RECEIVED BY DTIC AUG 30 1967

000-690-20

CESTI REICES

H.Q. 3.00 MN 65

MASTER

I. DENATURATION OF ENZYMES BY THE DIRECT
ACTION OF IONIZING RADIATION.

AND

II. ENZYMICALLY ACTIVE COMPONENTS OF
THE CRYSTALLINE CHYMOTRYPSINS

by

Louis Edwin Henderson

B.A., University of Omaha, 1956

A Thesis submitted to the Faculty of the Graduate
School of the University of Colorado in partial
fulfillment of the requirements for the Degree

Doctor of Philosophy

Department of Chemistry

1966

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

phil

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

This Thesis for the Ph.D. degree by

Louis Edwin Henderson

has been approved for the

Department of

Chemistry

by

Bert M. Tolbert

Robert Alberici

Date October 8, 1965

Acknowledgment

Sincere thanks are extended to Dr. Bert Tolbert, Dr. Peter Albersheim, and Dr. Mancourt Downing for their guidance and encouragement.

Henderson, Louis Edwin (Ph.D. Chemistry)

I. Denaturation of Enzymes by the Direct Action of Ionizing Radiation
and II. Enzymically Active Components of the Crystalline
Chymotrypsins

Thesis Directed by Professor Bert M. Tolbert

Part I. The inactivation of alpha-chymotrypsin by the direct action of gamma radiation was shown to be an all-or-none process. The enzymic specific activity and the operational active sites of the enzyme were lost with the same D_{37} dose. No chemically altered but enzymically active molecules produced by irradiation could be detected by ion exchange chromatography or gel electrophoresis. Alpha-Chymotrypsin requires an average dose of 42.1 eV/molecule to inactivate the enzyme in the solid state.

The direct action of gamma radiation on alpha-chymotrypsin results in sufficient alterations of the ordered conformation of the molecule to account for loss of enzymic activity. The λ_o determined by optical rotatory dispersion decreases from 236.6 m μ for the native protein to about 224 m μ for the radiation denatured protein. The radiation denatured protein shows no "buried" chromophores which can be exposed by heating the protein through the heat induced transition of alpha-chymotrypsin.

The "hard to exchange amide hydrogens" (HEAH) of irradiated alpha-chymotrypsin are lost by a multi-hit process. About 1.65 eV are required to expose one HEAH. This suggests that about 19 HEAH are exposed per hit molecule.

The protein irradiated to 50% inactivation showed no change in the amino acid analysis or disulfide analysis. There was no evidence for specific chemical changes produced by radiation in the electrophoretic patterns of the reduced and non-reduced protein.

The doses required to inactivate a molecule of chymotrypsinogen, delta-chymotrypsin and papain are also reported. These doses do not differ significantly from the dose required to inactivate alpha-chymotrypsin.

Part II. An ion exchange column is described which is capable of resolving the crystalline chymotrypsins into their respective components. Each crystalline chymotrypsin contains contamination of the other chymotrypsins. 3X crystallized alpha-chymotrypsin contains about 16% beta or gamma-chymotrypsin.

"Disc" gel electrophoresis of flurodiisopropyl phosphate P³²-labeled chymotrypsins in 8M urea reveals a different electrophoretic mobility for alpha, beta, and gamma-chymotrypsin. However, these proteins all yield the same electrophoretic pattern following reduction with thioethanol. This suggests that these proteins may differ only in their disulfide arrangement.

This abstract of about 600 words is approved as to form and content. I recommend its publication.

Signed Burt M. Tolbert
Instructor in charge of dissertation

LIST OF FIGURES

FIGURE		PAGE
PART I		
1.	Change in Specific Enzymic Activity of Irradiated Alpha-Chymotrypsin	9
2.	Loss of Active Sites of Irradiated Alpha-Chymotrypsin	11
3-a.	Chromatographic Elution Diagram of Enzymic Activity from Irradiated and Native Alpha-Chymotrypsin	13
3-b.	Chromatographic Elution Diagram of Protein from Irradiated and Native Alpha-Chymotrypsin	14
4.	Change λ_o of Irradiated Alpha-Chymotrypsin	18
5.	OD ²⁹³ vs Enzymic Activity	20
6.	Change in $\Delta E^{293 m\mu}$ of Irradiated Alpha-Chymotrypsin	22
7.	Change in HEAH of Irradiated Alpha-Chymotrypsin	24
8-a.	Electrophoretic and Radioactive Profile of Reduced Alpha-Chymotrypsin	29
8-b.	Electrophoretic and Radioactive Profile of Non-Reduced Alpha-Chymotrypsin	30
PART II		
1.	Chromatographic Elution Diagram of Alpha-Chymotrypsin	52
2.	Chromatographic Elution Diagram of Component III from 3X crystallized Alpha-Chymotrypsin	55
3.	Chromatographic Elution Diagram of Beta-Chymotrypsin.	56
4.	Chromatographic Elution Diagram of Gamma- Chymotrypsin	57
5.	Chromatographic Elution Diagram of Delta-Chymotrypsin	59
6.	Chromatographic Elution Diagram of "Slow" Activation Mixture of Chymotrypsinogen-A.	60

FIGURE

PAGE

7. Chromatographic Elution Diagram of Alpha-Chymotrypsin Stored for Two Weeks at pH 7.75, 5°	61
8. Protein and Radioactivity Distributions in Electrophoretic Gels of DIP-32 Chymotrypsin .	63
9. Protein and Radioactivity Distributions in Electrophoretic Gels of Thioethanol Reduced DIP-32 Chymotrypsins	64
Expanded legends for Figures 1 through 9 . .	70

LIST OF TABLES

TABLES		PAGE
PART I		
I.	λ_o of Irradiated Alpha-Chymotrypsin	16
II.	Amino Acid Composition of Irradiated Alpha-Chymotrypsin	27
III.	Sulfhydryl and Disulfide Groups of Irradiated Alpha-Chymotrypsin	28
IV.	The Average Energy Necessary to Inactivate Selected Enzymes	32
PART II		
I.	Protein and Enzymic Composition of 3X Crystallized Alpha-Chymotrypsin	53

TABLE OF CONTENTS

CHAPTER		PAGE
PART I. DENATURATION OF ENZYMES BY THE DIRECT ACTION OF IONIZING RADIATION		
ACKNOWLEDGMENTS		i
ABSTRACT		ii
1. INTRODUCTION		1
2. EXPERIMENTAL		3
Enzymes and Substrates		3
Reagents		3
Radiation Conditions		3
Enzyme Assay		3
Active Site Titrations		4
Ion-Exchange Chromatography		4
Hard-to-Exchange Amide Hydrogens		5
Heat Perturbation Spectrum		5
Optical Rotatory Dispersion		6
Amino Acid Analysis		6
Sulfhydryl and Disulfide Analysis		7
"Disc" Gel Electrophoresis		7
3. NATURE OF ENZYME INACTIVATION BY IONIZING RADIATION		8
Enzymic Activity		8
Titration of Active Sites		10
Column Chromatography		10
4. CONFORMATIONAL INTEGRITY, ENZYMIC ACTIVITY AND IONIZING RADIATION		15
Optical Rotatory Dispersion		15
Ultraviolet Light Absorption Effects		19
Hard-to-Exchange Amide Hydrogens		23

CHAPTER	PAGE
5. COVALENT CHANGES IN IRRADIATED PROTEINS	26
Amino Acid Analysis	26
Disulfide and Sulfhydryl Analysis	26
"Disc" Gel Electrophoresis	26
Radiation Denaturation of Selected Enzymes	31
6. PROPOSED MECHANISMS OF RADIATION DENATURATION OF ENZYMES	33
7. DISCUSSION	39
8. SUMMARY	42

PART II. ENZYMICALLY ACTIVE COMPONENTS OF THE CRYSTALLINE CHYMOTRYPSINS

INTRODUCTION	48
EXPERIMENTAL PROCEDURE	49
Enzymes, Proteins, and Materials	49
Ion Exchange Chromatography and Column Preparation	49
Protein and Enzyme Assays	50
Desalting	50
Gel Electrophoresis	50
RESULTS	51
Alpha-Chymotrypsin	51
Beta-Chymotrypsin	54
Gamma-Chymotrypsin	54
Delta-Chymotrypsin	58
"Slow" Activation Mixture of Chymotrypsinogen-A	58
Conversion of Alpha-to Beta- and Gamma-Chymotrypsin	58
Gel Electrophoresis	62

CHAPTER	PAGE
DISCUSSION	65
SUMMARY	67
REFERENCES	68

PART I

DENATURATION OF ENZYMES BY THE DIRECT ACTION OF IONIZING RADIATION

1. INTRODUCTION

Solid state enzymes lose enzymic activity when subjected to ionizing radiation. The quantitative aspects of the process indicate a single ionization anywhere within the molecule inactivates the enzyme (1). This concept has been used to estimate the molecular weight of enzyme from radiation inactivation data (2,3,4).

The experimental evidence to support the single ionization theory is that many enzymes absorb about 30 to 40 eV of ionizing energy per molecule inactivated (5). In gaseous systems an average of 35 eV of ionizing energy are required to create an ion pair (6). If these data can be compared, it would appear that a single ionization anywhere within the solid state enzyme results in enzyme inactivation. The model suggests that enzyme inactivation by the direct action of ionizing radiation is an all-or-none process; the energy absorbing molecules are always inactivated and no altered but enzymically active molecules are produced.

This need not be true, some ionizations outside the molecule may inactivate the enzyme, while other ionizations, inside the molecule may not. To determine the point, experimental evidence on irradiated samples should show:

- (A) All molecules with enzymic activity have not been ionized;
and
- (B) All enzymically inactive molecules have been ionized.

