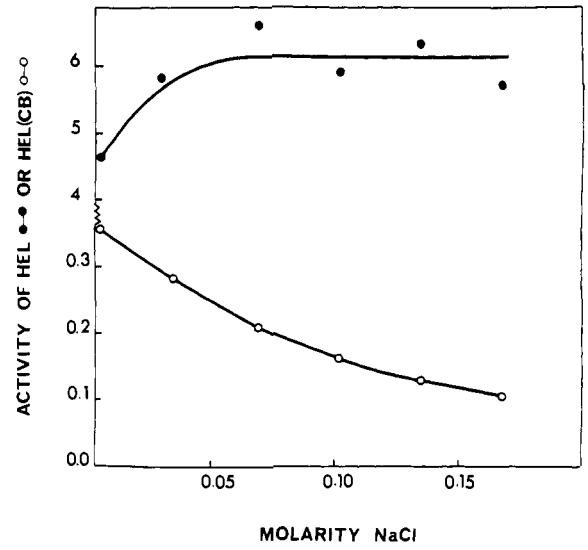

FIGURE 4: Effect of NaCl on the initial rate of enzymatic activity of HEL and HEL(CB). Activity is in arbitrary units derived from measurement of the initial rate of decrease in absorption at 500 mμ of the standard *Micrococcus* cell suspension (see Methods). All reactions were carried out in 0.02 M sodium acetate (pH 6.2).

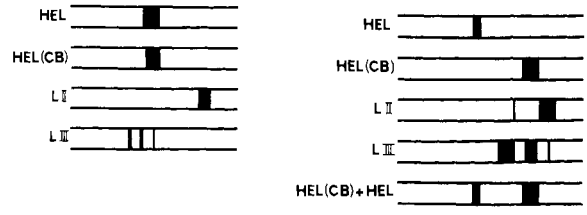

FIGURE 5: Diagrammatic representation of acrylamide gel electrophoresis (A) run at pH 4.5 using the conditions described by Reisfeld *et al.* (1962) for the separation of basic proteins. (B) Run at pH 8.0 using the conditions described by Reisfeld and Small (1966).

Immunological Characterization. To try to assess the extent of the alteration in HEL by CNBr cleavage, we made use of several immunological methods designed to show whether HEL(CB) is recognized as different from HEL by antibody reactivity or by immunogenicity.

INHIBITION OF ANTIGEN BINDING. To assess the quantitative extent of the cross reaction between HEL(CB) and HEL, these proteins were compared by inhibition analysis in an ^{125}I-HEL rabbit-anti-HEL serum precipitation system using the Farr (1958) technique as modified by Reichlin *et al.* (1966) (Figure 8). Inhibition of precipitation of ^{125}I-HEL by varying concentrations of native HEL or formic acid treated HEL (not shown) gives values which fall on the theoretical curve with 50% inhibition occurring at a 1:1 ratio. In contrast, a 6:1 ratio of HEL(CB) to ^{125}I-HEL is needed to obtain 50% inhibition. Complete inhibition of the ^{125}I-HEL–anti-HEL reaction by HEL(CB) is not reached at a ratio of 100:1; the plateau of the inhibition curve in numerous experiments is 70%. This suggests that certain determinants are present on HEL which are no longer present or available on HEL(CB).

