MASTER

RECEIVED BY DTIC APR 23 1970

CHEMICAL AND CONFORMATIONAL STUDIES
OF GAMMA IRRADIATED LYSOZYME

by

Sarah Mary Herbert

B.A., College of Notre Dame, 1964

M.S., Georgetown University, 1966

A thesis submitted to the Faculty of the Graduate
School of the University of Colorado in partial
fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry

1969

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

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 Doctor of Philosophy Degree by

Sarah Mary Herbert

has been approved for the

Department of

Chemistry

by

Bert M. Tolbert

Bert M. Tolbert

Mancourt Downing

Mancourt Downing

Date Dec. 8, 1969

Herbert, Sarah Mary (Ph.D., Chemistry)

Chemical and Conformational Studies of Gamma Irradiated Lysozyme

Thesis directed by Professor Bert M. Tolbert

The phosphate soluble fraction of gamma irradiated lysozyme can be separated by column chromatography into a number of protein components. Detailed information on the nature of these fractions is essential in understanding chemical damage of irradiated enzymes. The chemical and physical properties of a major fraction, P3-I, eluting very close to native lysozyme was examined in depth in this study. It was of interest to determine whether P3-I was undamaged lysozyme, one kind of damaged material, or a mixture of molecules which has been damaged in various ways. If this protein was damaged it was of interest to see how it differed from native lysozyme. In addition, the free radical distribution in the irradiated phosphate soluble fractions including P3-I was determined by labeling with tritiated hydrogen sulfide (HTS).

The protein fraction, P3-I, showed considerable homogeneity on Bio-Rex 70 chromatography and could not be separated from the native protein. The disc gel electrophoresis experiments performed on the irradiated fraction P3-I under a variety of conditions indicate a small distribution of either charge or molecular weight. The results of the reaction of the phosphate soluble portion of irradiated lysozyme with HTS show that the proteins resulting all have about the same level of radical formation even though they differ widely in

enzyme activities. Sedimentation velocity measurements on native lysozyme and P3-I indicate that the latter has a distinctly different average shape than the native protein. The enzyme activity of the irradiated protein P3-I is 95% of that of the native enzyme. Optical rotatory dispersion spectra show very little difference between P3-I and native lysozyme down to 220 nm. Ultraviolet absorption spectroscopy shows several differences between P3-I and native lysozyme. There are changes in the environment of the peptide backbone as well as of the aromatic chromophores.

These results suggest that P3-I is not a homogeneous material but consists of molecules which have been damaged in many ways, and in which no one molecule has suffered extensive damage.

Both heat and pH perturbation spectra of the irradiated protein, when compared to native lysozyme, show that part of the conformational damage to this irradiated molecule can be "corrected" by denaturation and renaturation. The heat denaturation curves confirm this result. These data, together with the UV absorption spectra after denaturation by heat and alkaline pH, indicate that there are two levels of conformational damage to P3-I. There is conformational damage which is not reversible and presumed caused by chemical changes and there is that which is reversible and presumed not caused by chemical effects.

Two mechanisms are proposed for the reversible conformational damage. One involves the formation of a "hot spot" during irradiation whereby very limited regions of the protein are subject to a rise in effective temperature sufficient to give atomic mobility within the solid molecule. The other mechanism involves the radiation

formation of charged groups in hydrophobic centers of the protein which result in rearrangement of the hydrophobic groups to accommodate the charge. A mechanism for chemical damage from gamma irradiation of proteins is proposed involving independent cumulative inactivation of amino acid residues.

This abstract is approved as to form and content. I recommend its publication.

Signed Bert M. Tolbert
Faculty member in charge of dissertation

ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to Dr. Bert M. Tolbert for his guidance and continuing support during this investigation, and to Dr. Mancourt Downing for acting as advisor in Dr. Tolbert's absence.

The author also wishes to thank Dr. William Bauer for help in running the ultracentrifugation experiments, and Mr. Dante Marciani for many helpful discussions.

Financial assistance from the University of Colorado and the United States Atomic Energy Commission is gratefully acknowledged.

TABLE OF CONTENTS

CHAPTER	PAGE
I. Introduction	1
Biochemistry of Lysozyme	1
Ionizing Radiation and Macromolecules.	10
II. Experimental Procedures.	23
Materials.	23
Methods.	23
Column Chromatography of Lysozyme	23
Irradiated Lysozyme Samples	25
Enzyme Assay.	26
Disc Gel Electrophoresis.	27
Optical Rotatory Dispersion	27
Absorption Spectra.	28
Heat Denaturation Curves.	28
Ultracentrifugation of Lysozyme	29
Reaction with HTS	29
III. Results.	33
IV. Discussion	65
V. Bibliography	78

LIST OF TABLES

TABLE	PAGE
I. Specific activity and enzyme activity of pooled peaks from reaction of lysozyme and irradiated lysozyme with HTS	64
II. Amino acid content of a fraction from lysozyme receiving 30 Mrads.	66

LIST OF FIGURES

FIGURE	PAGE
1. The three dimensional model of the area around the active site of lysozyme.	7
2. Apparatus for the HTS reaction.	30
3. Pentex lysozyme chromatographed on Bio-Rex 70.	34
4. Phosphate soluble irradiated lysozyme chromatographed on Bio-Rex 70	35
5. Irradiated protein, P3-I, rechromatographed on Bio-Rex 70	37
6. Drawings of polyacrylamide gels resulting from disc gel electrophoresis of native lysozyme and the irradiated protein, P3-I	39
7. Optical rotatory dispersion spectra of native lysozyme and P3-I.	41
8. Visible region, 360-600 nm, of optical rotatory dispersion spectra of native lysozyme and P3-I	42
9. Absorption spectra of native lysozyme and P3-I. in citrate-phosphate buffer.	43
10. Absorption spectra, 240-360 nm, of native lysozyme and P3-I in water.	45

LIST OF FIGURES

FIGURE	PAGE
11. Absorption spectra, 180-250 nm, of native lysozyme and P3-I	46
12. Absorption spectra, 240-360 nm, of lysozyme in water at different temperatures.	48
13. Absorption spectra, 240-360 nm, of P3-I in water at different temperatures	49
14. Absorption spectra, 180-250 nm, of lysozyme in water at different temperatures.	50
15. Absorption spectra, 180-250 nm, of P3-I in water at different temperatures	51
16. Absorption spectra, 180-250 nm, of lysozyme at various pH values.	53
17. Absorption spectra, 180-250 nm, of P3-I at various pH values	55
18. Absorption spectra, 240-360 nm of lysozyme at various pH values.	56
19. Absorption spectra, 240-360 nm, of P3-I at various pH values	57
20. Heat denaturation curves of lysozyme and P3-I.	59

LIST OF FIGURES

FIGURE	PAGE
21. Second heat denaturation curves of lysozyme and P3-I	60
22. Elution pattern from column of Bio-Rex 70, of native lysozyme after reaction with HTS	62
23. Elution pattern from column of Bio-Rex 70, of irradiated lysozyme after reaction with HTS	63

CHAPTER I

INTRODUCTION

Biochemistry of Lysozyme

Lysozyme from egg white of the common domestic chicken is probably one of the best characterized proteins, readily available in pure form. These factors make lysozyme ideal for a study of the effects of ionizing radiation on proteins. It is the purpose of this thesis to describe some of the effects of gamma radiation on the physical and enzymic properties of this molecule and to develop a theory to explain these observations.

In 1922 Fleming discovered that a component of nasal mucous had the property of lysing bacteria¹. The component of the mucous which has this property is a protein which he called lysozyme. Since that time lysozymes have been found in the organs and secretions of vertebrates, invertebrates, bacteria, phages, and even plants. These enzymes all catalyze the same reaction, however, the amount of activity in each lysozyme varies. Lysozymes from different species may have quite different amino acid compositions and chromatographic behavior. In addition, differences have been found between lysozymes from two organs or secretions of the same species. Jollès² has defined lysozyme as a basic protein of low molecular weight, stable at acidic pH without significant loss of activity, unstable at alkaline pH, and active against suspensions of *Micrococcus lysodeikticus*.

liberating reducing sugars and N-acetylhexosamines, namely N-acetylglucosamine and N-acetylmuramic acid. The molecular weights of the majority of lysozymes are about 15,000, but some are higher, for example papaya lysozyme has a molecular weight closer to 25,000. Lysozyme is also called muramidase, β -glucosamidase, or N-acetylmuramidine glycanohydrolase, (E.C.3.2.1.17). The most common lysozyme studied to date has been hen egg white lysozyme, and any further reference to lysozyme in this thesis will refer to this material.

Lysozyme is a single chain of 129 amino acids and has a molecular weight of 14,307 based on this sequence³. Analytical data give a molecular weight of $14,500 \pm 200$. Sophianopoulos *et al.*⁴ give a sedimentation coefficient of $s_{20,w} = 1.91 \times 10^{-13}$ sec when extrapolated to infinite dilution, and a partial specific volume of 0.703 ml/g at 20°. These data correspond to a molecular weight of $14,400 \pm 100$.

The complete amino acid sequence of lysozyme was published simultaneously by Jollès and coworkers⁵ and Canfield and Anfinsen^{3,6,7} in 1963. There were originally several discrepancies between the work from these two laboratories but these have since been resolved by X-ray crystallography. Lysozyme contains eight half-cystine residues³. A combination of the work from three laboratories^{5,8,9} gives the positions of the disulfide bonds as 6-127, 30-115, 64-80, and 76-94.

The complete X-ray structure of lysozyme at 2 Å resolution was published by Phillips and co-workers in 1965¹⁰. From this and the complete amino acid sequence Phillips has constructed a model of the lysozyme molecule. The molecule is roughly ellipsoidal (45 by 30 by

30 Å) with a deep cleft running up one side. As is to be expected, the polar side chains are distributed on the surface of the molecule while the interior contains mostly hydrophobic residues. In addition the cleft is partially lined with hydrophobic residues. Secondary structure in the form of α -helix is found between residues 5-15, 24-34, and 88-96. These helices are somewhat distorted from the classical form. The peptide groups are rotated slightly so the CO groups point outward from the helix axis and the NH groups point inward. There are two groups of residues, 80-85 and 119-122, which are helical but with a three-fold symmetry axis. Optical rotatory dispersion measurements give 35% α -helix¹¹ whereas hydrogen-deuterium exchange gives 45% readily-exchangeable amide hydrogens¹². From the X-ray structure one can calculate 24% α -helix if only the classical form is considered, and 32% if the three-fold symmetry axis helical portions are included. Lysozyme also has some residues which are in the so-called β -helix or antiparallel pleated sheet conformation. Residues 41-45 and 50-54 are in this conformation connected by residues 46-49 which are folded into a hairpin bend. These portions of β -helix probably account for the high helical content values calculated from hydrogen-deuterium exchange measurements.

One of the best pieces of evidence which showed that a protein structure is essentially the same in solution as it is in the crystal was obtained with lysozyme. Although the crystal used for the X-ray structure contained 35.5% water of crystallization¹³ this is not substantial enough evidence to prove that the two states of lysozyme have the same structure. Sternlicht and Wilson¹⁴ employed NMR to

obtain evidence that the enzyme structure in solution and in the crystal state are very similar, if not identical. These workers used the X-ray crystallographic coordinates and aromatic ring current data to reconstruct the upfield resonances and these results were in good agreement with the observed spectra.