Since one cannot observe ionizations in the solid state, it is necessary to assume each ionization will result in some chemical or physical change. Therefore, it must be shown that all enzymically active molecules have not changed physically or chemically, and all enzymically inactive molecules have changed physically or chemically.

Many enzymes are inactivated by what appears to be a single ionization process (5). Evidently some general feature of enzymes necessary for enzymic activity is altered by the absorbed energy. It is generally suggested that the absorbed energy alters the conformation of hit molecules (7). However, it has never been conclusively shown that the conformation of hit molecules is sufficiently altered to account for loss of biological activity.

The experimental work presented in this report is directed toward investigating aspects of the one ionization model. Alpha-chymotrypsin is shown to be inactivated by an all-or-none process and the ordered structure of inactivated molecules is altered sufficiently to account for loss of biological activity.

Alpha-chymotrypsin was selected as a model enzyme for this study because previous work by Pollard (3) indicated this enzyme is inactivated by a single ionization process. The enzyme has a molecular weight of 24,300, a known amino acid sequence and disulfide arrangement (8), a well-defined substrate (9, 10), and is available commercially. Amino acid residues directly involved in the active site are known and their position in the sequence has been determined (11, 12). The enzyme is composed of three peptide chains held together by disulfide bonds. Amino acids of the active site are located in two or three chains. More detailed information about the enzyme can be found in references cited.

2. EXPERIMENTAL

Enzymes and Substrates. Alpha-chymotrypsin, papain, trypsin and chymotrypsinogen were obtained from Worthington Biochemical as the 3X crystallized enzymes and used without further purification. Beta-, gamma-, and delta-chymotrypsins were obtained from Sigma Biochemical as the 2X crystallized preparations and used without further purification. N-acetyl-L-tyrosine ethyl ester (ATEE), N-benzoyl-L-arginine ethyl ester (BAEE) and N-trans-cinnamoyl imidazole (TCI) were obtained from California Biochemical. Fluorodiisopropyl phosphate-³²P (FDIP-32) was obtained from New England Nuclear Corp. Unlabeled FDIP was obtained from Sigma Biochemical.

Reagents. Deuterium oxide (99.8%) and Bio-Rex 70 were obtained from Bio-Rad Company. The Sephadex was obtained from Pharmacia Corp. The urea, obtained from Sigma Biochemical was de-ionized and recrystallized immediately before use. Tris-hydroxymethylaminomethane (Tris) was obtained from Sigma Biochemical. All other chemicals were reagent grade preparations.

Radiation Conditions. All samples to be irradiated were sealed under 10 micron vacuum in ampules made of 3 mm pyrex glass tubing. The samples were weighed into the tubes, attached to a vacuum line, evacuated overnight, and sealed off. The samples were then irradiated in the University of Colorado kilo-curie Cs¹³⁷ source. The dose rate was calibrated at 2.65×10^{19} ev/g/hr with a Fricka dosimeter solution. The temperature in the source was constant at 24°. The irradiated samples were allowed to stand for at least 48 hours at -20°C before opening for analysis.

Enzyme Assay. Chymotryptic activity was determined by the spectrophotometric method of Schwert and Takenaka (10) using ATEE as

the substrate. The assay solution was 1×10^{-3} M ATEE in 0.05 M Tris-HCl buffer at pH 7.90; 0.01 to 0.001 mg of enzyme was added to the test cell in about 100 λ of solution. The test cuvette was read at time zero and the time required to produce a 0.050 decrease in absorbance at 237 $m\mu$ was recorded. The exact concentration of enzyme was determined by weight and dilution techniques or by its 280 $m\mu$ absorption (taking $E_{1\%}^{1cm} = 19.8$). The change in the absorbance per minute per mg of protein was taken as a measure of the specific enzyme activity.

Papain activity was determined by the same procedure, only BAEE was used as the substrate. The substrate solution was 1×10^{-3} M BAEE, 2×10^{-2} sodium citrate, pH 6.00, 1×10^{-3} M EDTA and 5×10^{-3} M cysteine.

Chymotrypsinogen was assayed as chymotrypsin following a rapid tryptic activation by methods described in the literature (13).

Active Site Titrations. Chymotrypsin active site titrations were performed by the method of Schondaum (18). A solution of chymotrypsin was allowed to react with TCI at pH 5.00. The cinnamoylated derivative was measured spectrophotometrically at 315 $m\mu$. The concentration of cinnamoylated enzyme was calculated using $\Delta E^{315} = 1.0 \times 10^4$. This value was determined on unirradiated 3X crystallized alpha-chymotrypsin and agrees with the 1.09×10^4 literature value.

Spectrophotometric measurements were made on a Cary Model 14 recording spectrophotometer. Recording was started 30 seconds after adding the enzyme and extrapolated back to zero time to correct for hydrolysis. Absorbance at extrapolated zero time was used to calculate the present remaining operationally active sites.

Ion-exchange Chromatography. Separation of the irradiated samples of alpha-chymotrypsin by ion exchange chromatography was performed by the techniques described in detail in Part II of this thesis.

Hard-to-Exchange Amide Hydrogen (HEAH). The HEAH were determined by the method of Blout et al. (25). The irradiated and unirradiated protein was dissolved in 99.8% D_2O to make a 5% solution. This solution was introduced into a 0.05 mm CaF_2 cell and the infrared spectrum was taken between 5.750 and 7.250 microns at such a rate as to observe the 6.400 micron region to 10 minutes after solution.

Cell blanks were run before each determination. Separate samples of unirradiated protein were incubated in D_2O for 24 hours at room temperature and from 8 to 12 hours at $45^\circ C$. These samples were used to establish the base line for completely deuterated protein. The amide I band at 6.150 microns remained constant during exchange reaction and was used as an internal standard to determine protein concentrations. The amide II band at 6.500 decreased with deuteration. Blout determined that the ratio of $\frac{\text{amide II}}{\text{amide I}}$ was 0.40 for the undeuterated protein.

The amide II to amide I band ratio for the completely deuterated alpha-chymotrypsin was 0.196. The HEAH were defined as all the hydrogen which did not exchange in 10.0 min. The percent remaining HEAH were calculated from absorbance of the 6.500 band after base line and concentrations corrections were made taking the unirradiated specific absorbance as 100%.

Heat Perturbation Spectrum. The change in the extinction coefficient at $293\text{ m}\mu$ produced by heating the protein above its denaturation temperature (about $55^\circ C$) was determined by the following procedure (22). The irradiated or unirradiated samples were dissolved in sufficient 0.05 M sodium acetate-acetic acid buffer pH 3.52 (0.1 M in KCl) to bring the protein concentration to 1.68 mg/ml. This solution was placed in a 1.00 cm quartz cell and read in a thermostated Beckmann D. U. spectrophotometer. A pyrex glass cell filled with buffer was used as a blank. The instrument was adjusted to read zero at $293\text{ m}\mu$ with the blank cell, the protein solution read about 0.440 O.D.

The temperature was regulated with a water bath. A thermometer was fitted into the cell compartment so that the cell compartment temperature could be read directly. The water bath was first cooled then heated at 1°/min. The O.D. at 293 mμ of the sample was determined every 1° from 5° to 65°.

The change in absorbance from 30° to the denaturation temperature (about 55°C) was taken as a measure of enzyme conformation and reported as percent of the change in absorbance for the unirradiated enzyme.

Optical Rotatory Dispersion. All measurements were made on a Rudolph Model 200A Oscillating Polarizer with a zirconium arc light source. Measurements were made on 1.0% protein solution in 0.05 sodium acetate-acetic acid buffer pH 3.52 (0.1 M in KCl) at 25°. Cells were 1.6 mm bore x 5 cm length with quartz end plates. Readings were taken at nine wave lengths from 650 mμ to 340 mμ and the λ_o calculated by means of the Drude equation

$$[\alpha] = \frac{a\lambda_o^2}{\lambda^2 - \lambda_o^2}$$

where $[\alpha]$ is the specific rotation at a wave length λ , and a is a constant. λ_o is obtained from a plot of $1/[\alpha]\lambda^2$ versus $1/\lambda^2$.

Amino Acid Analysis. The amino acid analyses were performed by the method of Moore (14). Five mg of protein was dissolved in 5 ml of a constant boiling HCl, frozen and sealed under vacuum. The sealed tubes were heated at 110° for 12, 24 and 48 hours. Each determination was run in duplicate. The hydrolyzates were lyophilized to dryness and analyzed on a Beckmann automatic amino acid analyzer. The value for serine and threonine was determined by extrapolating back to zero time of hydrolysis. Tryptophan was determined separately by spectrophotometry (15).

Sulfhydryl and Disulfide Analysis. Sulfhydryl and disulfide analyses were performed by a modification of the methods of Boyer (16) and Brown (17).

Approximately 3 mg of protein was weighed accurately and dissolved in 0.5 ml of 1.0 M sodium borohydride in 8 M urea. After 3 hours at room temperature, the reaction was stopped with 0.5 ml 3 M acetic acid. The reduced protein was titrated spectrophotometrically with p-chloromercuribenzoate (16) in the presence of 0.05% sodium dodecyl sulfate. Sulfhydryl groups were determined by omitting the reduction step. This method consistently gave results for lysozyme and chymotrypsin which were 20% too high. The source of this error was undetermined.

Disc Gel Electrophoresis. Gel electrophoresis was performed by methods described in Part II of this thesis.

3. NATURE OF ENZYME INACTIVATION BY IONIZING RADIATION

Enzymic Activity. Samples of alpha-chymotrypsin were weighed into tubes, sealed under vacuum and irradiated in a kilocurie Cs^{137} source at 2.65×10^{19} eV/hr/g. Samples were assayed for enzymic activity with N-acetyl-L-tyrosine ethyl ester (ATEE) as a substrate by methods described in the experimental section (10).