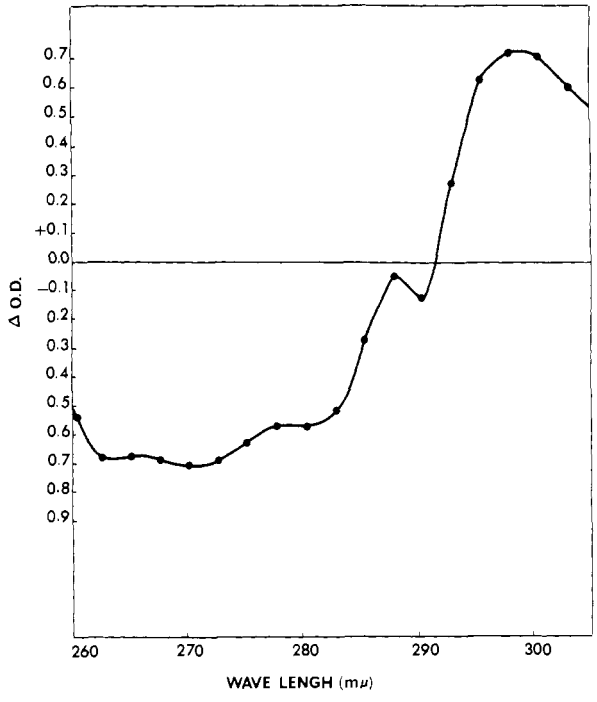

FIGURE 6: Difference spectrum of HEL in 0.1 M sodium acetate, pH 4.5, *vs.* HEL(CB) in the same solvent. The concentration of each protein was 0.025%. The spectra were measured separately in a Cary Model 15 spectrophotometer and the difference spectrum was calculated.

Two other derivatives of HEL, HEL(NBS_{62}), and heptasuccinyl hen egg-white lysozyme, were analyzed in the same way as HEL(CB). HEL(NBS_{62}) is a molecule in which tryptophan residue 62 is modified (Hayashi *et al.*, 1965); it is enzymatically inactive. As shown in Figure 8, no immunological differences are detected between HEL(NBS_{62}) and HEL.

Heptasuccinyl hen egg-white lysozyme, in which all six lysine residues are modified, behaves as a weaker inhibitor than HEL(CB). The maximum inhibition reached is only 42% (Figure 8).

GEL DIFFUSION ANALYSES. To study the immunological relationship between HEL and HEL(CB), double diffusion analysis in agarose has been performed with HEL, HEL(CB), and their respective antisera. Extensive cross reaction can be observed between the HEL and HEL(CB) as a line of partial identity (Figure 9) with an anti-HEL serum. A spur is formed with HEL indicating that there are determinants on HEL, antibodies which cannot be absorbed out with HEL(CB). Likewise, evidence for immunological differences between HEL(CB) and HEL can be shown in gel diffusion analyses with anti-HEL(CB) serum. Antibodies exist in anti-HEL(CB) which are not removed by HEL, thus permitting spur formation with HEL(CB) (Figure 9).

IMMUNOLOGICAL UNRESPONSIVENESS. To determine whether HEL(CB) was recognized by the antibody-forming system as being different from HEL, use