Much information about the structure of lysozyme has been obtained from studies of the physical properties of the molecule and by denaturation experiments. Tanford and Wagner¹⁵ found that the carboxyl region of the titration curve of native lysozyme was abnormal. Donovan *et al.*¹⁶ showed that the three "masked carboxyls" are titrated normally only in the presence of high molarities of guanidine hydrochloride. Tanford and Wagner¹⁵ also showed that the ionization profile of the tyrosines is abnormal but regains normal character in 8 M guanidine hydrochloride.

Studies related to the tryptophan residues also contribute information about the structure and function of lysozyme. Steiner¹⁷ measured difference spectra of lysozyme after denaturation by urea and low pH. He concludes that the positive peak at 303 nm and the negative peak at 292 nm which appear on denaturation are due to changes in the environment of all or some fraction of the indole groups of the tryptophan residues. Hayashi and co-workers¹⁸ showed that four of the six tryptophan residues in lysozyme are accessible to the solvent and two are buried in the interior of the molecule. These workers also showed that one of the accessible tryptophanys becomes buried when the enzyme-substrate complex is formed. They showed that this tryptophanyl is important in the binding of the

substrate to the active site.

Hamaguchi *et al.*¹⁹ showed that the physicochemical properties of lysozyme do not change in ethylene glycol and the change in the enzyme activity in the presence of organic solvents could not be correlated with a change in conformation. These same workers contend that neither ethylene glycol nor polyethylene glycol disrupt the helical structure or the hydrophobic structure of the lysozyme molecule. Hamaguchi and Sakai²⁰ measured the enthalpy change from the temperature dependence of the specific rotation and concluded that it represents the heat necessary to disrupt the antiparallel pleated sheet portion of the molecule rather than a helix-coil transition. These same workers showed that increasing the hydrocarbon portion of the solvent molecule increases its ability to reduce the transition temperature. Foss²¹ uses specific rotation to follow the conformational transition which takes place upon heating the molecule, and found that the transition temperature is lowered in 8 M urea. These results are consistent with the present belief that hydrophobic bonds make a great contribution to the stability of protein molecules.

In 1952 Salton²² showed that the substrate for lysozyme in *Micrococcus lysodeikticus* was the cell wall mucopolysaccharide. This mucopolysaccharide is a $\beta(1-4)$ polymer of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). Lysozyme acts on cell wall saccharides exclusively as an N-acetylmuramidase, that is, it cleaves only glycosidic bonds of N-acetylmuramic acid residues. Lysozyme also attacks chitin, a linear $\beta(1-4)$ polymer of N-acetylglucosamine, where it acts as an N-acetylglucosamidase..

After the X-ray crystallographic studies of Phillips and co-workers were published the study of the mechanism of action of lysozyme attracted many more workers. Johnson and Phillips²³ published the three-dimensional structure of a lysozyme-inhibitor complex at 6 Å resolution. The inhibitor, (NAG)₃, was bound in the cleft of the enzyme and filled nearly half its length. The nonreducing terminal was bound at the top of the cleft in a region called subsite A (see Figure 1, taken from Chipman and Sharon²⁴ after the model of Phillips). The next two sugar residues occupied subsites B and C. Several hydrogen bonds and numerous van der Waals contacts are formed between the enzyme and the sugar residues. These X-ray studies²³ also showed that parts of the lysozyme molecule move with respect to one another when the enzyme-substrate complex is formed, consistent with the induced fit theory of Koshland. From the three-dimensional structure of lysozyme and the lysozyme-inhibitor complex a model was constructed containing a substrate six saccharide residues long. From this model several inferences were made. It was found that six sugar moieties fill the cleft, and that the sugar unit in subsite D must be distorted from the chair conformation into a less stable "half chair" because of steric interactions with the enzyme. If the cell wall oligosaccharides are bound in the same manner as the chitin polymers, then the NAM residues are not bound in subsites A, C, or E because of steric factors. Since cleavage of the oligosaccharides occurs only between NAM and NAG then the linkage cleaved by the enzyme is either between B and C or D and E. The X-ray crystallographic studies were performed on a saccharide molecule filling

FIGURE 1

The three dimensional model of the area around the active site of the lysozyme-substrate complex [taken from Chipman and Sharon²⁴ after the model of Phillips]. The pyranose rings of the substrate are shown in heavy lines, with the monosaccharide units of the lysozyme-(NAG)₃ complex (A-C) solid and those placed by model building (D-F) dashed. The light dashed lines are the six hydrogen bonds between (NAG)₃ and the enzyme. The R groups are hydrogens in the case of chitin substrates, and lactyl groups in cell wall saccharides. The arrow indicates the bond cleaved.

subsites A, B, and C, and this complex was stable, therefore Phillips suggested that the linkage cleaved by lysozyme is between sugar residues D and E¹³. The potentially reactive groups for the hydrolysis of a glycosidic bond in the region of this D-E bond are glutamyl-35 and aspartyl-52. From the crystallographic model alone, Phillips¹³ proposed that the mechanism could be a concerted action of these two reactive groups on the enzyme. The glutamyl-35 donates a proton to the glycosidic oxygen while the negative charge on aspartyl-52 stabilizes the carbonium ion at C₁ of sugar D. He also proposed that the distortion of the sugar residue at subsite D played a part in the catalysis. Since that time chemical evidence has accumulated in agreement with Phillips' model. Rupley and Gates²⁵ acquired kinetic data in support of this mechanism. The chain-length dependence of the reaction rate and the preferential attack by the enzyme on certain bonds in polymeric substrates supported the idea that cleavage occurred between sugar residues D and E. These same workers used ¹⁸O to confirm that the bond broken is the C₁-O bond. Kinetic evidence also confirms that the sugar in subsite D has to be distorted in order to bind. Rupley and co-workers²⁶ published further evidence for the formation of a carbonium ion where glutamyl-35 participates as an acid catalyst and aspartyl-52 stabilizes the developing ion. Dahlquist and Raftery²⁷ used NMR to study the effects of temperature and pH on the enzyme-inhibitor association. These workers concluded that one ionizable group with a pK_a of $7.0 \pm .05$ affects the binding of β -methyl-N-acetylglucosamine. This group is probably the glutamyl-35 residue which is in a nonpolar environment and has an abnormally

high pK. One of the most conclusive pieces of evidence of the involvement of glutamyl-35 and aspartyl-52 was obtained by Lin and Koshland²⁸ who modified the carboxyl groups by the carbodiimide-nucleophile procedure. Their results eliminate all carboxyl residues as potential catalytic groups or essential binding groups except glutamyl-35 and aspartyl-52, which are both necessary for enzyme activity. Parsons and Raftery²⁹ extended the work of Lin and Koshland and have direct chemical evidence that the catalytic action of lysozyme occurs in the region of aspartyl-52. The work from these two studies together strongly indicates that aspartyl-52 and glutamyl-35 are the important carboxyls in the active site of lysozyme and this work lends strong support to the mechanism of Phillips.

The above chemical studies were obtained using both water and other molecules as acceptors of the product in the enzyme-product complex. In work with the saccharide acceptors Rupley found that the rates are considerably enhanced over water presumably because the saccharides have favorable interactions with the enzyme. The transfer reaction is stereo-specific even if saccharides are not used as acceptors, for example, the transfer of (NAG)₂ to methanol yields only β -methylglycoside.

From both crystallographic and chemical data it is proposed that the mechanism of action of lysozyme is the following. Glutamyl-35 forms a hydrogen bond with the bridge oxygen of the D-E glycosidic bond. The carbonyl oxygen of aspartyl-52 makes contact with C₁ and the ring oxygen of sugar D. Cleavage of the D-E bond

then proceeds by protonation of the bridge oxygen, rupture of the C₁-O bond to form a C₁ carbonium ion with the reaction rate being enhanced by the neighboring negative charge of aspartyl-52, then dissociation of the aglycone and its replacement by water or a saccharide acceptor.

Ionizing Radiation and Macromolecules

The effects of ionizing radiation on biological macromolecules are commonly discussed in terms of direct and indirect effects. Indirect effects of ionizing radiation are those caused by excited solvent species rather than by direct interaction with the radiation. In solution, direct effects on macromolecules take place, but it is often difficult to separate these effects from solvent mediated changes. With solid state irradiations one can study the direct effects of the ionizing radiation on the macromolecule. The present work will be concerned with the direct effects of ionizing radiation on lysozyme.

The two primary processes for interaction of ionizing radiation with matter are via ionization and excitation. In the earlier work it has often been assumed that ionization plays the dominant role in radiation damage of macromolecules³⁰, and that excitations were ineffective in damaging the molecules. The reasons for this assumption were the logarithmic relationship between loss of function and dose, the quantitative agreement between molecular weights measured by physicochemical methods and the radiation sensitive volume or target volume calculated using an ionizing model. Pollard *et al.*³¹ show

that the target sizes of molecules from penicillin (~ 350) to the DNA of *pneumococcus* transforming principle ($\sim 6 \times 10^6$) agree within a factor of two with the size determined by other physicochemical methods. From these arguments the target theory was formulated. Classical target theory contends that an ionization releases a localized amount of energy in a molecule which disrupts a limited region and inactivates a given molecule. The inactivation of the biological molecule is caused by the interaction of a particle or photon to give an ionization. This does not mean that each particle or photon inactivates each molecule through which it passes, only that inactivation is due to the action of a single particle³¹.

Augenstein *et al.*³² showed in more recent work that the agreement between target volume and molecular weight was not as good as was first suggested. Examination of the data shows large discrepancies exist for a number of molecules. Augenstein and co-workers show the presence of extensive intermolecular migration of energy and/or charge and these workers conclude that excitations may play the predominant role³². Brustad³³ shows that the radiosensitivity of enzymes can be modified by the presence of foreign molecules, the temperature during exposure, and the presence or absence of oxygen and he concludes from this that molecular interactions are a contributing factor in the inactivation of enzymes in the dry state. Fluke³⁴ measured the temperature dependence for radiation inactivation of several enzymes and concludes that the Lea target hypothesis is not complete and needs to be extended to include temperature dependence. At this point it is still not clear whether ionization or

excitation as primary processes in radiation damage to biological macromolecules plays the predominant role, and whether the classical target theory has physical significance in radiation chemistry of macromolecules.

Following ionization and excitation, secondary processes take place which cause the disruption of the macromolecule. It is through these secondary processes that energy from the radiation is used to alter chemical bonds and induce conformational changes. Partial mechanisms for destruction of biological activity through secondary effects are numerous; and there is no one proposal or even combination of proposals which seems to fit all the available data. One of the theories, discussed by Hutchinson³⁵ and by Hutchinson and Pollard³⁶ is that the ionization energy migrates along the covalent structure of the molecule to the weaker bonds. Mass spectrometer patterns of electron bombarded hydrocarbons show that bonds that are weaker, thermodynamically, are the ones most likely to break³⁶. In a protein the end result of this energy transfer to some point in the molecule would be a change in the covalent structure. The bonds of a protein molecule which are considered to be weakest are the disulfide bonds. Although some disulfide bond breakage is found it does not account for all of the damage.