Specific enzymic activities were based on the weight of the sample and rate of ATEE hydrolysis. Percent remaining specific activities were based on the specific activities of unirradiated controls. These data are presented in Figure 1. The log percent remaining specific activity is plotted versus dose in eV. The linear relationship predicted by the one ionization theory is obtained (1). The inactivation process follows the relationship $\log \frac{A}{A_0} = \frac{-2.203}{D_0} D$: Where $\frac{-2.203}{D_0}$ is a constant, A and A_0 are the specific enzymic activities before and after dose D. D_0 is the average radiation energy required to inactivate a molecule. The D_{37} value is the energy required to inactivate 63% of the original enzyme and represents the amount of energy required to inactivate all the molecules if repeat hits did not occur. The D_{37} value for alpha-chymotrypsin is 98×10^{19} eV/g which corresponds to 42.1 eV/molecule. This value is consistent with the present data on the energy required to create an ionization in organic solids.

In order to equate the number of inactivated molecules with loss of enzymic activity it must be assumed that all active molecules after irradiation have the same turnover number.

It should be shown that the operationally active sites of the irradiated protein have decreased by the same amount as enzymic activity.

Figure 1
CHANGE IN SPECIFIC ENZYMIC ACTIVITY OF
IRRADIATED ALPHA-CHYMOTRYPSIN

Titration of Active Sites. Operationally active sites of alpha-chymotrypsin can be titrated by the methods detailed in the experimental section (18). The method takes advantage of the fact that catalysis by alpha-chymotrypsin occurs in several steps (19).

Steps 1 and 2 are independent of pH between pH 3 and 8. Step 3 is dependent upon an un-ionized imidazole and is very slow below pH 6. At pH 5 the reaction with N-trans-cinnamoyl imidazole is blocked at step 3. All molecules which undergo step 2 form a stable acylated enzyme. The cinnamoylated enzyme can be measured spectrophotometrically and used to titrate the "operationally active sites."

Figure 2 is a plot of the log percent remaining active sites vs dose. The D_{37} is in good agreement with the D_{37} for the hydrolysis of ATEE. It can be concluded that all enzymically active molecules after irradiation have the same turn-over number.

Column Chromatography. The possible production of altered molecules which retain enzymic integrity was investigated with cation exchange chromatography. The procedure is the most sensitive method available for purification of alpha-chymotrypsin. In the experiment, 10 mg of irradiated protein was eluted from the column.

Protein was detected by absorbance at 280 m μ and enzymic activity was measured by ATEE hydrolysis. Specific activity was calculated assuming all protein to have a specific absorbance at 280 m μ equivalent to alpha-chymotrypsin.

IRRADIATED ALPHA-CHYMOTRYPSIN
LOSS OF ACTIVE SITES OF

Figure 2

Figures 3a and 3b are elution diagrams of 3X crystallized alpha-chymotrypsin and irradiated alpha-chymotrypsin. Figure 3a is an enzymic activity elution diagram and Figure 3b is a protein elution diagram. No new enzymically active components are evident in the irradiated protein. Most of the radiation-inactivated protein is eluted in an unresolved slur before the enzymically active protein. The specific enzymic activity of the major enzymically active component in the irradiated sample is equal to the specific enzymic activity of unirradiated alpha-chymotrypsin. All of the enzymic activity of the irradiated protein was recovered from the column.

Enzymic Activity Elution Diagram
of Irradiated and Native Alpha-Chymotrypsin

ml of Eluate
Protein Elution Diagram of Irradiated and
Native Alpha-Chymotrypsin

4. CONFORMATIONAL INTEGRITY, ENZYMIC ACTIVITY AND IONIZING RADIATION

Several experimental parameters are currently used to study protein conformation. They include optical rotatory dispersions, spectral shifts of ultra-violet light absorption, and the rate of hydrogen exchange at amide bonds of the protein. Theories and principles of the techniques are discussed in the literature and only a brief treatment of each technique will be presented here. These techniques were used to compare radiation inactivated alpha-chymotrypsin with a native enzyme.

Optical Rotatory Dispersion. Proteins exhibit a dispersion of optical rotation as a function of the wave length of light used (20, 21). At shorter wave lengths specific rotation decreases through a minimum, then increases. The wave length of the minimum has been termed the λ_0 of the protein and may be related to the alpha-helix of the protein. The λ_0 of alpha-chymotrypsin irradiated to various dose levels was determined, these values are given in Table I. The concentration of the unirradiated protein was determined by absorbance at 280 m μ . Radiation produces a chromophore which absorbs slightly in the 280 m μ region. Therefore, the concentration of irradiated samples was determined by weight of the protein, this introduced a random error.

It is possible to obtain information about the effect of radiation denaturation on the conformation of enzymes by assuming the D_{37} value for change in conformation to be equal to the D_{37} for inactivation of the molecule. This assumption will permit a quantitative description of conformational parameter associated with radiation inactivated molecules. The all-or-none nature of radiation inactivation of alpha-chymotrypsin makes this a tenable assumption.

TABLE I
 λ_0 of Irradiated Alpha-Chymotrypsin

Dose eV/g	λ_0 m μ	Average λ_0 m μ
0	244	236.6
	235	
	232	
	239	
	234	
	236	
31.8×10^{19}	237	234.0
	233	
	232	
64.8×10^{19}	232	230.6
	228	
	232	
95.5×10^{19}	231	228.3
	228	
	226	
Heated to 60°C	224	224.0
	224	

The following is a general scheme for relating change in conformational parameters of irradiated alpha-chymotrypsin to the conformational parameters of a single hit molecule.

then

$$\frac{[\text{Unknown conformation}]}{[\text{Native conformation}]} = \frac{[\text{Inactive protein}]}{[\text{Active alpha-chymotrypsin}]}$$

and

$$[\text{Unknown conformation}] = [\text{Native conformation}] e^{-kD}$$

The average λ_o for the radiation inactivated molecules was calculated from data in Table I using the following equation and based on the above scheme.

$$\frac{(\lambda_o)_{\text{native}} - (\lambda_o)_D}{(\lambda_o)_{\text{native}} - (\lambda_o)_{\text{irr}}} = e^{-kD}$$

where $(\lambda_o)_D$ is the λ_o experimentally determined for a sample which had received a dose D , k is the slope of the loss of enzymic activity vs dose curve, $(\lambda_o)_{\text{native}}$ is the λ_o of the native alpha-chymotrypsin and $(\lambda_o)_{\text{irr}}$ is the average λ_o of radiation denatured molecules. The average $(\lambda_o)_{\text{irr}}$ was 224 m μ .

The log percent change of λ_o between 236.6 m μ and 224.0 m μ with increasing dose is shown in Figure 4. The value of 236.6 m μ was taken as 100% and 224 m μ was taken as zero % λ_o . The average value of three independent determinations was taken to calculate the percent remaining λ_o for the irradiated protein. Points fall very close to the straight line for loss of enzymic activity.

Alpha-chymotrypsin undergoes a heat induced conformational transition at about 54° which inactivates the molecule (22). The λ_o of alpha-chymotrypsin which has been heated to 60° was determined

Figure 4
CHANGE IN τ_0 OF IRRADIATED
ALPHA-CHYMOTRYPSIN

and found to be 224 $m\mu$ (Table I). The equivalence of λ_o for the two states of the protein indicates that radiation denatured protein has assumed a conformation in solution which is similar to the conformation of heat denatured protein.

Ultraviolet Light Absorption Effects. The heat induced conformational transition of alpha-chymotrypsin is accompanied by a blue shift in the UV absorption spectra with a maximum change in the absorption at 293 $m\mu$ (22). This spectral shift is presumably associated with movement of a number of tryptophan residues from a less polar to a more polar environment (23).

This spectral shift can be directly correlated with the loss of enzymic activity of the heated sample. Figure 5 shows the change in the 293 $m\mu$ absorbance for a sample of alpha-chymotrypsin heated through this temperature transition. Samples were periodically withdrawn and assayed by the rate of ATEE hydrolysis.

The change in specific activity with temperature is also plotted on the same temperature coordinate. As the enzyme undergoes the structural transition the enzymic activity is lost. In this solvent system the change in conformation is irreversible.

Since radiation denatured protein and heat denatured protein have a λ_o of about 224 $m\mu$ it is reasonable to suspect that radiation denatured protein will be unable to undergo further temperature induced spectral transitions. However, this is not necessarily true. λ_o is presumably a measure of alpha-helix and the spectral transition reflects a change in environment of tryptophan residues. The two properties need not be directly related. The ΔE^{293} for radiation denatured protein could be equal to, greater than, or less than that of native protein. If ΔE^{293} for radiation denatured protein is zero, the protein may be in a conformational state similar to heat denatured protein.

OD₂₉₃ vs. Enzymic Activity

Figure 5

In the special case where ΔE^{293} for the radiation denatured protein is zero;

$$\frac{\Delta E_{irr}^{293}}{\Delta E_{native}^{293}} = e^{-kD} = \frac{A}{A_0}$$

where A and A_0 refer to the specific enzymic activities of protein irradiated to dose D and unirradiated protein respectively.

Figure 6 is a plot of the log percent remaining ΔE^{293} vs dose. The D_{37} is identical to the D_{37} for loss of enzymic activity indicating ΔE^{293} for radiation denatured protein is zero. These data were obtained by methods outlined in the experimental section.