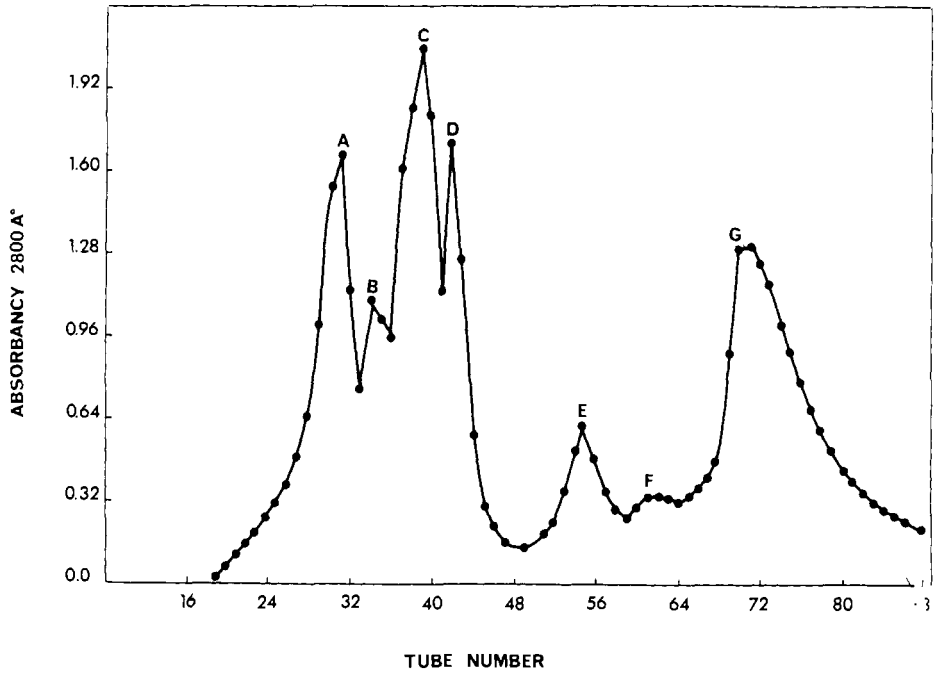

FIGURE 7: Gel filtration of the products obtained by treating HEL(CB) with trypsin on a Sephadex G-25 column (3 × 80 cm); flow rate was 30 ml/hr. HEL(CB) was suspended in 1% NH_4HCO_3 at pH 8.0 and trypsin was added at a ration of 1/100 to that of HEL(CB). The reaction mixture was stirred during incubation at 37° for 12 hr. The tubes were read at 2800 Å. Amino acid analyses have indicated that peaks A and B are of high molecular weight components. Peak G was found to correspond to a heptapeptide of amino acid composition equivalent to that of sequence 106–112 of HEL molecule. Notice that peak G has a high absorption of 2800 Å due to the presence of two tryptophan residues, 108 and 111 (Canfield, 1963).

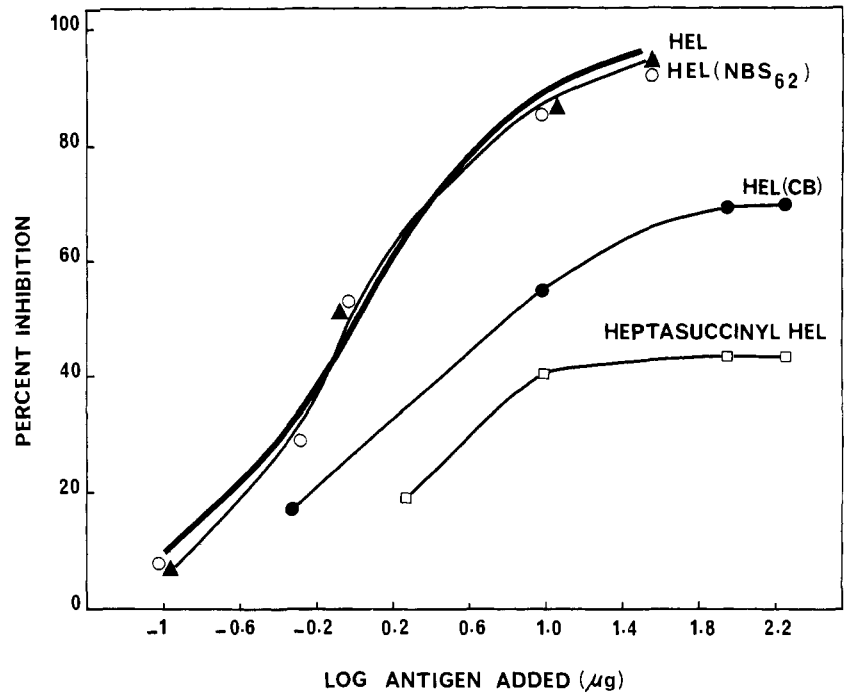

FIGURE 8: Inhibition analyses using the ^{125}I-HEL–anti-HEL system in the presence of various inhibitors. The amount of ^{125}I-HEL used was constant, as well as the volume of the antihen egg-white lysozyme serum. Increasing amounts of inhibitors were used and plotted in log micrograms. The per cent inhibition was calculated from the number of radioactive counts precipitated. The theoretical curve which is expected is represented by a heavy line (—). Inhibitors used are hen egg-white lysozyme (▲—▲); N-bromosuccinimide-treated hen egg-white lysozyme (○—○); cyanogen bromide treated hen egg-white lysozyme (●—●); and heptasuccinyl hen egg-white lysozyme (□—□).

was made of the phenomenon of immunological unresponsiveness, or "paralysis." Cinader and Dubert (1955) and Weigle (1961, 1962) have shown that paralysis of a given antigen may be overcome by presenting a naturally cross-reacting antigen or by altering the antigen through chemical modification. For example, bovine serum albumin paralyzed animals could be given either human serum albumin or arsanilated–sulfanilated bovine albumin to overcome paralysis. Thus, mice paralyzed to HEL(CB) could be tested for their ability to recognize HEL as immunogenic, which would indicate the existence of some determinants on the native lysozyme not present on HEL(CB). A typical experiment is shown in Table VI. HEL(CB) paralyzed mice, which are unable to respond to HEL(CB), do form antibodies to HEL. Thus, there is clear recognition by the immune system that HEL(CB) is a different entity from HEL.