It has been observed by ESR that free radicals are formed when proteins are irradiated. Henriksen and co-workers³⁷ reported from their ESR data that at room temperature two types of radicals are formed in irradiated proteins, the glycylglycine type and the sulfur type. Stratton³⁸ found that the ESR pattern changes with time and

temperature and postulates on this basis that thermal energy is required for the formation of sulfur radicals in ribonuclease and lysozyme. In addition, admission of oxygen has no effect on the ESR spectrum at 77° K however, at room temperature a change to an oxygen-type spectrum takes place, with some competition from sulfur radical formation. If the sample is allowed to remain at room temperature *in vacuo* until most of the sulfur radical spectrum has developed then exposure to air causes no change in the sulfur spectrum. This worker also found that the radical yield was dependent on dose, and that at the same dose rate the Bragg peak proton radiation was less efficient than gamma radiation for radical production. At the same dose rate (22.0 krads/min) and with the same kind of radiation (Bragg peak protons) the G_{radical} value for ribonuclease is 2.9 whereas that for lysozyme is 2.3, at 77° K. The radical yield is also dependent on temperature. Henriksen and co-workers³⁷ find that at room temperature the G_{radical} values for ribonuclease and lysozyme are 4.5 and 3.6 respectively, when they are irradiated with X-rays. These workers also found that radical yields were not significantly altered by heat denaturation of the proteins. Henriksen and collaborators suggest an explanation of these data. The free radicals induced in proteins by radiation are in part formed by homolytic rupture of chemical bonds and that unpaired spins are transferred to specific structures partly by intermolecular processes. These specific structures are the glycine and cysteine/cystine residues.

Riesz and co-workers³⁹ have developed another method for determining the distribution of part of the free radicals in gamma

irradiated proteins, using tritiated hydrogen sulfide as a radical scavenger. This method has been employed in the present work to determine the distribution of free radicals in the chromatographic fractions of gamma irradiated lysozyme. Riesz and co-workers^{39,40} consider the reactions which can take place by gamma irradiation.

In order to eliminate the possibility of abstraction of hydrogen by sulfhydryl radicals to form radicals on the protein which do not result from gamma radiolysis, as is shown in the following equation

tritiated hydrogen iodide was used as a radical interceptor⁴¹. With this reagent reaction (6) cannot occur because of the low bond dissociation energy of hydrogen iodide. It was found that the incorporation of tritium with hydrogen iodide was of the same order of magnitude as for hydrogen sulfide so these workers conclude that abstraction of hydrogen by sulfhydryl radicals is not a significant process. In the free radical interceptor method the irradiated protein is exposed to HTS gas and the carbon free radicals become labeled with tritium by the process shown in equation (2). The protein is then hydrolyzed and non-exchangeable labeling of tritium on each kind of amino acid is determined. These workers have shown that contrary

to the interpretation of the ESR experiments there is not a preponderance of glycine radicals. Riesz and co-workers have examined the free radical distribution in ribonuclease³⁹, chymotrypsinogen⁴¹, lysozyme, and insulin⁴⁰, as well as the carboxymethylated derivatives of these proteins. They found that the free radical distribution depends on the conformational integrity of the protein since the carboxymethylated derivatives give radical distributions that are more nearly alike than the parent proteins. In a later paper White⁴² reports the distribution of radicals in the chymotryptic peptides of lysozyme. He found distinct spatial relationship when the more heavily labeled residues were related to the X-ray diffraction model of lysozyme. One group of heavily labeled residues was near a strongly hydrophobic region, another in the helical portion of the molecule (both α and β), and in addition, both the amino and carboxyl ends of the molecule were heavily labeled.

The limitations of this method are discussed by White and co-workers⁴⁰. Incorporation of tritium by exposure of a nonirradiated protein to HTS is called β -labeling and must be included in analysis of the data. Loss of tritium during acid hydrolysis is not the same for all amino acids. Counting errors must also be taken into account since a flow-counting technique was employed. In addition these workers found that analyses of all proteins studied showed radioactive components that were not detectable by ninhydrin color. As yet, no studies have been reported on the distribution of label in any given amino acid. In the present work all of these limitations are excluded except β -labeling, which has to be taken into account. However, there

is an additional limitation of excluding exchangeable hydrogens since acid hydrolysis is not used.

This free radical interceptor technique provides an independent experimental technique for the study of free radical formation in irradiated proteins.

Although free radicals are formed upon irradiation of proteins there is no evidence that radical formation can be directly related to loss of biological-activity. Brustad and co-workers⁴³ tried to correlate the formation of radicals, as detected by ESR, to the inactivation of the enzyme trypsin. These authors concluded that there was no correlation. Although radical formation is thought to be an important consideration it is not known at present just what role the formation of free radicals plays in the disruption of macromolecules.

Most workers have attributed ultimate radiation damage to breakage of covalent bonds. Garrison and co-workers⁴⁴ propose that the main pathway of radiation damage in proteins is by the radiolytic oxidation of amides in solution and have applied this theory to proteins in the solid state. The intermediate species proposed by Garrison are:

In Garrison's proposal an imine is formed which undergoes spontaneous hydrolysis upon dissolution in water. This results in a new amide end group and a new carbonyl end group in the protein.

Hydrolysis of the amide produces ammonia. Production of ammonia has been detected in irradiated proteins in solution, and after hydrolysis of proteins irradiated in the solid state, but breakage of the peptide bonds which would result in fragments has not been observed in any appreciable amount, even at very high doses.

Other workers contend that selective loss of specific amino acids is responsible for radiation inactivation of enzymes. Mee⁴⁵ suggests from changes in the absorption spectrum following irradiation of chymotrypsin that tryptophan residues are destroyed. Dosé and collaborators⁴⁶ give a G value of 7 for tryptophan. These workers give G values for all the amino acids in lysozyme. Their work shows that there are several amino acids with considerably higher G values than tryptophan, among these are methionine, tyrosine, phenylalanine, and the half-cystine residues. Drake and collaborators have studied the effects of gamma radiation on the amino acid content of insulin⁴⁷. These workers found that at low doses the most radiosensitive amino acids were cystine, tyrosine, proline, phenylalanine, and histidine. However, insulin contains no methionine, cysteine, or tryptophan so this work cannot be directly related to that of Dosé. Shimazu and Tappel⁴⁸ have studied the radiolability of solutions of free amino acids and of amino acids in proteins. They found that the radiolability was similar in both cases with the most radiosensitive amino acids being cystine, methionine, phenylalanine, and histidine. Tolbert *et al.*⁴⁹ report on the relative radiation stabilities of some crystalline amino acids as determined by their G values. Their

results in the solid state are quite different from those where the G value is determined in the protein as in the work of Dosé. It appears that the radiosensitivities of the amino acids are not equal, and in fact, there will be a selective destruction of specific amino acids. However, it is not clear whether this can account totally for the inactivation of proteins.

An often recurring chemical theory for the secondary effects of ionizing radiation is the breakage of disulfide bonds. In fact disulfide cleavage is included in some of the theories outlined above. Stevens and co-workers⁵⁰ contend that 10-20% of radiation inactivation in lysozyme can be attributed to disulfide bond damage, however, these workers believe it is unlikely that disulfide interchange can take place in the solid state.

Radiation inactivation has also been attributed to conformational effects. However, some workers contend that all conformational damage is caused by chemical changes in the macromolecules, and there are no direct effects of ionizing radiation on the conformation of a molecule without concomitant chemical effects.

A purely conformational theory for the secondary effects of radiation which produces disruption of macromolecular structure was proposed by Norman^{51,52} and is called the thermal spike model. In this model the material within the heavy-ion tracks is raised to a high temperature called a "hot spot". These "hot spots" exert sufficient pressure to deform nearby molecules, since the heated material expands and the rates of physical and chemical processes will be greatly increased by the heat. This theory has been generally discredited

since it has been shown that because of rapid heat dissipation the amount of heat introduced could not create a temperature increase of more than a few degrees. This would be insufficient to cause denaturation in a dry protein.

Another conformational theory has been proposed for damage to macromolecular structure. This mechanism has been proposed by Tolbert and Strickler⁵³ to account for conformational damage to proteins without concomitant chemical damage. This theory suggests that ionizing radiation will cause the formation of many low energy singlet and triplet excited states, and many of the singlet states will undergo internal conversion to triplet states. The triplet states could be located in the carbonyl-amide link of the protein backbone. The bond angles of the excited structure would be changed appreciably from normal since the carbonyl would change from an sp^2 system with 120° bond angles to an sp^3 system with bond angles of $\sim 109^\circ$. This would affect both the α -helical and pleated sheet portions of the protein in question. Adequate energy is available for this process since the triplet energy may be 4-6 ev and the hydrogen bond energy is less than 0.1 ev.

Platzman and Franck⁵⁴ have proposed a "polarization wave" theory. The inactivating event is due to the instantaneous appearance of an electric charge within the medium which produces a polarization wave, that is, a rearrangement of any polar groups in the vicinity of this charge. This rearrangement could destroy the secondary structure of the molecule by breaking hydrogen bonds. This theory has not been generally used in recent years.

It would be of interest now to discuss some of these theories in relation to actual data resulting from irradiation of particular proteins in the dry state. Garrison's work in support of his theory has been done mainly in solution⁴⁴ but in one paper⁵⁵ he reports on irradiated solid pepsin, which yields high molecular weight products on dissolution in water. Alexander and co-workers⁵⁶ have found changes in solubility, molecular weight by light scattering, sedimentation, and accessibility of disulfide groups upon irradiation of bovine serum albumin in the solid state in the absence of oxygen. These workers postulated three stages of radiation damage in their work with BSA. First, the "primary ionization" causes a shape change in the molecule. This is evidenced by a 25% revelation of S-S bonds and by a different ultracentrifugation pattern. At "two events per molecule" BSA shows decreased solubility and there are small aggregates present. Further irradiation causes extensive aggregation which is attributed to the formation of intermolecular hydrogen bonds. It is doubtful that the damage process is this simple. Although Garrison uses Alexander's results to support his theory⁴⁴, Alexander does not attribute his results to Garrison's mechanism⁵⁶.

Tolbert and co-workers have studied the effects of ionizing radiation on trypsin and trypsin-inhibitor compound⁵⁷, lysozyme⁵⁸, and chymotrypsin⁵⁹. Trypsin was shown to lose its esterase and protease activities at different rates when exposed to ionizing radiation in the dry state, *in vacuo*, and the D₃₇ values are concomitantly different. The trypsin-inhibitor complex makes the enzyme more sensitive to radiation, and the two types of enzyme activity are still

lost at different rates. Lysozyme and chymotrypsin were found to be very different when the loss of enzyme activity was compared with the loss of hard-to-exchange amide hydrogens⁵⁹. With lysozyme the hard-to-exchange amide hydrogens are lost two to three times as fast as the enzyme activity, whereas with chymotrypsin these two parameters are lost at about the same rate. These data indicate that the conformational integrity of the whole molecule is not essential for the activity of lysozyme. This is apparently not the case for chymotrypsin. The concept of target volume or radiation cross section presupposes that the entire molecule is necessary for activity.

Stevens and co-workers found that several lysozyme species could be separated on an ion exchange column after irradiation⁵⁸. Maksimov *et al.*⁶⁰ have studied the chromatographic properties of irradiated lysozyme using Sephadex. They find two fractions, one of which has 80% activity. The elution volume of this protein coincides with that of native lysozyme, and the total intensity of fluorescence is 80% of that of native lysozyme.

The kinetic parameters of chymotrypsin were studied after X-irradiation⁴⁵ and it was found that K_M increases and v_{max} decreases. Mee concludes that the increase in K_M represents a decreased affinity for the substrate which indicates the formation of a modified but still active enzyme molecule. It is hard to interpret these kind of data because the experimental substrate is a mixture of components.