The temperature induced transition of alpha-chymotrypsin shown in Figure 5 is relatively sharp, occurring over a temperature range of about 5°. The temperature induced transition for irradiated samples was less sharp. The samples which received high doses began their transition at temperatures as low as 35°. To obtain the total ΔE_{irr}^{293} it was necessary to take the difference between E^{293} at 30°C and the E^{293} at the peak temperature. This change in slope of the temperature transition curve implies some enzymically active but heat labile molecules are present (24). This evidence is contradictory to the conclusion that radiation inactivation of alpha-chymotrypsin is an all-or-none process.

Temperature induced structural transitions of proteins may be thought of as melting out ordered conformation of some parts of the molecule. This probably involves breaking some hydrogen and hydrophobic bonds. The temperature of transition can be lowered by a number of additives. For example, in separate experiments with alpha-chymotrypsin it was found that 5×10^{-3} M cyclohexanol was sufficient to reduce the transitions temperature by 10°. Irradiated protein represents a heterogeneous mixture as evidenced by chromatography. The

effect of the products of radiation upon the temperature induced transition of unhit molecules cannot be neglected. Therefore, the observation of heat labile enzymic activity in irradiated protein does not necessarily reflect the presence of altered but enzymically active molecules.

Hard-to-Exchange Amide Hydrogens. It is possible to obtain an estimate of the number of hydrogen bonds still intact after irradiation by the method of Blout (25) using D_2O . Blout has noted that the infrared N-H deformation band of proteins undergoes a shift to longer wave lengths when the hydrogen is exchanged for deuterium. There is an initial rapid exchange of protons which is complete in 3 to 5 minutes followed by a slow exchange which takes over 24 hours to complete. The hard-to-exchange amide hydrogens (HEAH) are believed to be involved in hydrogen bonds and hydrophobic centers. A change in conformation of the protein may permit the rapid exchange of the exposed amide hydrogens.

Figure 7 is a plot of log percent remaining HEAH vs dose for irradiated alpha-chymotrypsin. All hydrogens which do not exchange in 10 minutes have been designated as HEAH. The total HEAH of the native enzyme was found to be 128 and was taken as 100 percent.

These data do not yield a straight line. The initial ionizing event resulted in the loss of some but not all of the HEAH of the protein. Subsequent ionizations are necessary to further reduce the HEAH of inactive molecules. The initial slope of the curve was extrapolated to the D_{37} for the first ionizing event and the number of electron volts absorbed per HEAH lost was found to be 1.65 eV/HEAH. Since an average of 42.1 eV are absorbed per molecule inactivated, the inactivated molecules have lost an average of 19 HEAH/molecule.

CHANGE IN HEAH OF IRRADIATED
ALPHA-CHYMOTRYPSIN

Figure 7

This figure represents the average number of HEAH exposed by conformational alterations in hit molecules. These hydrogens may have been involved in hydrogen bonds or hydrophobic centers. Hit molecules may not have completed their conformational change in the solid state. Further change could take place after solution which may permit the exchange of additional hydrogen atoms. For these reasons the figure 1.65 eV/HEAH must be considered a rough estimate.

5. COVALENT CHANGES IN IRRADIATED PROTEINS

Amino Acid Analysis: Table II shows the amino acid analysis for alpha-chymotrypsin before and after irradiation to 50% inactivation. The samples were run in duplicate by methods outlined in the experimental section. There is no significant loss of any amino acid residue. Gamma radiation is not highly specific for the destruction of amino acid residues in solid state proteins (27). Chemical changes may take place at any of several amino acid residues.

Disulfide and Sulfhydryl Analysis: Table III shows the disulfide and sulfhydryl analysis of alpha-chymotrypsin before and after irradiation. These figures are averages of triplicate runs performed by methods outlined in the experimental section. The method routinely gave values for alpha-chymotrypsin and lysozyme which were 20% too high. The reason for this discrepancy is unknown but the method is of value for comparing the product and starting materials. There is an increase of titratable sulfhydryl groups following reduction of irradiated protein but the increase is too small to account for the total enzyme inactivation.

"Disc" Gel Electrophoresis: Irradiated and native alpha-chymotrypsin was labeled at the active site with FDIP-32.

Thioethanol-reduced and non-reduced DIP-32 labeled protein was electrophoresed in 8 M urea through polyacrylamide gels. Figure 8-a shows the distribution of radioactivity in the non-reduced gels.

The significance of each band and the distribution of radioactivity in the unirradiated protein is discussed in Part II of this thesis. The techniques take advantage of the fact that enzymically active alpha-chymotrypsin will react with FDIP-32 at the active site

TABLE II
Amino Acid Composition of Irradiated Alpha-Chymotrypsin

Amino Acid	Amino Acid Residues ^(a) per Molecule ^(b)	
	Control	63.5×10^{19} eV/g
Lysine	12.5	12.3
Histidine	1.6	1.6
Ammonia	25.4	25.2
Arginine	2.4	2.2
Aspartic Acid	18.6	18.9
Threonine	17.4	17.4
Serine	23.7	22.2
Glutamic Acid	13.0	13.0
Proline	7.2	7.4
Glycine	18.6	18.2
Alanine	18.6	19.0
Valine	16.3	16.0
Methionine	1.6	1.6
Isoleucine	6.8	6.5
Leucine	14.8	15.1
Tyrosine	3.0	3.0
Phenylalamine	4.6	4.6
Tryptophan	7.7	6.7

(a) Each value is the average of duplicate determinations that agree within 3%.

(b) Molecular weight taken as 24,800.

TABLE III
Sulfhydryl and Disulfide Groups of
Irradiated Alpha-Chymotrypsin

Groups ^(a) per Molecule ^(b)		
Dose eV/g	Sulfhydryl	Disulfide
0	0	6.4
79.5×10^{19}	0	7.5
278×10^{19}	0	7.0

(a) Each value is the average of triplicate determinations.

(b) Molecular weight taken as 24,800.

Figure 8-a

ELECTROPHORETIC AND RADIOACTIVE PROFILE
OF REDUCED ALPHA-CHYMOTRYPSIN

Figure 8-b
ELECTROPHORETIC AND RADIOACTIVE PROFILES
OF NONREDUCED ALPHA-CHYMOTRYPSIN

serine to form the serylphosphate ester. In the non-reduced electrophoretic pattern, the radioactivity is associated with enzymically active protein. In the reduced pattern it is associated with the C-chain.

Comparison of the non-reduced, irradiated and native, protein electrophoretic patterns and distributions of radioactivity shows no new protein bands and no new radioactive bands in the irradiated protein. The absence of distinct new protein bands indicates no specific chemical changes occurred. There is a protein background over the entire electrophoretic region in the irradiated samples which may be due to nonspecific chemical changes in the irradiated protein.

The reduced protein always gave a much clearer electrophoretic pattern than unreduced material; if any significant amount of specific chemical change did occur they should be more evident in the reduced electrophoretic pattern. Comparison of the reduced patterns of the irradiated and native protein reveals no new electrophoretic band in the irradiated protein.

Radiation Denaturation of Selected Enzymes. Information regarding the mechanism of radiation denaturation of enzymes may be gained by examining the energy required to inactivate different enzymes. Table IV lists the energies required to inactivate several enzymes. These data were determined by the method already discussed. Irradiations were conducted in the University of Colorado cesium-137 source. This is a consistent set of data that eliminate calibration and many analytical errors involved in comparing such values from literature references.

TABLE IV
The Average Energy Necessary to Inactivate
Selected Enzymes

Enzyme	Inactivation Energy eV/Molecule
Alpha-Chymotrypsin	42
Chymotrypsinogen	35
Delta-Chymotrypsin	30
Papain	49
Lysozyme ^(a)	86
Ribonuclease ^(b)	65

(a) Data taken from reference no. 45

(b) Data taken from reference no. 39, 50

6. PROPOSED MECHANISMS OF RADIATION DENATURATION OF ENZYMES

An enzyme may be inactivated by altering amino acid residues directly or indirectly involved in the active site or by altering the ordered conformation necessary for maintaining the catalytic center. The inactivation of enzymes by ionizing radiation may proceed by either one or both of these general mechanisms. In dilute solution the radiolytic products of the solvent may react with amino acid residues on the surface of the enzyme and promote inactivation by chemical changes (7). This process is generally referred to as inactivation by indirect effect of ionizing radiation. The inactivation of enzymes by direct effects of ionizing radiation would not be expected to show a preference for the surface amino acid residues. Most authors have described enzyme inactivation by direct effect of ionizing radiation in terms of non-specific changes in the ordered conformation of the molecule.

Augenstine has recently reviewed the proposed mechanisms for enzyme inactivations by direct effects of ionizing radiation (7). All of the proposed mechanisms consider the loss of ordered conformations as the main mode of enzyme inactivation by a single ionizing event. He and other workers are unable to define whether such changes are the result of radiation induced covalent changes, or are directly induced by the absorption of radiation energy.

Pollard (5) suggests that "excitations" may move along the peptide backbone, occasionally opening a covalent bond and disrupting a number of hydrogen bonds. The end result would be a degraded peptide chain with random configuration.

There is little-or-no evidence for a degraded peptide chain in irradiated lysozyme (26) and bovine serum albumin (27). The one, two and three chain systems of the chymotrypsins are inactivated by about the same energy per molecule. This suggests that much of the inactivation energy does not travel along the entire length of the peptide chain.

Platzman and Franck (28) presented an analysis of the electronic events which should occur in a protein immediately following its ionization. They concluded that the formation of a single point charge in a region of high dielectric constant should result in a polarization wave through the medium of a sufficient magnitude to disrupt as many as 14 secondary bonds. Their postulate would predict the formation of many altered but enzymically active molecules, greater radiation sensitivity at elevated temperatures and a dependency of the radiation sensitivity upon the conformation of the molecule.