Discussion

We have demonstrated that the cleavage of two methionyl peptide bonds, even in a disulfide-stabilized protein such as HEL, has distinct effects on both its enzymatic and antigenic activity. Proof of cleavage by cyanogen bromide was afforded by the demonstration that in the treated protein (a) no methionines remained, (b) the expected new residues, homoserine or homoserine lactone, were found, (c) the expected new N-terminal lysine and aspartic acid appeared, and (d) upon reduction, three fragments, with the

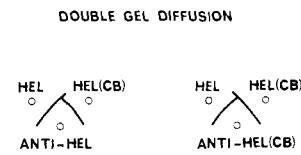

FIGURE 9: Diagrammatic representation of diffusion analyses in 1% agarose, 0.1 M sodium acetate (pH 5.5), and 0.05 M glycine. Left: cross reaction between HEL and HEL(CB) with rabbit antihen egg-white lysozyme serum. Right: pattern of cross reaction between HEL and HEL(CB) with rabbit anti-HEL(CB) serum.

appropriate amino acid composition (residues 1–12, 13–105, and 106–129), could be demonstrated.

Within the limits of our detection, no native HEL remains after cyanogen bromide cleavage. This is shown by acrylamide gel electrophoresis of HEL(CB) and HEL performed under conditions where their migration rates differ (see Figure 5B); no trace of a component migrating to the HEL position appears in our HEL(CB) preparation. As another criterion of homogeneity, in immune inhibition experiments similar to that depicted in Figure 8, a plateau is reached in the inhibition curve in an anti-HEL system with the addition of sufficient HEL(CB). Despite further increments of HEL(CB), the plateau remains flat at the 70% inhibition level. Enough HEL(CB) can be added to exclude the presence of even 0.2% admixture with native HEL.

Cleavage of HEL with CNBr did not occur, even in the presence of 2 N HCl. The lysozyme was recovered with unchanged enzymatic activity. The failure to obtain reaction in HCl can be explained if it is assumed that the conformation of HEL in solution closely resembles that found in crystals. The indications are that both methionines are buried in the interior of the HEL molecule and presumably would be shielded from attack by CNBr (Blake *et al.*, 1965). It was necessary to use 70% formic acid to obtain maximum peptide-bond cleavage of β-galactosidase (Steers *et al.*, 1965). Provided that more than 25% formic acid was used in our CNBr reaction mixture, no methionine residues were detectable in HEL(CB) on amino acid analysis. It is conceivable that at formic acid concentrations between 15 and 25%, only one of the two methionines on each molecule is cleaved, but this must be studied further. Although formic acid treated HEL was indistinguishable enzymatically and immunologically from native HEL, it is probable that in the presence of formic acid, reversible unfolding of the protein occurs providing easier access of the CNBr to the two methionines.

HEL(CB) is a unique type of modified protein, the three peptide fragments of which are held together in the unreduced molecule by four disulfide bonds (see Figure 1). In a protein such as myoglobin, the fragments produced upon cleavage with cyanogen bromide immediately separate (Edmundson, 1963). Ribonuclease has been treated with CNBr; in this case, part of the molecule is removed upon cleavage leaving a disulfide-bound residue (Gross and Witkop, 1963). Likewise, rabbit and human immunoglobulin G have been cleaved at the sensitive region which separates the antibody-active Fab fragments from the inactive Fc fragments (Cahnman *et al.*, 1966; Lahav *et al.*, 1967). Other peptide-bond scissions in the Fab and Fc fragments have been studied (Waxdal *et al.*, 1968) but nothing is known about the conformation of the macromolecular product.

Except for the two methionines, the primary structure of HEL(CB) is presumably unchanged. There is no indication of bromine formation from cyanogen bromide and possible oxidation of HEL tryptophan residues in the presence of 70% formic acid (E. Gross, 1968, personal communication). Furthermore, comparison of HEL(CB) and HEL discloses no difference in their tryptophan/tyrosine ratio.

However, indications of an alteration in the tertiary structure arise from several independent types of evidence: spectral, trypsin sensitivity, enzymatic activity, and immunological activity. The red shift in the spectrum which occurs when HEL is converted into HEL(CB) resembles that found upon solvent perturbation of HEL (Hamaguchi *et al.*, 1963; Williams *et al.*, 1965) and is almost identical with that obtained after treatment of HEL with cationic detergents (Hayashi *et al.*, 1968). The above authors have attributed this shift to a change in tryptophan absorption as a consequence of transfer from a relatively aqueous to a nonpolar environment.