These data, along with the data to be presented here, show that the classical target theory, as presented by Lea³⁰ is inadequate to explain the direct effects of ionizing radiation on proteins. It is

believed that not any one of the theories discussed for the secondary effects is sufficient to explain the damage to biological macromolecules caused by ionizing radiation, but that it is a combination of effects. A study of the formation of free radicals in gamma irradiated lysozyme will be presented. In addition, one species of irradiated lysozyme is separated by chromatography and the properties of this molecule are studied in detail. If this irradiated protein is damaged it is of interest to see how this protein differs from native lysozyme in its physical and chemical properties. These changes can then be correlated either with the existing theories of radiation inactivation or a new theory can be formulated.

CHAPTER II

EXPERIMENTAL PROCEDURES

Materials

The lysozyme used in all of the experiments referred to as native lysozyme was crystallized hen egg white lysozyme, Pentex, lot 19, used without further purification. Tritiated hydrogen sulfide was obtained from New England Nuclear Corporation, lot 345-283, with a specific activity of 225 millicuries per millimole. All the chemicals used were obtained commercially and were Reagent grade.

Methods

Column Chromatography of lysozyme: Bio-Rex 70, 200-400 mesh, sodium form, from Bio-Rad Laboratories was used for column chromatography. The resin was changed to the hydrogen form using the method of Stevens and Bergstrom⁶¹. The resin (1 kg) was stirred with 4 liters 3 N HCl overnight at room temperature, followed by extensive washing with distilled water until the filtrate reached pH 6. The resin was then suspended in 0.2 M potassium phosphate buffer, pH 7.09, and the pH of the suspension was adjusted to 7.09 with 20% potassium hydroxide with continuous stirring. The resin was then washed with 0.2 M potassium phosphate buffer, pH 7.09, 10 liters per kg original resin

weight. Fines were removed by suspending the resin in buffer, allowing to settle for 20 minutes, then decanting the supernatant. This step was repeated until no cloudiness remained in the decanted solution. The resin was then suspended in buffer and poured into the columns. Two column sizes were employed in this work. The large preparative columns were 3 cm by 50 cm. This column contained a fritted glass disc on top of which was placed a half-inch layer of glass wool before the resin was added. The small analytical column, 1 cm by 40 cm, was a commercial Glenco column fitted with a plastic porous disc and did not require glass wool. Protein samples up to 400 mg were put on the large column and 40 mg on the small one. The flow-rate for the large column was 1 ml per minute and that for the small column was 0.5 ml per minute. The enzyme samples were dissolved in 2 ml and 10 ml 0.2 M potassium phosphate buffer, pH 7.09, for the small and large columns respectively. The samples were washed onto the columns with two aliquots of buffer then the top of the column was filled with buffer and the pump turned on. A Beckman Accu-flo pump was used to regulate the flow rate. Fraction sizes were 3 ml for the small column and 6 ml for the large one. The absorbance of each fraction was read at 280 nm on a Beckman DB spectrophotometer using a Gilson automatic transferator. If the column was a preparative one, the fractions were pooled and put into dialysis tubing which had been treated previously so that a minimum of lysozyme would escape through the pores. This treatment involved heating the dry tubing for 3 days at 80° in an oven. Immediately upon removal from the oven the tubing was placed in distilled water and stored at 4°. Before use the

dialysis tubing was boiled in distilled water for one hour to remove substances which leach out of the tubing and absorb in the ultra-violet. The pooled peaks were exhaustively dialyzed against distilled water until the water on the outside of the bag no longer gave a positive phosphate test. The phosphate test employed was the occurrence of a yellow color when added to a saturated solution of ammonium molybdate in 6 N HCl. When all the phosphate had been removed the protein was lyophilized and stored in the freezer.

One sample of the irradiated protein was desalted using column chromatography on Bio-Gel P2 from Bio-Rad Laboratories. The glass column was treated first with dichlorodimethyl silane to prevent streaming. A solution of 1% dichlorodimethyl silane in benzene was heated to 60° then poured into the column and allowed to stand until cool. The column was then rinsed with absolute methanol and dried with ether. The resin was hydrated by adding slowly with constant stirring to acetic acid-pyridine buffer (5.72 ml glacial acetic acid, $d = 1.050$; 24.2 ml pyridine, $d = 0.982$, distilled water to make 1 liter, pH 5.5). The suspension was allowed to stand overnight. The column was filled with buffer and a funnel placed on top. The slurry of resin was put into the funnel all at once and allowed to settle. Column size was 1.5 cm by 45 cm. The flow-rate was 1 ml per minute. Fractions were read at 280 nm and the ammonium molybdate test for phosphate was used on each fraction. This method was only useful for desalting small amounts of protein.

Irradiated Lysozyme Samples: Irradiations of the evacuated lysozyme samples were performed at 25° using a kilocure ^{137}Cs source. The

dose rate was 0.383 megarads per hour determined using a Fricke chemical dosimeter⁶². A value of 15.7 was used for $G(\text{Fe}^{+3})$ for ^{137}Cs gamma rays⁶². The difference in molar extinction coefficients between ferric and ferrous ions at 304 nm was taken as $\Delta\epsilon = 2148$. Each dose was approximately 22.6 megarads delivered in 59 hours. The irradiated protein which was the object of this study was obtained by chromatography of the whole irradiated substance on Bio-Rex 70 according to the method outlined above. The "native" peak, that which falls in approximately the same position as native lysozyme, was pooled, dialyzed exhaustively to remove phosphate, and then lyophilized. This irradiated protein, which was chromatographically homogeneous, will be called P3-I.

Enzyme Assay: The enzyme activity of the different lysozyme samples was determined using Worthington *Micrococcus lysodeikticus* cells as a substrate. For each assay a fresh solution of *M. lysodeikticus* cells was made up (approximately 19 mg per 50 ml) so that the absorbance reading of the blank (2.5 ml *M. lysodeikticus* + 0.5 ml buffer) would be 0.8 to 0.9 at 645 nm. This reading was used as the absorbance at zero time. Solutions of lysozyme of 120, 90, 60, and 30 μg per ml are used for the calibration curve. To a 2.5 ml sample of *M. lysodeikticus* is added 0.5 ml protein so the final concentrations of lysozyme for the calibration curve are 20, 15, 10, and 5 μg per ml. The lysozyme samples to be assayed were kept within this concentration range. The protein solution was added to the solution of bacteria in a spectrophotometer cell, the solution stirred, and placed

in the spectrophotometer. The decrease in absorbance (turbidity) was measured as a function of time using a Cary 16K recording spectrophotometer. The absorbance was read continuously for 5 minutes. The data were plotted as a first-order reaction, i.e. log Absorbance vs. time; k is calculated from the initial slope then plotted versus enzyme concentration. The resulting curve was not a straight line, but has an empirical basis. The k for each sample being assayed was calculated and from the calibration curve the corresponding lysozyme concentration was determined. This concentration was compared to that determined by absorbance at 280 nm to give the percent activity. A calibration curve was run each time the assay was performed.

Disc Gel Electrophoresis: Disc gel electrophoresis was performed by modifying the method of Reisfeld *et al.*⁶³ for basic proteins. It was found that the small-pore solution polymerized too rapidly and bubbles formed in the gels so in this work 30 mg $K_3Fe(CN)_6$ was added to retard polymerization. Gels of 5, 7.5, and 10% cross-linking were used. A saturated sucrose solution was used for the protein sample rather than a stacking gel. The tray buffer was β -alanine and acetic acid, pH 4.5. The gels were routinely run at 3 mamp per tube for 2 hours. After electrophoresis was complete the gels were removed from the glass tubes and placed in test tubes containing 0.25% Coomassie brilliant blue in 10% acetic acid for staining. After one hour in the staining solution the gels were removed and placed in 7% acetic acid overnight for destaining.

Optical Rotatory Dispersion: The optical rotatory dispersion spectra

were run in 0.06 M citric acid - 0.12 M disodium phosphate buffer, pH 6.0. These spectra were kindly run by Dr. Jacqueline A. Reynolds at Washington University using a Cary 60 spectropolarimeter.

Absorption Spectra: The absorption spectra of the different samples of lysozyme and irradiated lysozyme were run using a Cary 16K recording spectrophotometer. Quartz cells from Precision Cells, Inc. were used which have higher than 85% transmission at 200 nm. Since the spectrophotometer was not equipped with an automatic slit drive the slit was changed manually to give the optimum sensitivity of the instrument at various wavelengths. Each time the slit width was changed the instrument was re-zeroed. The extinction coefficient of the irradiated protein, P3-I, was measured using the microbiuret test from "Techniques in Protein Chemistry"⁶⁴. A protein sample containing 0.1 to 2.0 mg protein dissolved in 4 ml of 3% sodium hydroxide was added to the reagent solution which contained sodium citrate, sodium carbonate and copper sulfate. After 15 minutes the absorbance was read at 330 nm. A calibration curve over the entire concentration range was made with native lysozyme, since the extinction coefficient was known ($\epsilon_{1\%}^{1\text{ cm}} = 26.0$). From this the extinction coefficient of P3-I was determined, $\epsilon_{1\%}^{1\text{ cm}} = 29.7$.

Heat Denaturation Curves: The heat denaturation curves were followed at 280 nm with a Cary 16K spectrophotometer. Three milliliters of each solution to be denatured was placed in a spectrophotometer cell. The Multi-Zero Suppression accessory of the Cary 16K was used so that each sample could be monitored with a different full-scale absorbance

if necessary. The smallest full-scale absorbance that would cover the denaturation was used in each case. This was usually 0.1 absorbance full scale. The electronics of the instrument were kept at 25° using a Haake water bath while the temperature of the cell compartment was raised using a Neslab Instruments water bath. The temperatures were measured by means of a thermocouple immersed in a buffer-filled cell in the sample compartment. The temperature was read directly by having the thermocouple connected to a Leeds and Northrop Speedomax W multipoint recorder and using an ice bath and a boiling water bath to calibrate the recorder. In a typical experiment the spectrophotometer was set for a 15 second scan of each sample then a 2 minute dwell. During the time that the samples were being scanned the temperature was read and recorded manually on the chart paper.

Ultracentrifugation of Lysozyme: Sedimentation velocity experiments were carried out with a Spinco model E ultracentrifuge, equipped with a UV scanner. The rotor speed used was 56,000 rpm. Both proteins were dissolved in M/15 sodium phosphate buffer, pH 6.0, containing 0.25 M sodium chloride. Scans were made every 8 minutes at 280 nm.