The HEAH of alpha-chymotrypsin are lost with an energy requirement of 1.65 eV/HEAH. 42.1 eV are required (on the average) to inactivate the molecule. Thus, the actual number of HEAH lost per single ionization would be 19 HEAH/single ionized molecule. This figure is surprisingly close to the 14 secondary bonds predicted by Platzman and Franck. However, no altered but enzymically active molecules were found. This suggests that the breakage of a few hydrogen bonds in the solid state is sufficient to produce a conformational rearrangement which is not reversible.

G. Buggel working in this laboratory showed that the D_{37} for trypsin remained constant though the λ_0 for the protein was varied from 219 m μ to 236 m μ . The simplest interpretation of these data is that the denaturation reaction is independent of the conformation of the molecule.

Augenstine (7) proposes that the breakage of specific covalent bonds necessary for the maintenance of the ordered conformation may play an important role in enzyme inactivation. He proposes that disulfide bonds may be particularly radiation-sensitive and that the energy once deposited in the molecule may migrate to the disulfide bond causing its rupture. Data supporting this theory comes from solution U.V. studies and it has been suggested this effect could extend to solid state gamma radiation.

There is no evidence of disulfide cleavage in irradiated alpha-chymotrypsin, lysozyme or bovine serum albumin. Disulfide cleavages have been noted in irradiated ribonuclease(29) but the effect could only account for a small fraction of the denatured molecules. Other workers fail to find any evidence of disulfide cleavage in irradiated ribonuclease (30).

Norman and Speigler (31) have proposed that energies associated with an ionization should be transferred into vibrational energies of the molecule. This would result in a "hot" molecule which may denature before the energy can be dissipated to the surrounding medium. One can only estimate the temperature of the molecule because the rate of heat loss is unknown for a molecule in the crystalline state. Our experiments with alpha-chymotrypsin indicate that the enzyme can be denatured in the solid state by heating to 155°. The ionizing event supplies more than enough energy to thermally denature the enzyme in the solid state. However, the rate of heat loss in the solid state may be much faster than the time necessary for a conformational rearrangement to take place. There is no data available which will decide this point.

Several authors have proposed radiation produced free radicals as important intermediates in radiation denaturation of enzymes. Free radicals produced in proteins by ionizing radiation have been studied with ESR measurements (32, 35). These radicals seem to be composed

of three classes: 1. a non-specific radical detected at very low temperatures; 2. a free radical on an alpha-carbon called a glycine like radical; and 3. a radical on a sulfur atom called a cystine like radical. If the irradiated system is observed at 77°K only type 1 radicals are observed, as the sample is warmed to room temperature the type 1 radicals are converted to a mixture of types 2 and 3 with some loss in signal strength presumably due to recombination. This is direct evidence for free radical migration through the solid state system. The activation energy for migration has been estimated at 2 to 3 kcal/mole (36). The presence of cystine like radicals at room temperature provides direct support for Augenstine's hypothesis that the disulfide bonds may be especially radiation sensitive.

Several authors have noted a temperature dependence for the inactivation of enzymes by ionizing radiation (37,38). This temperature dependence yields an apparent activation energy for the denaturation reaction of about 2 to 3 kcal/mole (36). The initial ionizing event is not expected to show a temperature dependence but chemical or physical changes induced by the ionization would be expected to have an activation energy. This activation energy is difficult to interpret because each ionized molecule has received an amount of vibrational energy which may in some way aid the denaturation process. Free radical migrations and the denaturation reaction have comparable apparent activation energies. This has been interpreted as evidence for a free radical mediated denaturation reaction (36).

Any proposed mechanism for a reaction must take into account the nature of the starting material, the products and the known effects of the reaction environment. At the start of this work very little was known about the nature of the products of inactivation of enzymes by ionizing radiation. Alexander (27) had shown that none of the component amino acids of bovine serum albumin were particularly radiation

sensitive. He did note an increase in the ammonia yield from hydrolyzates of irradiated protein. Later Garrison (39, 40) followed up this work and showed that the increased ammonia was accompanied by an increased yield of carbonyl groups. These observations led him to postulate the formation of an acid labile intermediate at the peptide bond which subsequently hydrolyzes to NH_3 and alpha-keto acids. He has proposed a substituted imino acid (a dehydropeptide) as the acid labile intermediate but has failed to observe it directly in irradiated proteins.

The evidence for some acid labile intermediates which can hydrolyze to yield ammonia and carbonyl groups is good. However, its relevance to the denaturation reaction has not been established. It is difficult to believe that dehydropeptide formation will inevitably result in enzyme inactivation, since this would imply that the proper bond angles and distances must be maintained at every peptide bond.

Sedimentation experiments on irradiated bovine serum albumin indicate that the products have the same molecular weight as the starting material but a lower diffusion constant. Alexander (27) interpreted this as evidence for ionized molecules having an expended structure which one would expect if the ionization induced significant changes in the ordered conformation of the molecule. These data were not correlated with biological activity and represent a single observation which may be subject to many errors of interpretation. The data presented in this report supports the conclusions of Alexander and relate the conformational effects to the loss of biological activity.

Numerous investigations have been conducted into the possibility of altering the radiation sensitivity of enzymes by adding selected compounds to the system. In general it has been found that the addition of copious quantities of sulfur containing compound to a lyophilized preparation of protein will cause some radiation "protection" for the

protein. These data and ESR measurements indicate that some energy transfer process is involved whereby energy is transferred from the protein to the additive (35).

Other compounds, particularly biradical gases such as oxygen, will serve as radiation "sensitizers" when added to the system (41, 42). These compounds have the effect of lowering the average energy necessary to inactivate an enzyme molecule and will quench the stable free radicals of the system. It is difficult to interpret these results without first knowing more about the fundamental principles underlying the radiation denaturation of proteins.

7. DISCUSSION

The single ionization theory of enzyme inactivation by ionizing radiation states that a single ionization anywhere within the molecule is sufficient to inactivate the enzyme (1,7). This theory predicts enzyme inactivation should be an all-or-none process. Alpha-chymotrypsin is inactivated in the solid state by the direct action of gamma radiation with an average dose of 42.1 eV per molecule. This dose is compatible with previous studies on this enzyme (43), and is consistent with average energies required to create an ion pair in the solid state. All enzyme activity present in irradiated samples can be attributed to molecules which are indistinguishable from native alpha-chymotrypsin. Comparison of the D_{37} for enzymic activity and active sites shows all enzymically active molecules have a turnover number equivalent to that of native enzyme. Chromatographic separation of irradiated mixtures shows all molecules with enzymic activity have chromatographic mobilities equivalent to the native enzyme. Most of the enzymically inactive protein is chromatographically distinguishable from native alpha-chymotrypsin. Apparently each ionization in a native molecule results in an alteration of chromatographic mobility, and loss of the functional active site.

Optical rotatory dispersion studies reveal that radiation denatured alpha-chymotrypsin has a λ_0 equivalent to that of heat denatured enzyme. Heat denatured enzyme has undergone an irreversible UV absorption spectra shift. The D_{37} for this spectra shift in irradiated enzyme is equivalent to the D_{37} for loss of enzymic activity. These data show that radiation denatured enzyme assumes a

conformation in solution which is comparable to that of heat denatured enzyme. A single ionization in the solid state enzyme results in a conformational change of sufficient magnitude to inactivate the enzyme.

Platzman and Franck (28) theorized that a single ionization in a protein should result in a breakage of about 14 secondary bonds. The HEAH of irradiated alpha-chymotrypsin are lost by a multi-hit process requiring 1.65 eV/HEAH. This corresponds to a loss of about 19 HEAH per ionization. This is in surprisingly good agreement with the Platzman and Franck hypothesis. Evidently the exposure of 19 HEAH is sufficient to cause a conformational change which will inactivate alpha-chymotrypsin.

Although irradiated alpha-chymotrypsin showed no change in the amino acid composition, or disulfide content and the electrophoretic experiments showed no evidence of specific peptide bond breakage, this does not rule out general chemical changes. Both the reduced and non-reduced irradiated samples showed a protein slur over the entire electrophoretic region. This may be due to non-specific chemical changes in irradiated protein.

The energy required to inactivate selected enzymes was determined. Chymotrypsinogen, delta-chymotrypsin and alpha-chymotrypsin have almost the same amino acid analysis and sequence but represent one, two, and three peptide chains respectively (9). They are all inactivated with energies which cluster around 35 eV per molecule. There is no apparent dependence upon the number of peptide chains. Papain can be degraded with leucine amino peptidase to a third of its original molecular weight without loss of enzymic activity (44). Although papain is more stable to radiation denaturation than alpha-chymotrypsin it is not three times more stable. An ionization in any part of the molecule will apparently cause inactivation.

Ribonuclease and lysozyme are unusually stable to radiation denaturation. These enzymes lose HEAH with about the same energy requirement per HEAH as alpha-chymotrypsin (30, 45). Altered but enzymically active molecules have been detected in the radiation products of both enzymes (29, 46). Both ribonuclease and lysozyme are anomolous enzymes in that they are stable to heat (47, 48) denaturation. Conformational alterations in these enzymes are partly or completely reversible. Conformational changes in radiation denatured ribonuclease and lysozyme may be reversible provided chemical changes do not restrict the process.

The evidence presented in this report indicates that a single ionization in a solid state protein will result in conformational changes which are sufficient to inactivate the enzyme. These conformational changes may or may not be reversible depending on the nature of the enzyme and any covalent changes induced by the radiation. The free radicals produced in radiation denatured proteins (32, 34) may initiate covalent changes such as dehydropeptide (40) formation or migrate to other molecules (35). If the free radicals are transferred to other molecules the denatured enzyme may have an increased probability of reforming an enzymically active conformation. The free radical acceptor would then act as a radiation "protection" molecule (41, 49). The "migratory" free radicals of the system may not normally inactivate the ionized molecules, but may do so when they react with oxygen or other gases added to the system.