Since tryptophan is perhaps the most hydrophobic of the amino acids (Tanford, 1962), it follows that the partial exposure of tryptophan to water in native HEL results from restriction of the position which the tryptophan may assume because of other energetic considerations which impose a particular, rigid conformation on HEL. It may be that the introduction of two new scissions in the single peptide chain of HEL, especially that at position 105, allows the exposed tryptophans (at position 108 and possibly at position 111) to assume an energetically more favorable conformation than in the parent molecule.

A change in conformation in the region of position 105 would be consistent with increased sensitivity of arginine 112 in HEL(CB) to attack by trypsin. An increase in flexibility of HEL(CB) relative to HEL, restricted to the point of cleavage near position 105, suggests a possible, though clearly not unique, explanation for the reduced enzymatic activity of HEL(CB) and for the alteration of antigenic properties of HEL(CB).

It is of some interest that the difference spectrum of HEL in the presence or absence of chitotriose (Dahl-

TABLE VI: Antibody Response in Mice Paralyzed to Cyanogen Bromide Treated Hen Egg-White Lysozyme and Challenged with Hen Egg-White Lysozyme and Cyanogen Bromide Treated Hen Egg-White Lysozyme.

Mice	Tolerogen	Immunogen	μg of ^{125}I Hen Egg-White Lysozyme Bound/ml of Serum[a]
3	HEL(CB)	HEL	12
3	HEL(CB)	HEL(CB)	0.5
3	HEL(CB)		0.5
3		HEL	36
3		HEL(CB)	28

[a] The Farr assay was used to calculate the amount of labeled ^{125}I hen egg-white lysozyme that is precipitated with the antiserum. The numbers represented in the last column are averages of three antisera tested.

quist *et al.*, 1966) closely resembles that obtained upon solvent perturbation of HEL (Hamaguchi *et al.*, 1963, 1964; Williams *et al.*, 1965). Chitotriose is thought to bind in a manner similar to the polysaccharide substrate and to interact with tryptophan 108, shielding it from water (Blake *et al.*, 1965). X-Ray analysis of lysozyme after interaction with the inhibitor, chitotriose, suggests that very little conformational change results upon formation of the enzyme–substrate complex. It may be that HEL(CB), in the region of the cleft, may assume a conformation similar to HEL only in the presence of substrate; thus, the substrate may induce a larger conformational change with HEL(CB) than with HEL. Some proof for this notion may be obtained by examination of immunological properties and trypsin sensitivity of HEL(CB) in the presence and absence of chitotriose.

HEL(CB) differs immunologically from HEL in both having gained determinants not present on HEL, and in having lost determinants present on HEL. HEL(CB) cross-reacts with only 70% of the rabbit anti-HEL serum used in the present inhibition experiments. It is interesting that the largest product obtained after treatment of HEL(CB) with trypsin (peak A, Figure 7), which contains no native HEL(CB), still inhibits an anti-HEL system two-thirds as well as HEL(CB). Presumably, the loss of several amino acids in HEL(CB), due to the cleavage at newly disclosed trypsin-sensitive sites, has not fundamentally altered epitopes on the remainder of the molecule, which can still react with anti-HEL. The initial products of tryptic digestion of HEL(CB) are now being compared with native HEL(CB) with HEL-absorbed anti-HEL(CB) serum to locate epitopes which are specific to HEL(CB).

The antibodies formed in the experiment on immune recognition of HEL in HEL(CB) paralyzed mice may be useful in determining the extent of the deformation caused by cleavage of the HEL peptide chain. For example, the anti-HEL serum made in HEL(CB) paralyzed mice may be directed only against portions of the HEL molecule which have been altered by cyanogen bromide treatment.

There is suggestive evidence from experiments with HEL derivatives that the region surrounding methionine 12 in HEL is not a major antigenic determinant region. Modification of histidine 15 on native HEL by combination with 3-nitro-4-hydroxybromoacetaniline does not alter HEL's immune reactivity (B. Bonavida, 1968, unpublished data). Likewise, oxidation of the two methionine residues has no immunological effect (L. Kanarek, personal communication). However, disruption of the helical region surrounding methionine 12 would be expected to be expressed by an immunological change. More work is clearly required to specify whether this is one of the locations of the new HEL(CB) epitopes.

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