Reaction with HTS: The reactions of native and irradiated lysozyme with tritiated hydrogen sulfide (HTS) were performed in special glass vessels which allow the entire reaction to take place in the absence of air, with the partial pressure of HTS 0.2 atmosphere. Figure 2 shows a drawing of this apparatus. For the control experiment 40 mg lysozyme was evacuated overnight and sealed under vacuum, and the reaction with HTS performed. For the irradiated sample 40 mg lysozyme

FIGURE 2

Apparatus for the reaction of HTS with lysozyme and irradiated lysozyme.

is evacuated, sealed under vacuum, and then irradiated in the ^{137}Cs source as discussed above. When the desired dose has been obtained the sample is removed from the source and the HTS reaction proceeds. The HTS reaction is performed on this material approximately 12 hours after the enzyme sample is removed from the source. The lysozyme and HTS vessels were connected to the Y-tube and the system was evacuated. After evacuation was complete, as determined using a Tesla coil, the system was sealed under vacuum and the break-seals were broken using a magnet to guide the steel breakers. The HTS gas was drawn down into the lysozyme by immersing the lysozyme vessel in liquid nitrogen. After ten minutes the HTS vessel was sealed off from the system and the liquid nitrogen was removed. The reaction was allowed to proceed for four hours. After this time any unreacted HTS gas was removed by immersing the Y-tube in liquid nitrogen thereby drawing down the unreacted gas. This was sealed off and discarded. The lysozyme was then dissolved in distilled water, allowed to stand, lyophilized, and redissolved in water until the specific activity of the lysozyme was constant. Each time 100 ml distilled water was added to the lysozyme and the solution was allowed to stand for at least 24 hours. The washing procedure was repeated ten times before the specific activity of the lysozyme remained constant. When all readily-exchangeable hydrogens were removed the lysozyme-HTS was dissolved in potassium phosphate buffer and put onto a Bio-Rex 70 column. The protein in each fraction was monitored as before, by absorbance at 280 nm. Tritium counts per minute in each fraction were determined using a Beckman LS 250 Liquid Scintillation System. A 50 microliter sample of each

fraction was added to 15 ml scintillation cocktail of the following composition: 7.0 g 2,5-diphenyloxazole, 0.42 g 1,4-Bis[2-(4-methyl-5-phenyloxazole)]benzene, brought to 1 liter with toluene. To this solution was added 200 ml Beckman Bio-SolvTM solubilizer, Formula BBS-1. The tritium efficiency was determined by spiking with a ³H-toluene standard from New England Nuclear Corporation.

CHAPTER III

RESULTS

It has been demonstrated that gamma irradiation of crystalline lysozyme *in vacuo* produces phosphate soluble and phosphate insoluble components⁵⁸. The phosphate soluble material can be resolved into a number of components by column chromatography on Bio-Rex 70⁵⁸. These results have been confirmed in preliminary work in this study. Column chromatography of Pentex crystalline lysozyme on Bio-Rex 70 gives one major peak with a small secondary component preceeding the major peak. Both fractions contain enzyme activity. Figure 3 shows an experiment where 40 mg lysozyme was chromatographed on a column 1 x 40 cm. If a much larger column is used the secondary component can be resolved into a number of peaks. Each of these peaks shows enzyme activity and Stevens and Bergstrom⁶¹ have shown that each peak has approximately the same molecular weight as native lysozyme. Gamma irradiation of lysozyme produces several new chromatographic peaks, again the number that can be resolved depends in part on the column size. Figure 4 shows the elution pattern when the phosphate soluble fraction from irradiated lysozyme is chromatographed on a column 1 x 40 cm. Approximately 40% of the irradiated material is not soluble in the buffer system, thus the absorbance readings in Figure 4 are less than in Figure 3. Stevens and co-workers have found that part of this insoluble material contains disulfide cross-links⁵⁰.

FIGURE 3

Pentex lysozyme (40 mg) on a 1 x 40 cm column of Bio-Rex 70.

Solvent is 0.2 M potassium phosphate buffer, pH 7.09.

FIGURE 4

Phosphate soluble fraction of irradiated lysozyme on a column (1 x 40 cm) of Bio-Rex 70. Solvent is 0.2 M potassium phosphate buffer, pH 7.09.

The third peak shown in Figure 4 is the major component of the soluble fraction of irradiated lysozyme. In previous studies of irradiated lysozyme it has been considered undamaged protein. In this study it is called P3-I. The exact nature of this material is of considerable interest in understanding the mechanism of radiation inactivation of lysozyme. It is of importance to know if P3-I is actually undamaged native lysozyme or a single specie of damaged molecule or a mixture of molecules which have been damaged in a variety of different ways. In addition, if the lysozyme in P3-I is damaged it is of interest to determine what kind of changes have taken place in the molecules as a result of gamma irradiation.

To determine whether P3-I was a stable species and did not exist in equilibrium with the other irradiated chromatographic components, a small amount of P3-I was desalted on Bio-Gel P2 then rechromatographed on Bio-Rex 70. As is shown in Figure 5, P3-I eluted in approximately the same position as before and no other peaks are apparent. This irradiated enzyme, therefore, is a stable species and is chromatographically homogeneous on Bio-Rex 70.

Two differences between the native enzyme and P3-I can be observed by comparing Figures 3 and 4. The irradiated protein elutes slightly earlier than the native and the shapes of the peaks are different. The half width at half height is greater for P3-I (1.9 cm) than for the native protein (1.1 cm). A Bio-Rex 70 column was run with a mixed sample of Pentex lysozyme and P3-I. The elution pattern was very much like the native protein, with the peak having a half width at half height of 1.1 cm.

FIGURE 5

Irradiated fraction, P3-I, desalted on Bio-Gel P2 then re-chromatographed on Bio-Rex 70, using 0.2 M potassium phosphate buffer, pH 7.09.

The enzyme activity of P3-I has been measured many times using the method outlined in the experimental section. The activity of this enzyme is found to be 90-100% of the native enzyme. The variation of 10 percent is as good as is normally possible with the lysozyme assay employing *Micrococcus lysodeikticus* as a substrate. The assay with *Micrococcus lysodeikticus* is quite reproducible if duplicate lysozyme samples are assayed at the same time. However, if these same samples are reassayed at a later time the results will not necessarily check the earlier value, even if a new calibration curve is made with pure lysozyme. Reproducibility for duplicate samples is usually $\pm 2\%$ whereas the long-term reproducibility of a given material is not better than $\pm 10\%$. The reason for this variability is not known but is probably related to the heterogeneous nature of the *Micrococcus lysodeikticus* suspensions used in the assay. The conclusion from a large number of experiments is that although P3-I has considerable enzyme activity it is consistently lower than 100% of the native enzyme, and falls between 85 and 95%. A statistical analysis of the available data gives $94 \pm 8\%$.

Disc gel electrophoresis was performed on both native lysozyme and P3-I using 5, 7.5, and 10% cross-linked polyacrylamide gels. The results of these experiments are shown in Figure 6. It can be seen from these data that the only difference between the native protein and P3-I using this method is the band width. Disc gel electrophoresis experiments were also done as a function of loading and the band broadening phenomenon was still observed with P3-I. The band broadening, therefore, is not a function of either cross-linking or protein

FIGURE 6

Drawings of polyacrylamide gels resulting from electrophoresis at pH 4.5 using β -alanine-acetic acid buffer. Gels were stained with 0.25% Coomassie brilliant blue in 10% acetic acid then de-stained with 7% acetic acid.

NATIVE P3-I

5 % Gels

NATIVE P3-I

7.5% Gels

NATIVE P3-I

10% Gels

concentration. Separation on disc gel electrophoresis depends on charge and molecular sieving (size) effects. These disc gel electrophoresis experiments indicate that P3-I is probably not a homogeneous species and has either a distribution of molecular weights or a distribution of charges.

The optical rotatory dispersion spectra of native lysozyme and the irradiated protein are shown in Figure 7. Very little difference between these two enzymes can be detected by this method in this wavelength region. The solvent system used for these experiments, i.e. a citrate-phosphate buffer, absorbs strongly below 220 nm and spectra could not be run at shorter wavelengths. This is unfortunate since the absorption spectra of native lysozyme and P3-I show the most interesting differences below 220 nm. There is a slight difference between the native protein and P3-I in the visible region. This can be seen more easily in Figure 8 where the scale has been expanded.

The ultraviolet absorption spectra of lysozyme and the irradiated species P3-I have been measured in different solvents, at various pH values, and at different temperatures. Figure 9 shows the absorption spectra of the two proteins in citrate-phosphate buffer, pH 6.0. It can be seen that the same phenomenon which prevented taking the ORD spectra below 220 nm is again present here, i.e. the solvent absorbs strongly below this wavelength. A buffer composed of M/15 disodium phosphate and M/15 sodium phosphate was also tried but in this case one can only go to 215 nm without solvent absorption interfering. In citrate-phosphate buffer, however, absorption by

FIGURE 7

Optical rotatory dispersion spectra of lysozyme (——), and the irradiated material (- - - -) in 0.06 M citrate - 0.12 M disodium phosphate buffer, pH 6.0.

FIGURE 8

Upper portion, 360-600 nm, of the optical rotatory dispersion spectra of lysozyme (—) and the irradiated material (- - -) in citrate-phosphate buffer.

FIGURE 9

Absorption spectra of native lysozyme, ○, and the irradiated protein, ○, in 0.06 M citrate - 0.12 M disodium phosphate buffer, pH 6.0.

P3-I is increased in the 280 maximum and the 290 nm shoulder. The irradiated protein also shows an increased absorption in the trough at 250 nm but the minimum is not shifted. These spectra were repeated using distilled water as a solvent. Figure 10 shows the range 240-360 nm; the irradiated protein has an increased absorbance at the 280 peak and 290 shoulder. The P3-I also shows an increased absorbance above 300 nm which has been described in denaturation difference spectra of proteins⁶⁵. The minimum at 250 nm remains at the same wavelength but there is an increase in ϵ_{MOLAR} at this wavelength. The spectra below 240 nm are in Figure 11. This portion of the curve is particularly interesting since the absorption band of the irradiated material shows a small blue-shift from 190 to 188 nm, and an increase in ϵ_{MOLAR} at this wavelength. In fact, the increase in the 190 nm band is probably the most interesting aspect of the spectrum since this band has been assigned to the secondary structure of the protein⁶⁶.

Since the irradiated material shows this possible difference in secondary structure it is of interest to see if perturbation of this protein in some manner would allow it to reconform to a structure more like the native protein. If radiation provides enough energy to change the conformation of P3-I so it is different than the native conformation then upon denaturation and renaturation the protein should return to the most favorable thermodynamic state which is the native conformation. To test this theory the irradiated protein was subjected to heat and conditions of alkaline pH. At the same time the native protein was subjected to the same conditions as a control. The absorption spectra of lysozyme and P3-I have been measured at

FIGURE 10

Absorption spectra, 240-360 nm, of native lysozyme, ○, and the irradiated protein, ○, in water.

FIGURE 11

Absorption spectra, 180-250 nm, of native lysozyme, ○, and the irradiated protein, ○, in water.

80°, which is above the "melting point", and again after cooling to 20°. These data are shown in Figures 12, 13, 14, and 15 along with the data at 20° before denaturation. In Figure 12 it is evident that heating and cooling native lysozyme has an effect on all features of the spectrum above 240 nm. Above 300 nm the curve is increased at 80° as would be expected if denaturation is taking place. After cooling back to 20° this portion of the curve is increased even more. Both the 280 peak and the 290 shoulder are increased by heating and further increased by cooling, indicating that the heat denaturation of lysozyme is not completely reversible. After heating and cooling the tyrosine and tryptophan groups are more exposed to the solvent than before. The 250 trough absorbance is increased by heating and further increased by cooling back to 20°, and at the same time the minimum is shifted slightly from 250 to 252 nm. These data for the native lysozyme show different trends than the data for P3-I. In Figure 13 it can be seen that the irradiated protein shows a decrease in the curve above 300 nm at 80° then an increase upon cooling back to 20°. Both the 280 peak and the 290 shoulder show a decrease upon heating to 80° then both portions of the curve increase after cooling to a value between "native" P3-I and denatured P3-I. The 250 trough shows the same shift to 252 upon denaturation and renaturation but the size of the trough does not show the same trend as native lysozyme. Rather, the trough absorbance is decreased upon heating, then increased upon cooling back to 20°. The absorption spectra below 240 nm for native lysozyme and P3-I are shown in Figures 14 and 15 respectively. The 210-230 shoulder of the native protein is increased slightly by

FIGURE 12

Absorption spectra, 240-360 nm, of lysozyme in water at different temperatures: ○, 20°; □, 80°; ●, upon cooling to 20° after having been heated to 80°.