8. SUMMARY

Inactivation of alpha-chymotrypsin by direct action of gamma radiation was shown to be an all-or-none process. All enzymically active molecules present after irradiation were shown to be indistinguishable from native alpha-chymotrypsin by enzymic properties, column chromatography, and gel electrophoresis. This evidence supports the single ionization theory for enzyme inactivation by ionizing radiation.

The radiation inactivated molecules were shown to have an altered conformation. The change in the conformational parameters would be correlated with the loss of enzymic activity. The conformational changes were detected by optical rotary dispersion, ultraviolet light absorption and hard-to-exchange amide hydrogen content. The first ionization appears to result in an inactive molecule with a λ_o of 224 m μ , no "buried" chromophores, and the exposure of about 19 hard-to-exchange amide hydrogens.

Radiation denatured molecules showed no specific loss of amino acid residues or disulfide bounds. There was no evidence of specific chain cleavage. These data are discussed in terms of currently proposed mechanisms of enzyme inactivation by direct action of ionizing radiation.

9. REFERENCES

1. Lea, D. E., Action of Radiations on Living Cells, Cambridge Press (1962), pp. 69-99.
2. Pollard, E. C., Buzzell, A., Jeffreys, C. and Forro, F., Arch. Biochem. Biophys., 33, 9 (1951).
3. Pollard, E. C., Adv. Biol. Med. Phys., 3, 153 (1953).
4. Adams, E., J. Biol. Chem., 209, 829 (1954), Ibid., 217, 325 (1955).
5. Pollard, E. C., Guild, W. R., Hutchinson, F., and Setlow, R. B., Progr. Biophys. and Biophys. Chem., 5, 72 (1955).
6. Friedlander, G., and Kennedy, J. W., Nuclear and Radiochemistry, Wiley and Sons, Inc., N. Y. (1955), pp. 186.
7. Augenstine, L. G., Adv. Enzymology, 24, 359 (1962).
8. Hartley, B. S., Nature, 201, 1284 (1964).
9. Desnuelle, P., in The Enzymes, Vol. 4, Boyer, P. D., Lardy, H., Myrback, K., (Editors), Academic Press, Inc., New York, (1960), pp. 93.
10. Schwert, G. W., Takenaka, Y., Biochim. Biophys. Acta., 16, 570 (1955).
11. Schoellmann, G., Shaw, E., Biochemistry, 2, 252 (1963).
12. Koshland, D. E., Strumeyer, D. H., Ray, W. J., Brookhaven Symp. Biol., 15, 101 (1962).
13. Laskowski, M., Methods in Enzymology, Vol. 2, Colowisk, S. P., and Kaplan, N. O., (Editors), Academic Press, Inc., New York, (1955), p. 8.

14. Moore, S., Spackman, D. H. and Stein, W. H., Anal. Chem., 30, 1185, 1190 (1958).
15. Goodwin, T. W. and Morton, R. A., Biochem. J., 40, 628 (1946).
16. Boyer, P. D., J. Am. Chem. Soc., 76, 4331 (1954).
17. Brown, W. D., Biochim. Biophys. Acta., 44, 365 (1960).
18. Schonbaum, G. R., Zerner, B., Bender, M. L., J. Biol. Chem., 236, 2930 (1961).
19. Bender, M. L., J. Am. Chem. Soc., 84, 2582 (1962).
20. Moffitt, W., J. Chem. Phys., 25, 467 (1956).
21. Moffitt, W., and Yang, J. T., Proc. Natl. Acad. Sci. U.S., 42, 596 (1956); Moffitt, W., Ibid., 42, 736, (1956); Fitts, D., Kirkwood, J. G., Ibid., 43, 33 (1957); Moffitt, W., Fitts, D., and Kirkwood, J. G., Ibid., 43, 723 (1957).
22. Foss, J. G., Biochim. Biophys. Acta., 47, 569 (1961).
23. Donovan, J. W., Laskowski, M. and Scheraga, H. A., J. Am. Chem. Soc., 83, 2686 (1961).
24. Braams, R., Inter. J. Radiation Biol., 6, 297 (1963).
25. Blout, E. R., DaLoze, G. and Asadourian, A., J. Am. Chem. Soc., 53, 1595 (1961).
26. Stevens, C. O., Personal Communication (1963).
27. Alexander, P. and Hamilton, L. D. G., Radiation Res., 12, 510 (1960); et al., Ibid., 13, 214 (1960).
28. Platzman, R. and Franck, J., Symp. Information Theory Biol., 262-270, Paragon Press (1958).
29. Hunt, J. W. and Williams, J. F., Radiation Res., 23, 26 (1964).
30. Hayden, G. A. and Friedberg, F., Radiation Res., 22, 130 (1964).
31. Norman, A. and Speigler, P., J. Appl. Phys., 33, 2658 (1962).

32. Gordy, W. and Shields, H., Radiation Res., 22, 29 (1964).
33. Patten, R. A. and Gordy, W., Radiation Res., 22, 29 (1964).
34. Henriksen, T., Sanner, T., and Pihl, A., Radiation Res., 18, 147 (1963).
35. Henriksen, T., Sanner, T., and Pihl, A., Radiation Res., 18, 163 (1963).
36. Augenstine, L. G., and Mason, R., Biological Effects of Ionizing Radiation at the Molecular Level, Published by International Atomic Energy Agency (1962).
37. Setlow, R. and Doyle, B., Arch. Biochem. Biophys., 46, (1953).
38. Pollard, E. G., Powell, W. F., Reaume, S. H., Prac. Natl. Acad. Sci. U.S., 38, 173 (1952).
39. Garrison, W. M., and Weeks, B. M., Radiation Res. 17, 341, (1962).
40. Garrison, W. M., Jayko, M. E., and Bennett-Corniea, W., Science, 146, 250 (1965).
41. Hutchinson, F., Radiation Res., Supplement, 2, 49 (1960).
42. Butler, J. A. V. and Robins, A. B., Radiation Res., 17, 63 (1962).
43. Butler, J. A. V., Robins, A. B. and Rotblat, J., Proc. Roy. Soc. A, 256, 1 (1960).
44. Smith, E. L., Hill, R. L. and Kimmel, J. R., Symp. on Protein Structure, Nueberger, A. (Editor), London Methuen and Co. LTD. (1958) pp. 182.
45. Stevens, C. O., Tolbert, B. M. and Reese, F. E., Arch. Biochem. Biophys., 102, 423 (1963).

46. Stevens, C. O., Henderson, L. E. and Tolbert, B. M., Arch. Biochem. Biophys., 107, 367 (1964).
47. Anfinsen, C. B., and Haber, E., J. Biol. Chem., 236, 1361 (1961).
48. Hayashi, K., Hamaguck, Z. and Funatsu, M., J. Biochem. (Tokyo), 53, 374 (1963).
49. Braams, R., Radiation Res. 12, 113 (1960).
50. Blat, G., Unpublished Data (1964).

Part II
Enzymically Active Components
of the Crystalline Chymotrypsins

by

Louis Edwin Henderson

Chymotrypsinogen-A is an enzymically inactive protein which can be crystallized in good yields from beef pancreas. The amino acid sequence and disulfide arrangement of chymotrypsinogen-A has recently been published (2). This protein is a single peptide chain with five internal disulfide bonds. The action of trypsin on this protein results in a mixture of proteolytic enzymes known as the chymotrypsins (3). By selecting the proper conditions, this mixture of enzymes can be fractionated into four crystalline enzyme preparations. These preparations have been designated as alpha-, beta-, gamma- and delta-chymotrypsin.

Delta-chymotrypsin is an enzymically active two chain molecule derived from chymotrypsinogen-A by the enzymic release of a dipeptide unit. Alpha-chymotrypsin is an enzymically active three chain molecule derived from delta-chymotrypsin by the enzymic release of a second dipeptide unit. Beta- and gamma-chymotrypsin are derived from alpha-chymotrypsin with no apparent change in their molecular weights (4), amino acid analysis or, N- and C-terminal groups (5-7). They differ in sensitivity to urea denaturation, crystal structure and conditions for crystallization. The salient features of the chymotrypsins have recently been reviewed (8).

In this report a chromatographic procedure capable of separating some of the chymotrypsins is described. The technique is used to investigate the heterogeneity of crystalline chymotrypsin preparations. Some of the chymotrypsins can be also distinguished by their electrophoretic mobilities through polyacrylamide gels in 8 M urea. Alpha-, beta- and gamma-chymotrypsin have different electrophoretic mobilities in this system but their reduced chains have identical electrophoretic mobilities. This suggests that the three forms of the enzyme differ in their disulfide arrangements.

EXPERIMENTAL PROCEDURE

Enzymes, Proteins and Materials: All chemicals employed were reagent grade. Alpha-chymotrypsin, chymotrypsinogen-A and trypsin were obtained from Worthington Biochemical Company as 3X crystallized salt-free preparations. Beta-, gamma-, and delta-chymotrypsin were obtained from Mann Biochemical Company as 2X crystallized preparations. N-acetyl-L-tyrosine ethyl ester (ATEE) was obtained from California Biochemical Company. Bio-Rex 70 (100-200 mesh) was from Bio-Rad Laboratories. Fluorodiisopropyl phosphate (FDIP) was obtained from Mann Biochemical and ^{32}P labeled FDIP (FDIP-32) was obtained from New England Nuclear Corp.

Ion Exchange Chromatography and Column Preparation: Bio-Rex 70 (100-200 mesh) is a polyacrylic acid cation exchange resin. The resin was first washed with 1 N NaOH, then with distilled water, then with 1 N HCl and again with distilled water. The washed resin was suspended in 0.10 M citric acid and titrated to $\text{pH } 5.60 \pm 0.02$ at 25° with 50% NaOH. The resin was allowed to settle and the fines were removed by decanting the supernatant and resuspending the resin in fresh elution buffer. This operation was repeated several times. The pH of the resin in the elution buffer was closely adjusted to 5.60 ± 0.02 at 25° . The washed, equilibrated resin was packed into 220 cm pyrex glass columns. The packing was continued until a 200 cm resin bed was obtained. The packed column was placed in a 4° room, and the citrate elution buffer was allowed to flow through the column overnight. The pH of the eluate should be 5.60 ± 0.02 at 25° . The elution buffer was prepared by titrating 5 l. of 0.10 M citric acid to a pH of 5.60 ± 0.02 at 25°C with 50% NaOH. Analytical (0.8 x 220 cm) columns will take up to 300 mg of protein although the runs reported here did not exceed 50 mg. The protein was applied to the column in 1.0 ml of elution buffer and washed

in with 2 to 3 ml of buffer. The columns were run at flow rates not exceeding 6 ml/hr. Three ml fractions were collected. A 4 x 220 cm column prepared by the above technique was used to separate 3 grams of protein.

Protein and enzyme assays: The protein eluted from the columns was detected by its 280 m μ absorbance. Relative enzymic specific activities were calculated from the rate of hydrolysis of ATEE and the protein concentration calculated using 1.98 for the absorbance of 1 mg/ml alpha-chymotrypsin. All enzymic specific activities are reported as relative values taking the specific activity of 3X crystallized alpha-chymotrypsin as 1.00. Enzyme assays were performed by the method of Schwert and Takenaka (9) using ATEE as substrate and following the rate of hydrolysis in a thermostated Beckmann DU spectrophotometer.

Desalting: The fraction collector tubes containing a single protein component were pooled and desalted on a 4 x 50 cm Sephadex G-25 column at 4°. The eluted protein was detected by 280 m μ absorbance. The desalted protein was lyophilized to dryness. One pass through the column usually reduced the citrate in the protein fraction to less than 1% by weight. Citrate determinations were performed by measuring the absorbance at 500 m μ of the red complex formed when citric acid is dissolved in a 50:50 mixture of pyridine: acetic anhydride.

Gel Electrophoresis: "Disc" gel electrophoresis experiments were performed by the method of Reisfeld, Lewis and Williams (10) as modified by Maizel (11). All electrophoretic runs were performed in 7.5% polyacrylamide gels which were 8 M in deionized urea. One mg of protein in 0.1 ml of spacer gel buffer 12 M in urea was layered on top of the spacer gel. Electrophoresis was carried out at 4° for four hours at 300 V.

Each enzyme sample was reacted with FDIP-32 to label the serine at the active site (12-14). The sample was then made 12 M in deionized urea. One half of the sample was set aside, the other half was reduced with thioethanol. These samples, labeled "reduced" and "non-reduced" were electrophoresed by the above technique. The gels were removed from the tubes and stained in a 0.5% solution of amino Schwartz black for 18 hours. Excess stain was removed by soaking the gels in 10% acetic acid over a bed of activated charcoal for 36 hours. The developed gels were placed next to a meter stick and photographed. The gels were then frozen with dry ice-acetone and sliced into discs 0.8 mm thick. The radioactivity in each disc was determined in a windowless planchet counter.

The tops of some of the gels were destroyed in removing them from the glass tubes. Therefore, it was necessary to measure the distance of each band from the "front." This "front" is visible during the electrophoretic run as a sharp diffraction band, and appears as a sharp blue band after staining. The position of this band was essentially constant from run to run. A scale sketch of each gel was prepared from the photographs and the distribution of radioactivity plotted under the sketch on the same scale. These data appear in figures 8 and 9.

RESULTS

Alpha-Chymotrypsin. Fig. 1 is the elution diagram obtained by chromatographing 3X crystallized alpha-chymotrypsin by the techniques described in the experimental section. The dotted line representing the enzymic activity has been adjusted to fit the 280 m μ curve at the indicated point. Three enzymically active components are evident, these have been designated as components I, II and III in order of elution. Table I gives the percent of each component present and their relative specific activities.

TABLE I

Protein and Enzymic Composition of
3x Crystallized Alpha-Chymotrypsin

	Component			
	I	II	III	Inactive Protein
Percent of Total Protein	4.4	74.5	16.3	2.8
Relative Specific Enzymic Activity	0.72	1.00	0.93	0

Protein corresponding to component III was isolated from a 3g preparative run, desalted, lyophilized and rechromatographed on an analytical column. Fig. 2 shows the elution diagram of the "purified" component III. The small amount of component II is probably due to the incomplete separation of these components on the preparative column. A substantial amount of component I is present indicating that some protein was converted to this component during the purification process. The major protein component of "purified" component III rechromatographed as component III. This is taken as evidence that this component of alpha-chymotrypsin is not a chromatographic artifact.

Beta-Chymotrypsin. Fig. 3 shows the elution diagram of the soluble fraction of 2X crystallized beta-chymotrypsin. A considerable amount of inactive protein is eluted in the early fractions. The major enzymically active fraction of beta-chymotrypsin is eluted in the same volume as component III of 3X crystallized alpha-chymotrypsin. This result was confirmed by co-chromatographing a mixture of crystalline alpha- and beta-chymotrypsin. The remainder of the protein and enzymic activity is eluted in broad band covering the elution volumes for components I and II of crystalline alpha-chymotrypsin. This broad region may represent many different enzymically active species.

Gamma-Chymotrypsin. Fig. 4 shows the elution diagram of crystalline gamma-chymotrypsin. The major active component appears in the same elution volume as component III of crystalline alpha-chymotrypsin. The remainder of the protein is eluted in a series of overlapping components covering the elution volumes of components I and II. All of the eluted protein from crystalline gamma-chymotrypsin was enzymically active. Elution volumes were checked by co-chromatographing a mixture of crystalline alpha- and gamma-chymotrypsin. The major active component of gamma-chymotrypsin appears to have a higher specific enzymic activity than the major active species of alpha-chymotrypsin. This result could be due to a lower specific absorbance at 280 m μ for this component of gamma-chymotrypsin.

Figure 2
Chromatographic
Elution Diagram of Component III
from 3x crystallized Alpha-chymotrypsin

Figure 4
Chromatographic
Elution Diagram of
Gamma-Chymotrypsin

Delta-Chymotrypsin. Fig. 5 shows the chromatographic elution pattern for crystalline delta-chymotrypsin. The major active component is eluted between components I and II of crystalline alpha-chymotrypsin. Approximately 50% of the eluted protein was present as components I, II and III. All of the eluted protein was enzymically active. The number over each component in Fig. 5 represent the relative specific enzymic activities taking the specific enzymic activity of 3X crystallized alpha-chymotrypsin as 1.00.

On the basis of the foregoing data we conclude that component II (Fig. 1) is alpha-chymotrypsin. Component III is either gamma- or beta-chymotrypsin and component I remains unidentified.

"Slow" Activation Mixture of Chymotrypsinogen-A. Fig. 6 shows the elution diagram obtained by chromatographing the mixture of enzymes produced when chymotrypsinogen-A is acted upon by trypsin under the conditions of "slow" activation (15). Under these conditions most of the protein should be alpha-chymotrypsin. Components corresponding to delta-, alpha-, and beta- or gamma-chymotrypsin are present. Alpha-chymotrypsin represents the major enzymically active component. The numbers over the peaks refer to the relative enzymic specific activities based on the 280 m μ absorbance and the rate of ATEE hydrolysis.

Conversion of Alpha- to Beta- and Gamma-Chymotrypsin. A solution of 3X crystallized alpha-chymotrypsin was allowed to incubate for two weeks under the condition for the conversion of alpha-chymotrypsin to beta- and gamma-chymotrypsin (2, 15). The entire incubation mixture was chromatographed on an analytical column. Fig. 7 shows the elution diagram obtained. An appreciable fraction of the protein has been converted to the beta- and gamma- forms but most of the protein remains as alpha-chymotrypsin.

Components II and III (Fig. 1) of crystalline alpha-chymotrypsin were isolated from a 3g run on a preparative column, desalted and lyophilized. These two components had the same amino acid analysis

which agreed well with the published amino acid analysis for alpha-chymotrypsin (8). They both gave tyrosine and leucine as the major products of carboxypeptidase-A digestion, and alanine and isoleucine as N-terminal groups by the method of Sanger (16). They had identical specific absorbance at $280\text{ m}\mu$ and identical K_m for ATEE. However, the $V_{\max}$ for component III appeared to be about 10% less than the $V_{\max}$ of component II with ATEE as a substrate. There was no reliable difference in the rates of casein hydrolysis. These data agreed with that published previously on alpha-, beta- and gamma-chymotrypsin.

Gel Electrophoresis. All electrophoretic runs were done in 8 M urea. Enzyme preparations were labeled in solutions of FDIP-32 at pH 7.00 (12). The solutions of chymotrypsin-DIP-32 were divided into two parts. One part was applied directly to the electrophoresis column. The other was reduced with thioethanol before electrophoresis. Since FDIP reacts selectively with the active site serine any protein in the electrophoresis gel which contained appreciable radioactivity must have originated from an active molecule.

Fig. 8 shows the distribution of radioactivity and non-reduced protein in the gels. Crystalline preparations of alpha- and gamma-chymotrypsin yield two radioactive bands. Most of the radioactivity incorporated into beta-chymotrypsin is associated with one broad band. Crystalline alpha-chymotrypsin contains appreciable amounts of either beta- or gamma-chymotrypsin. Crystalline gamma-chymotrypsin contains appreciable amounts of alpha-chymotrypsin while crystalline beta-chymotrypsin contains only small amounts of alpha-chymotrypsin but may contain other enzymically active species.