FIGURE 13

Absorption spectra, 240-360 nm, of the irradiated protein in water at different temperatures: ○, 20°; □, 80°; ⊙, upon cooling to 20° after having been heated to 80°.

FIGURE 14

Absorption spectra, 180-250 nm, of lysozyme in water at different temperatures: Δ , 20°; $\circ$, 80°; $\odot$, upon cooling to 20° after having been heated to 80°.

FIGURE 15

Absorption spectra, 180-250 nm, of the irradiated protein in water at different temperatures: $\circ$, 20°; Δ , 80°; $\odot$, upon cooling to 20° after having been heated to 80°.

heating but then returns to approximately the same position upon cooling. At 190 nm ϵ_{MOLAR} increases by 1.5×10^5 upon heating to 80°. Upon cooling this parameter falls back to a value intermediate between native and denatured lysozyme. In contrast, the irradiated protein shows a decrease in the 210-230 shoulder upon heating, and after cooling to 20° this portion of the curve remains the same as it was at 80°. At 190 nm ϵ_{MOLAR} decreases upon heating to 80° and is further decreased upon cooling back to 20°. These experiments suggest that the irradiated enzyme is in some metastable thermodynamic state and heating the molecule unfolds it. When it reconforms on cooling it goes to a conformation more like the native one, although not exactly the same.

Examination of the enzyme activity of native lysozyme and P3-I after heat denaturation indicates that the irradiated material is more resistant to heat denaturation than the native protein. The native protein following denaturation by heat has an activity of 77% of that of native lysozyme while the irradiated protein shows 89% activity. So heat denaturation causes a loss of 23% activity for the native protein and a loss of only about 6% for the irradiated material.

The alkaline pH spectral data show the same trends as the heat data. Figure 16 shows the absorption spectra of lysozyme below 240 nm at different pH values. As the pH is raised the 190 band increases although there is no blue shift. The pH 8 data show a small red shift at the 190 maximum which may only be an anomaly. The 210-230 shoulder increases up to pH 9.3 but shows a decrease

FIGURE 16

Absorption spectra, 180-250 nm, of lysozyme at various pH values. Solutions of lysozyme in water are adjusted to the desired pH with 0.06% NaOH: ○, pH 6.0; ○, pH 8.5; □, pH 9.3; △, pH 10.1.

when the pH is raised to 10. The irradiated protein (Figure 17) shows the opposite trend, the pH 6 solution has the highest ϵ_{MOLAR} at 190 nm. Upon raising the pH to 8 ϵ_{MOLAR} drops by about 2.5×10^5 then shows an increase as the pH is raised to 9 and a further increase upon raising the pH to 10. Again, the 210-230 shoulder shows the same trend with the "native" P3-I having the highest value, pH 8 P3-I having the lowest and the pH 9 and 10 solutions falling in between. These spectra are interesting since they indicate that alkaline pH denaturation of the irradiated protein brings its structure closer to that of native lysozyme. The portions of the pH perturbation curves above 240 nm are shown in Figures 18 and 19 for the native and irradiated proteins respectively. The 250 trough of the native protein shows the same trend upon denaturation by alkaline pH as was shown by heat denaturation. That is, as the pH is raised the absorbance at 250 nm increases. The 280 peak is almost the same for pH's 6, 9, and 10 but is increased slightly at pH 8. The irradiated protein on the other hand shows a distinct difference in absorbance at 290 and 280 for the different pH solutions. The pH 8 solution shows a decreased absorbance at 250, 280, 290, and above 300 nm. The pH 9 solution has a slightly higher absorbance above 300, about the same at 290, and less at 280. The curves cross at 265 and the absorbance at the 250 trough is greater for P3-I at pH 9 than at pH 6. At pH 10 the irradiated protein shows a higher absorbance than "native" P3-I in all portions of the spectrum.

The enzyme activities of solutions of native lysozyme and P3-I were measured after alkaline pH treatment. The solutions were

FIGURE 17

Absorption spectra, 180-250 nm, of the irradiated protein at various pH values. Solutions of P3-I in water are adjusted to the desired pH with 0.06% NaOH: ○, pH 6.0; ●, pH 8.3; □, pH 9.2; △, pH 10.0.

FIGURE 18

Absorption spectra, 240-360 nm, of lysozyme at various pH values. The same solutions as Figure 16 are used: ○, pH 6.0; ●, pH 8.5; △, pH 9.3; ▲, pH 10.1.

FIGURE 19

Absorption spectra, 240-360 nm, of the irradiated protein at various pH values. The same solutions as Figure 17 are used:
○, pH 6.0; ○, pH 8.3; □, pH 9.2; △, pH 10.0.

allowed to stand at alkaline pH for two hours then the pH was lowered to 6 and the activity measured. The results showed that a slight activation was present in the solutions over pH 8 both for native and irradiated lysozyme. The activity of the native protein is increased to 105% while that of the irradiated material is increased to 100%.

The heat denaturation curves of native lysozyme and P3-I are in Figure 20. It can be seen that native lysozyme shows a much smoother transition than the irradiated material. In addition, the irradiated material denatures at a lower temperature, indicating less conformational integrity. The native protein shows four transitions, the midpoints of which are at 31°, 37°, 57°, and 74°. The irradiated protein shows more than four transitions. The midpoint of only one of these coincides with the native protein. The second denaturation (Figure 21) shows fewer transitions for P3-I than before, however, the two materials are not the same by any means.

The sedimentation coefficient of lysozyme was calculated and found to be $s_{20,w} = 1.78$ S at 0.15 mg/ml protein concentration. At the same concentration the sedimentation coefficient of the irradiated protein was $s_{20,w} = 1.84$ S.

The entire phosphate soluble fraction of the irradiated protein was reacted with tritiated hydrogen sulfide to determine the carbon free radical content of each of the resulting chromatographic fractions.

Hydrogen sulfide is an excellent radical scavenger in irradiated proteins since in the gas phase it is known to have a high rate of reaction with alkyl radicals. In addition, sulfhydryl radicals will

FIGURE 20

Heat denaturation curves of lysozyme ○, and the irradiated protein, ●, in 0.06 M citrate-0.12 M disodium phosphate buffer, pH 6.0.

FIGURE 21

Second heat denaturation curves of lysozyme, ○, and the irradiated protein, ○, in 0.06 M citrate-0.12 M disodium phosphate buffer, pH 6.0.

not abstract hydrogen from primary, secondary, and tertiary carbon-hydrogen bonds of hydrocarbons. The tritium provides a convenient method for determining the location of radicals.

Column chromatography of native and irradiated lysozyme after reaction with HTS are shown in Figures 22 and 23 respectively. The bars over the peaks indicate where the divisions were made for pooling. The dose of β -irradiation was calculated and is 2.4×10^4 rads, just 0.1% of the gamma dose. Table I gives the specific information about the enzyme activity and specific activity of each of the pooled peaks. There are several interesting things to note about these data. First, in the control experiment all the peaks are labeled but to a lesser extent than for the irradiated protein. In addition, all the peaks in each case are labeled to approximately the same extent. The shoulder on the main peak in both Figure 22 and 23 appears consistently after reaction with HTS which leads us to believe that it is probably significant. This cannot be determined by the enzyme activity alone since there is only a few percent difference and the error in the enzyme activity is greater than this. One should also note that the activity of the main peak and its shoulder are less than what is found for P3-I, whereas the activity of the native protein after treatment with HTS is still 100%.

FIGURE 22

Elution pattern from column of Bio-Rex 70, of native lysozyme after reaction with HTS; Absorbance, (—); counts per minute, (- - - -). Solvent is 0.2 M potassium phosphate buffer, pH 7.09.

FIGURE 23

Elution pattern from column of Bio-Rex 70, of phosphate-soluble irradiated lysozyme after reaction with HTS:

Absorbance, (—); counts per minute, (- - - -). Solvent is 0.2 M potassium phosphate buffer, pH 7.09.

TABLE I

Specific Activities and Enzyme Activities of Pooled Peaks from
Reaction of Lysozyme and Irradiated Lysozyme with HTS

	P1	P2	P3	P4	P5	P6
Lysozyme-HTS						
Enzyme Activity (%)	0.0	2.1	1.8	3.5	100	106
Specific Activity (mc/mml)	3.0	5.8	2.0	2.6	2.2	2.3
Irradiated Lysozyme-HTS						
Enzyme Activity (%)	0.0	3	19	18.5	71.5	79.4
Specific Activity (mc/mml)	5.3	7.7	7.4	7.2	7.4	7.0

CHAPTER IV

DISCUSSION

A knowledge of the exact chemical and physical nature of P3-I is important in order to determine the mechanism of radiation damage to enzymes. This protein fraction shows considerable homogeneity on Bio-Rex 70 chromatography. The net charge on these molecules must be very close to that on native lysozyme. When P3-I is co-chromatographed with the native protein one peak is eluted which indicates that these two materials are not separable as to total cationic charge. The half width at half height of the P3-I peak is larger which may indicate some charge distribution in the P3-I molecules.

The disc gel electrophoresis experiments presented here for the irradiated material P3-I indicate a small distribution of either charges or molecular weights. The chromatography experiments with the mixed column of P3-I and native lysozyme on Bio-Rex 70 indicate that there is no difference in total cationic charge. In addition, it is probable that if the P3-I molecule had a substantially different net charge it would not chromatograph so close to the native protein. Stevens⁶⁷ has done the amino acid analysis of the chromatographic peak in question. The data are shown in Table II, along with the values for chromatographically pure lysozyme. In addition, the percent difference between these two values has been calculated and

TABLE II

Amino Acid Content of a Fraction from Lysozyme Receiving 30 Mrads⁶⁷
 (Residues Amino Acid per Mole Weight of Lysozyme¹)

Amino Acid	Chromatographically Pure Lysozyme	P-5 ²	Difference (%)
Lys	6.2	5.7	- 8
Hist	0.9	0.9	0
Ammonia	18.2	14.2	- 22
Arg	10.4	8.9	- 14
Asp	20.3	19.5	- 4
Thre	5.0	5.5	+ 10
Ser	8.9	7.9	- 11
Glu	4.9	4.4	- 8
Pro	1.6	1.4	- 13
Gly	11.2	9.1	- 19
Ala	11.1	10.5	- 5
Cys/2	2.6	2.1	- 19
Val	4.3	3.8	- 12
Meth	2.0	1.8	- 10
Isoleu	4.8	4.2	- 13
Leu	7.2	6.6	- 8
Tyr	2.8	2.6	- 7
Phe	2.7	2.4	- 11
Try	5.0	5.4	+ 8

¹Mole weight of lysozyme is taken as 14,5000

²P-5 of Stevens comparable with present P3-I. These data however are for 30 Mrads, whereas P3-I results from 22.6 Mrads.

included in the table. These data are undoubtedly in error since the analytical method of amino acid analysis contains considerable sources of error. The percent differences are extremely high and when the G values are calculated from the amino acid analysis data and compared to those of Dosé⁴⁶ the G values are concomitantly high. The difficulty lies in working with 30 Mrads where the differences in amino acid composition between the damaged and native protein are very small. The data of Stevens, however, are very useful for their qualitative value. One should note that although there is some selective destruction of amino acids, all the amino acids in lysozyme are affected with the exception of histidine. This agrees quite well with the G values of the amino acids in lysozyme reported by Dosé⁴⁶. However, Dosé does find histidine damage. It is unlikely that all these changes can be present in each molecule or this molecule would not show 95% enzyme activity. In addition, it is likely that these changes are widely distributed among all the molecules in P3-I or this material would not be chromatographically homogeneous and like the native protein. More likely, this chromatographic peak is not a homogeneous material and consists of molecules which have been damaged in various ways, and no one molecule has suffered extensive damage. Since Stevens' data show that several of the charged amino acids have been affected, it is not possible to conclude whether the disc gel electrophoresis experiments represent a distribution of molecules with different charges or different molecular weights.