Fig. 9 shows the distribution of radioactivity on the electrophoretic gels of reduced crystalline alpha-, beta-, and gamma-chymotrypsin-DIP-32. Relative distance of each band from the front band was used to construct the sketches. The radioactivity associated with the third electrophoretic band may represent non-specific labeling and

Figure 8

Figure 9

will not be considered further in this discussion. The major radioactivity is assumed to be associated with the C-chain. Thus, the second major band in each electrophoretic pattern represents the C-chain.

Polyacrylamide gels act as molecular sieves and larger protein molecules move more slowly under the influence of an applied field. Thus, the slowest moving major component in each of the gels should be the B-chain, the fastest major component should be the A-chain and the C-chain should have an intermediate mobility. The position of the C-chain is compatible with this scheme and we have tentatively assigned the order of B, C, A to the separated chains in order of increasing electrophoretic mobility.

The major reduced chains of alpha-, beta- and gamma-chymotrypsin have essentially identical electrophoretic mobilities in this system. Each preparation shows more than the expected three bands. Reduced alpha-chymotrypsin shows five bands suggesting that some of the molecules of this preparation may have an additional proteolytic cleavage in one of the three chains. Reduced beta- and gamma-chymotrypsin show at least six bands each and possibly more.

DISCUSSION

The heterogeneity of crystalline alpha-chymotrypsin has received renewed attention in recent publications (18-21). This report describes the first separation of the enzymically active components of chymotrypsin by ion exchange chromatography. The system described has been used to separate the enzymically active components of crystalline alpha-, beta-, gamma- and delta-chymotrypsin. Beta- and gamma-chymotrypsin have the same chromatographic mobilities in this system. Alpha- and delta-chymotrypsin can be separated from each other, and from beta- and gamma-chymotrypsin.

The major enzymically active components in the alpha family of chymotrypsins (alpha, beta and gamma) seem to be common to all preparations. Crystalline delta-chymotrypsin also contains contaminations by components of the alpha family. Each crystalline preparation investigated contained about 5% of the total protein as an enzymically active component which did not correspond to any of the known chymotrypsin. This heterogeneity of the alpha family is particularly interesting when one considers that the preparations have been shown to have the same amino acid analysis, N- and C- terminal end groups (5-7), molecular weight and enzymic properties (22). In non-denaturing solvents they have nearly the same isoelectric points (18). They differ in rate of urea denaturation and, as we have shown, in chromatographic mobilities.

The electrophoretic study shows that non-reduced protein containing the active site serine has different mobilities for alpha-, beta- and gamma-chymotrypsin. This observation could be explained by two possibilities. (a) Beta- and gamma-chymotrypsin may have additional proteolytic cleavages which result in the separation of part of the molecule from the remainder of the molecule in 8 M urea or (b) these molecules may have a disulfide arrangement which allows separation of one or more of the chains of the molecule from the C-chain in 8 M urea.

The first hypothesis should result in new N- and C-terminal groups and an altered electrophoretic mobility of one or more of the reduced chain of the molecules. This possibility is inconsistent with published results. The second hypothesis would not introduce new end groups and the reduced chains of these molecules would have identical electrophoretic mobilities. The reduced chains of alpha-, beta- and gamma-chymotrypsin have nearly identical electrophoretic mobilities. Therefore, beta- and gamma-chymotrypsin probably differ from alpha-chymotrypsin in the disulfide arrangements. Beta- and gamma-chymotrypsin may have a peptide chain which is associated with the remainder of the molecule only through non-covalent interactions.

It has been assumed that the disulfide arrangement of any protein molecule is determined by the amino acid sequence of the molecule, and represents the most thermodynamically stable state (23-25). In this discussion we have assumed that alpha-chymotrypsin has the same disulfide arrangement as chymotrypsinogen-A. Alpha-chymotrypsin does not have the same amino acid sequence as chymotrypsinogen-A. Two dipeptides have been removed resulting in the conversion of a single chain molecule to a three chain molecule. It may be that the disulfide arrangement of alpha-chymotrypsin is not the most thermodynamically stable state for the three chain molecule. More stable disulfide arrangements of the three chain molecule may be represented by beta and gamma chymotrypsin.

SUMMARY

An ion exchange chromatographic system has been developed which is capable of separating various crystalline chymotrypsins into enzymically active components. This technique was utilized to investigate heterogeneity of various crystalline chymotrypsins. Crystalline alpha-, beta-, gamma-, and delta-chymotrypsin were shown to contain several enzymically active components. Each crystalline preparation contained contaminations of other chymotrypsins.

Crystalline alpha-, beta-, and gamma-chymotrypsin were investigated with "disc" gel electrophoresis in 8 M urea. The major enzymically active component of these preparations have different electrophoretic mobilities in this system. The reduced chains of crystalline alpha-, beta-, and gamma-chymotrypsin have identical electrophoretic mobilities. These data suggest that these enzymes may differ from each other in their disulfide arrangements.

REFERENCES

1. Henderson, L. E., and Tolbert, B. M., Fed. Proc., **23**, 314,(1964).
2. Hartley, B. S., Nature, **4926**, 1284,(1964).
3. Kunitz, M., J. Gen. Physiol., **22**, 207,(1938).
4. Bartl, P., Sedlacek, B., and Kacena, V., Chem. Listy, **49**, 460,
(1955).
5. Gladner, J. A., and Neurath, H., J. Biol. Chem., **205**, 345,(1955);
Ibid, **206**, 911,(1954).
6. Röver, M., Fabre, C., and Desnuelle, P., Biochim. Biophys. Acta.,
10, 481,(1953).
7. Röver, M., Gabelotau, C., de Vernejoul, P., Guidoni, A., and
Desnuelle, P., Biochim. Biophys. Acta., **32**, 256,(1958).
8. Desnuelle, P., in Boyer, P. D., Lardy, H., and Myrback, K.,
(Editors), The Enzymes, Vol. 4, Academic Press, Inc., New York,
1960, p. 93.
9. Schwert, G. W., and Takenaka, Y., Biochim. Biophys. Acta **16**,
570,(1955).
10. Reisfeld, R. A., Lewis, U. J., and Williams, D. E., Nature, **195**,
281,(1962).
11. Maizel, J. V. Jr., Ann. New York Acad. Sci., **121**, 382,(1964).
12. Jansen, E. F., and Balls, A. K., J. Biol. Chem., **194**, 721,(1952).
13. Jansen, E. F., Jang, R., and Balls, A. K., J. Biol. Chem., **196**,
247,(1952).
14. Schaffer, N. K., May, S. C. Jr., and Summerson, W. H., J. Biol.,
Chem., **202**, 67,(1953); Ibid, **206**, 201 ,(1954).
15. Laskowski, M., in Colowick, S. P., and Kaplan, N. O., (Editors),
Methods in Enzymology, Vol. 2, Academic Press, Inc., New
York, 1955, p. 8.

16. Sanger, F., Biochem. J., 45, 563 (1949).
17. Raacke, I. D., Arch. Biochem. Biophys., 62, 184 (1956).
18. Egan, R., Michel, H. O., Schlueter, R., and Jandorf, B. J., Arch. Biochem. Biophys., 66, 354 (1957).
19. Hofstee, B. H. J., J. Biol. Chem., 238, 3235 (1963).
20. Erlanger, B. F., Cooper, A. G., and Bendich, A. J., Biochemistry, 3, 1880 (1964).
21. Kezdy, F. J., and Bender, M. L., Biochemistry, 4, 104 (1965).
22. Cunningham, L. W., and Brown, C. S., J. Biol. Chem., 221, 287 (1956).
23. Anfinsen, C. B., Comp. Biochem. Physiol., 4, 229 (1962).
24. Epstein, C. J., Goldberger, R. F., and Anfinsen, C. B., Cold Spring Harbor Symp. Quant. Biol., 28, 439 (1963).
25. Schachman, H. K., Cold Spring Harbor Symp. Quant. Biol., 28, 409 (1963).

Figure 1. Column Chromatographic Elution Diagram of 3X Crystallized Alpha-Chymotrypsin. The elution diagram was obtained by eluting 25 mg of protein from a 0.8 x 200 cm column of Bio-Rex 70 with 0.1 M sodium citrate pH 5.60 at 4°. The enzymic activity (·-·-·-·) was fitted to the 280 m μ absorbance (—) at point (O).

Figure 2. Column Chromatography Elution Diagram of Purified Component III of 3X Crystallized Alpha-Chymotrypsin: The same column conditions were employed here as for Figure 1 to elute 25 mg of protein. Numbers over the components represent the relative specific enzymic activity taking that 3X crystallized Alpha-Chymotrypsin as 1.00.

Figure 3. Column Chromatographic Elution Diagram of the Soluble Fraction of 50 mg of 2X crystallized Beta-Chymotrypsin: The same column conditions were employed here as for Figure 1. Enzymic activity (·-·-·) and 280 m μ absorbance (—) are plotted on the same coordinates as in Figure 1.

Figure 4. Column Chromatographic Elution Diagram of 50 mg of Gamma-Chymotrypsin: The same column conditions were employed here as for Figure 1. Enzymic activity (·-·-·) and 280 m μ absorbance (—) are plotted on the same coordinates as in Figure 1.

Figure 5. Column Chromatographic Elution Diagram at 50 mg of Delta-Chymotrypsin: The same column conditions were employed here as for Figure 1. Absorbance at 280 m μ (—) is plotted on a different scale than in Figure 1. Numbers over the components represent relative specific enzymic activity as in Figure 2.