The results of the reaction of the phosphate soluble portion of

irradiated lysozyme with HTS give information about the chemical changes which are present in these molecules. These results are of particular interest since they show that the proteins resulting from gamma irradiation all have about the same level of radical formation but widely different enzyme activities. In particular, the protein which is the major component and has been studied extensively here, has about the same degree of radical formation as the other irradiated species. This means that this protein has the same amount of chemical damage yet retains near normal enzyme activity. These results are in direct contradiction to the classical target theory where a "hit" constitutes an inactivation.

Several methods are available to study the secondary and tertiary structure of proteins. One of the most sensitive of these is optical rotatory dispersion. The present results show almost no difference in ORD between the native and irradiated proteins down to 220 nm. In view of the other optical changes noted below this is an extremely surprising result. In order to calculate B_0 and compare the α -helical content of these two proteins data below 220 nm are required. These data could not be obtained in the solvent system employed.

Ultraviolet spectroscopy is also an important tool of the protein chemist. Conformational changes in the protein can involve environmental changes of its chromophoric groups which result in alterations of the absorption spectrum. In the region 250-310 nm the ultraviolet absorption spectrum of a protein contains contributions from phenylalanine, tyrosine, and tryptophan residues. At

shorter wavelengths, from about 210-250 nm, the spectrum shows contributions from other groups such as the histidine residues and the peptide bonds. Below 210 nm one can study the helix to coil transition upon denaturation of the protein. The present work shows a variety of optical changes in all of the regions of interest for the irradiated protein P3-I. These changes can be attributed to various effects on specific chromophores, such as charge effects, medium effects, and hydrogen bonding effects.

When one compares the spectrum of native lysozyme with that of the irradiated protein it can be seen that the latter shows increased intensity in all portions of the spectrum. With the exception of the 190 nm band there is no change in position of any band. This effect is consistent with a model in which most parts of a molecule are very similar to native lysozyme but in which the changes that do occur are quite varied, since specific changes in intensity in a pure substance are usually accompanied by displacement of the maximum.

The 190 nm band increase and blue shift can be assigned to a helix to coil transition. This has been noted in synthetic polypeptides⁶⁸ and in proteins⁶⁶. A difference peak centering around 230 nm upon protein denaturation has been assigned by Glazer and Smith⁶⁹ to a change in environment of the peptide backbone. This region of the spectrum is due to an $n-\pi^*$ transition whereas the 190 nm band is due to a $\pi-\pi^*$ transition. Such a change in the peptide backbone environment is probably present in the irradiated protein since there is an increase in the calculated difference spectrum, $\epsilon_{P3-I} - \epsilon_{Native}$, at 230 nm.

The difference spectrum, calculated from $\epsilon_{P3-I} - \epsilon_{Native}$, is essentially zero above 340 nm, and positive below this wavelength. This difference spectrum is well-defined, significant, and clearly indicates a real conformation difference between P3-I and native lysozyme. It is useful to compare this difference spectrum with that obtained on denaturation of proteins. The latter normally have some negative components. Donovan and collaborators⁷⁰ have measured the difference spectrum of lysozyme and each of the aromatic chromophores upon pH perturbation. The lysozyme difference spectrum is positive from 295-320 nm and negative from 245-295. These workers attribute the lysozyme difference spectrum entirely to the indole chromophore, since they do not observe peaks at 280 and 287 nm characteristic of the phenolic chromophore, nor the peaks near 260 nm characteristic of the benzene chromophore. In the calculated difference spectrum, $\epsilon_{P3-I} - \epsilon_{Native}$ one can observe a shoulder at 290 nm which could be attributed to the indole chromophore, a broad peak centered at 278 nm which could be attributed to the phenolic chromophore, and peaks at 255 and 245 nm which could be attributed to the benzene chromophore. Thus pH perturbation and radiation perturbation do not produce comparable effects. It is likely that these observations in the difference spectrum are due to effects of surrounding groups on the chromophores and not destruction of the chromophores by ionizing radiation. If the latter were the case then greater changes in the original spectrum of P3-I should be present. It is also unlikely that radiation changes of chromophores would increase the absorbance of this protein at these specific wavelengths, as was

observed.

Since the near UV absorbance is extremely sensitive to charge effects, both from side groups of the protein and the medium, it is postulated that the irradiated protein P3-I has an altered charge environment in the region of the chromophores which affects the indole, phenolic, and benzene chromophores. In particular, changes in charge on the protein molecule are implicated by the fact that the irradiated protein at pH 8 has an absorption spectrum most like native lysozyme at pH 6.

One can conceive that conformational damage might be introduced into a protein by ionizing radiation in three ways. First, conformational changes could be a direct result of chemical changes induced by ionizing radiation. Second, in the transition state of a chemical change conformational changes could be induced and then trapped in the molecule. Third, conformational changes could be the result of a physical phenomenon caused by ionizing radiation, with no chemical intermediate.

Both the heat and alkaline pH perturbation spectra of the irradiated protein, when compared to those of native lysozyme, show that part of the conformational damage to this irradiated molecule can be "corrected" by denaturation and renaturation. From this result one can conclude that at least part of the conformational damage existing in P3-I is not a direct result of chemical changes. These results, however, cannot distinguish between conformational changes which take place while the enzyme is in a transition state, and those which are caused directly by ionizing radiation.

The heat denaturation curves also confirm that native lysozyme and P3-I are not the same. These curves show that the irradiated protein denatures at lower temperatures than native lysozyme, indicating loss of conformational integrity. These denatured materials were cooled and then heat denatured again. The curves resulting from the second denaturation indicate that the irradiated protein must have undergone changes which make this protein more like the native one. However, the P3-I still denatures at lower temperatures and retains many of the breaks in the curve observed in the first denaturation.

The heat denaturation results, along with the UV absorption spectra after denaturation by heat and alkaline pH, indicate that there are two levels of conformational damage to P3-I. There is conformational damage which is not reversible and presumed caused by chemical changes, and there is that which is reversible and presumed not caused by chemical effects. The latter result is rather surprising when one considers that these changes take place in the solid state and are not corrected by dissolution in water or another solvent. One can conceive of two possible mechanisms for the reversible conformational damage. One of these has been proposed by Norman^{51,52} for particulate radiation but is not usually proposed for gamma radiation. That is the thermal spike model discussed previously. One can conceive that a certain volume of the protein during irradiation experiences a rise in temperature sufficient to give atomic mobility within this solid molecule. Conformational changes which remain after irradiation is completed take place in

the "hot spot". Denaturation conditions of high temperatures or alkaline pH in the case of lysozyme are then needed to reverse these conformational changes. It is likely that this theory could be adapted to include the temperature dependence of inactivation.

Another mechanism that could explain conformational damage which is not a result of chemical damage is the formation of charged groups by gamma radiation in hydrophobic centers of the molecule. This would presumably result in rearrangement of the hydrophobic groups to accommodate the charge. If the charge was then neutralized the hydrophobic groups would not necessarily return to the original arrangement, however, this could be accomplished by denaturation and subsequent renaturation.

The sedimentation velocity measurements show a small increase in $s_{20,w}$ for the irradiated protein over native lysozyme. From the direction of the band broadening of the disc gel electrophoresis experiments one can conclude that the irradiated protein does not show any increase in molecular weight, but may show a slight decrease. These two results together indicate that on the average the irradiated protein has a distinctly different shape than native lysozyme. This shape change indicated by the increase in $s_{20,w}$ is in agreement with the trends which were observed in the UV spectrum of the irradiated protein. In particular, the helix to coil transition indicated by the increase in absorbance at 190 nm, would coincide with a shape change in the molecule. This particular change would presumably result in a less compact molecule, which is also indicated by the sedimentation velocity measurements.

The enzyme activity of P3-I is 95% of native lysozyme. It is not possible to state, at this point, whether this represents one in twenty molecules which have lost all activity, a mixture of molecules which have each lost one twentieth of their activity, or some mixture of these two extremes. In most molecules the tertiary structure of the irradiated protein, at least around the active site, must be much the same as native lysozyme since the enzyme activity is only changed by 5%. The specific amino acids which are necessary for enzyme activity must all be present, undamaged, and in the correct position or the activity would be more affected. The amino acids which are necessary for any activity include two tryptophan residues, one aspartic acid residue, and one glutamic acid residue. In addition, there are several amino acid residues in lysozyme which have some effect on the enzyme activity but their absence does not completely destroy activity as with the amino acids mentioned previously. These include two additional tryptophan residues, two methionine residues, and three of the four disulfide bridges. Since the enzyme activity of the irradiated protein is only lowered by 5% it is certain that none of the first group of residues cited is damaged, at least in the majority of molecules. It is probable that few, if any, in the second group are damaged. It seems likely that loss of activity is due to slight conformation changes of the binding site and, therefore, lowered activity. The binding of a trisaccharide substrate by lysozyme has been shown to involve six hydrogen bonds and numerous van der Waals contacts between the enzyme and the trisaccharide. Both of these forces involve specific geometry between enzyme and substrate, which could be affected.

ted by even a very small conformational change. This conformational change could be caused by gamma radiation. However, gamma radiation could also cause loss of the hydrogen bonding group on the protein, which would have the same effect on substrate binding, that is, make it less efficient, which would result in some loss of activity.

The results presented here confirm that the present theories of radiation inactivation are inadequate to explain the mechanism of radiation damage to proteins. These results show that with gamma irradiation of lysozyme the protein formed which has near normal enzyme activity has suffered both chemical and conformational damage. It is quite doubtful if any molecules are really undamaged. These results also show that the conformational damage is not totally due to chemical changes but in part is due directly to the ionizing radiation. However, the present results cannot distinguish between two mechanisms of direct conformational damage. In addition, the present results show that the different protein species resulting from gamma irradiation all suffer about the same degree of chemical damage and there is no correlation between chemical damage and loss of enzyme activity. Rather, it must be a random process where a given number of chemical changes take place with a given dose, and in some cases these changes are not critical to the biological activity of the molecule.

As was stated previously, several theories have been developed to explain the data on inactivation of enzymes by ionizing radiation. The classical target theory as formulated by Lea³⁰ states that an ionization releases a localized amount of energy in a molecule which

disrupts a limited region and inactivates a given molecule. In this theory a "hit" results in inactivation, although it is possible for a particle or photon to pass through a molecule without resulting in a "hit". The data presented here for the reaction of irradiated lysozyme with HTS contradict the classical target theory in the following manner. In these experiments the specific activity of tritium in each chromatographic peak is almost the same, although the enzyme activities vary from 0 to 80%. These experiments indicate the formation of carbon free radicals in molecules which are not completely inactivated. Radical formation is proportional to chemical damage, however, radical formation is not proportional to enzyme inactivation. From these results one can conclude that enzyme inactivation is not proportional to radiation damage and therefore, not proportional to "hits".

Previous work by Stevens and collaborators⁵⁹ showed that the hard-to-exchange amide hydrogens of lysozyme are lost two to three times as fast as the enzyme activity. These workers concluded that the integrity of the entire lysozyme molecule is not necessary for enzyme activity. The present work supports this conclusion. Classical target theory requires that the entire biological macromolecule is necessary for activity. It is believed that since the target theory does not include the possibility that the entire molecule is not necessary for activity it must be modified to do so. In addition, as stated previously, this theory has to be modified to include temperature dependence of inactivation. Further, since evidence exists that excitations are important, a theory of radiation

inactivation cannot exclude these.

The present work shows both conformational and chemical damage resulting from ionizing radiation in the irradiated lysozyme fraction retaining the most enzyme activity. It seems likely that there must be both chemical and conformational alterations in the more severely damaged molecules. Therefore, any theory for the secondary effects of radiation must include mechanisms for both chemical and conformational alterations which are not necessarily concomitant.

It is proposed that there are two types of effects of ionizing radiation on enzymes. One of these effects is a direct effect on the conformation of the molecules. These effects can take place either by the formation of a "hot spot" where the temperature is increased sufficiently to cause a conformational change in the solid state. Or, these effects can take place by the formation of a charge in the vicinity of a hydrophobic center which results in the rearrangement of the hydrophobic groups to accommodate this charge. The other type of effect of ionizing radiation on enzymes is a chemical one which results in concomitant conformational changes. It is proposed that each irradiated molecule has comparable numbers of chemical changes, however, the lethality of these changes is not the same. It has been proposed by Marciani, Herbert and Tolbert⁷¹ that inactivation of the enzyme results from independent cumulative inactivation of amino acid residues. For the present irradiated protein, P3-I, the amino acid residues which are inactivated are not necessary for enzyme activity.

CHAPTER V
BIBLIOGRAPHY

1. Fleming, A., *Proc. Roy. Soc. (London)*, Ser. B. 93, 306 (1922).
2. Jollès, P., *Angew. Chem. internat. Edit.*, 3, 28 (1964).
3. Canfield, R.E., *J. Biol. Chem.*, 238, 2698 (1963).
4. Sophianopoulos, A.J., Rhodes, C.K., Holcomb, D.N., and van Holde, K.E., *J. Biol. Chem.*, 237, 1107 (1962).
5. Jollès, J., Jauregui-Adell, J., Bernier, I., and Jollès, P., *Biochim. Biophys. Acta*, 78, 668 (1963).
6. Canfield, R.E., and Anfinsen, C.B., *J. Biol. Chem.*, 238, 2684 (1963).
7. Canfield, R.E., *J. Biol. Chem.*, 238, 2691 (1963).
8. Canfield, R.E., and Liu, A.K., *J. Biol. Chem.*, 240, 1997 (1965).
9. Brown, J.R., *Biochem. J.*, 92, 13P (1964).
10. Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Phillips, D.C., and Sarma, V.R., *Nature*, 206, 757 (1965).
11. Urnes, P., and Doty, P., in C.B. Anfinsen, Jr., M.L. Anson, Kenneth Bailey, and John T. Edsall (Editors), *Advances in protein chemistry*, vol. 16, Academic Press, New York, 1961, p. 401.
12. Blout, E.R., de Lozé, C., and Asadourian, A., *J. Am. Chem. Soc.*, 83, 1895 (1961).
13. Phillips, D.C., *Proc. Nat. Acad. Sci.*, 57, 484 (1967).
14. Sternlicht, H., and Wilson, D., *Biochem.*, 6, 2881 (1967).
15. Tanford, C., and Wagner, M.L., *J. Am. Chem. Soc.*, 76, 3331 (1954).
16. Donovan, J.W., Laskowski, M., Jr., and Scheraga, H.A., *J. Am. Chem. Soc.*, 82, 2154 (1960).
17. Steiner, R.F., *Biochim. Biophys. Acta*, 79, 51 (1964).

18. Hayashi, K., Imoto, T., Funatsu, G., and Funatsu, M., *J. Biochem.*, 58, 227 (1965).
19. Hamaguchi, K., Hayashi, K., Imoto, T., and Funatsu, M., *J. Biochem.*, 55, 24 (1964).
20. Hamaguchi, K., and Sakai, H., *J. Biochem.*, 57, 721 (1965).
21. Foss, J.G., *Biochim. Biophys. Acta*, 43, 300 (1960).
22. Salton, M.R.J., *Nature*, 170, 746 (1952).
23. Johnson, L.N., and Phillips, D.C., *Nature*, 206, 761 (1965).
24. Chipman, D.M., and Sharon, N., *Science*, 165, 454 (1969).
25. Rupley, J.A., and Gates, V., *Proc. Nat. Acad. Sci.*, 57, 496 (1967).
26. Rupley, J.A., Gates, V., and Bilbrey, R., *J. Am. Chem. Soc.*, 90, 5623 (1968).
27. Dahlquist, F.W., and Raftery, M.A., *Biochem.*, 7, 3277 (1968).
28. Lin, T., and Koshland, D.E., Jr., *J. Biol. Chem.*, 244, 505 (1969).
29. Parsons, S.M., and Raftery, M.A., *Biochem.*, 8, 4199 (1969).
30. Lea, D.E., *Actions of radiations on living cells*, Cambridge Univ. Press, Cambridge, 1962, p. 69.
31. Pollard, E.C., Guild, W.R., Hutchinson, F., and Setlow, R.B., in J.A.V. Butler and J.T. Randall (Editors), *Progress in biophysics and biophysical chemistry*, vol. 5, Pergamon Press, London, 1955, p. 80.
32. Augenstein, L.G., Brustad, T., and Mason, R., in Leroy Augenstein, Ronald Mason, and Henry Ouastler (Editors), *Advances in radiation biology*, vol. 1, Academic Press, New York, 1964, p. 227.
33. Brustad, T., *Radiation Res.*, Supp. 2, 65 (1960).
34. Fluke, D.J., *Radiation Res.*, 28, 677 (1963).
35. Hutchinson, F., *Radiation Res.*, Supp. 2, 49 (1960).
36. Hutchinson, F., and Pollard, E., in Maurice Errera and Arne Forssberg (Editors), *Mechanisms in radiobiology*, vol. 1, Academic Press, New York, 1961, p. 71.

37. Henriksen, T., Sanner, T., and Pihl, A., *Radiation Res.*, 18, 147 (1963).
38. Stratton, K., *Radiation Res.*, Supp. 7, 102 (1967).
39. Riesz, P., White, F.H., Jr., and Kon, H., *J. Am. Chem. Soc.*, 88, 872 (1966).
40. White, F.H., Jr., Riesz, P., and Kon, H., *Radiation Res.*, 32, 744 (1967).
41. Riesz, P., and White, F.H., Jr., *Nature*, 216, 1208 (1967).
42. White, F.H., Jr., *Radiation Res.*, 36, 470 (1968).
43. Brustad, T., Steen, H.B., and Dyrset, J., *Radiation Res.*, 27, 217 (1966).
44. Garrison, W.M., Jayko, M.E., and Bennett, W., *Radiation Res.*, 16, 483 (1962).
45. Mee, L.K., *Radiation Res.*, 21, 501 (1964).
46. Dosé, K., Risi, S., and Rauchfuss, H., *Biophysik*, 3, 202 (1966).
47. Drake, M.P., Giffey, J.W., Johnson, D.A., and Koenig, V.L., *J. Am. Chem. Soc.*, 79, 1395 (1957).
48. Shimazu, F., and Tappel, A.L., *Radiation Res.*, 23, 203 (1964).
49. Tolbert, B.M., Stansfield, R., and Krinks, M.H., in J. Sirchis (Editor), *Proceedings of the conference on methods of preparing and storing marked molecules*, Euratom, Brussels, 1964, p. 575.
50. Stevens, C.O., Sauberlich, H.E., and Bergstrom, G.R., *J. Biol. Chem.*, 242, 1821 (1967).
51. Norman, A., *Radiation Res.*, 16, 599 (1962).
52. Norman, A., *Radiation Res.*, Supp. 7, 33 (1967).
53. Tolbert, B.M., A.E.C. Annual Report, Contract No. AT(11-1) 600, Section VI, 1967, p. 1.
54. Platzman, R.L., and Franck, J., in H.P. Yockey, R.L. Platzman, and H. Quastler (Editors), *Symposium on information theory in biology*, Pergamon Press, New York, 1958, p. 262.
55. Jayko, M.E., and Garrison, W.M., *Nature*, 181, 413 (1958).

56. Alexander, P., Hamilton, L.D.G., and Stacey, K.A., *Radiation Res.*, 12, 510 (1960).
57. Shore, J.D., Tolbert, B.M., and Sauberlich, H.E., *Arch. Biochem. Biophys.*, 102, 125 (1963).
58. Stevens, C.O., Tolbert, B.M., and Reese, F.E., *Arch. Biochem. Biophys.*, 102, 423 (1963).
59. Stevens, C.O., Henderson, L.E., and Tolbert, B.M., *Arch. Biochem. Biophys.*, 107, 367 (1964).
60. Maksimov, V.I., Osipov, V.I., and Kaverzneva, E.D., *Biokhimiya*, 33, 451 (1968).
61. Stevens, C.O., and Bergstrom, G.R., *Proc. Soc. Exptl. Bio. Med.*, 124, 187 (1967).
62. Fricke, H., and Hart, E.J., in Frank H. Attix and William C. Roesch (Editors), *Radiation dosimetry*, vol. 2, Academic Press, New York, 1966, p. 191.
63. Reisfeld, R.A., Lewis, U.J., and Williams, D.E., *Nature*, 195, 281 (1962).
64. Bailey, J.L., *Techniques in protein chemistry*, Elsevier Publishing Co., Amsterdam, 1967, p. 341.
65. Foss, J.G., *Biochim. Biophys. Acta*, 47, 569 (1961).
66. Wetlaufer, D.B., in C.B. Anfinsen, Jr., M.L. Anson, Kenneth Bailey and John T. Edsall (Editors), *Advances in protein chemistry*, vol. 17, Academic Press, New York, 1962, p. 328.
67. Tolbert, B.M., in Wallace Snipes (Editor) *Electron spin resonance and the effects of radiation on biological systems*, Report No. 43, National Academy of Sciences, National Research Council, Washington, D. C., 1966, p. 107.
68. Imahori, K., and Tanaka, J., *J. Mol. Biol.*, 1, 359 (1959).
69. Glazer, A.N., and Smith, E.L., *J. Biol. Chem.*, 235 PC43 (1960).
70. Donovan, J.W., Laskowski, M., Jr., and Scheraga, H.A., *J. Am. Chem. Soc.*, 83, 2686 (1961).
71. Marciani, D.W., Herbert, S.M., and Tolbert, B.M., *Radiation Res.*, 39, 510 (1969).