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MASTER

FRACTIONATION AND ANALYSIS OF
GAMMA IRRADIATED LYSOZYME

by

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B.S., Universidad Nacional Mayor de San Marcos de Lima, 1962

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

1970

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Fractionation and Analysis of Gamma Irradiated Lysozyme

Thesis directed by Professor Bert M. Tolbert

The chemical and conformational properties of whole and fractionated lysozyme irradiated in the dry solid state by Cesium-137 gamma rays have been studied by a variety of procedures. The results have been used to extend our understanding of the basic mechanisms by which enzymes are inactivated by radiation.

Irradiated lysozyme (26 Mrad) was fractionated by salting out with 0.1 N acetic acid - 1 M NaCl. The salt insoluble fraction, F-I, accounts for 39% of the irradiated lysozyme, and the salt soluble fraction, F-II, the remainder. Chromatography of F-I on Bio-Gel P-60 shows components with hydrodynamic volumes up to 10 times the volume corresponding to native lysozyme. Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicates the presence of 13% of polymers which are formed by integral numbers of lysozyme monomers. The relationship between the amount of polymer and degree of polymerization is exponential, indicating a random process of polymerization. Reduction of irradiated lysozyme with 2-mercaptoethanol and alkylation with iodoacetamide show that polymers are formed by intermolecular disulfide bonds. Polyacrylamide gel electrophoresis of F-I in urea reveals the presence of different monomeric forms which differ from native lysozyme and from each other in charge, size and/or shape.

Difference spectra of F-I in 0.1 N versus 1 N acetic acid show that most of the increase in hydrodynamic volume is due to the unfolding of damaged molecules. Difference spectra of F-I components versus

native lysozyme in 5.4 M guanidine hydrochloride do not show significant destruction of tyrosyl residues but indicate that tryptophyl residues are destroyed by gamma rays. It is suggested that as a result of destruction of the hydrophobic tryptophyl residues, the folding of damaged molecules is different from that of native lysozyme. The altered conformation allows formation of intermolecular disulfide bonds, probably via a free radical process initiated by cystinyl radicals from solid irradiated lysozyme.

F-I fraction shows carbonyl groups, however, no chain breaks of the main polypeptide chain were observed as might be expected from Garrison's theory of carbonyl formation in peptides. An increase in ammonia was observed for F-I components which is more significant in fractions subjected to more fractionation steps, indicating a decomposition of damaged groups.

The specific lytic activity of F-I on cells of *M. lysodeikticus* was 10% of the activity of native lysozyme. The damaged enzyme is inhibited by the substrate at low and high concentrations of substrate, indicating alterations of distribution of electric charges on the surface of the protein molecule. Difference spectra of the enzyme-substrate complexes between F-I components and the artificial substrate, glycol chitin, show a decrease in the capacity of the enzyme to bind the substrate and formation of nonproductive complexes. The destruction of enzyme activity can be a result of the conformational damage and of the destruction of tryptophyl residues which play an important role in the binding of the substrate to the active site of lysozyme.

The salt soluble fraction, F-II, does not show a significant increase in hydrodynamic volume, and sodium dodecyl sulfate

polyacrylamide gel electrophoresis shows that F-II is a monomeric fraction. Spectral characteristics of this fraction are very similar to those of native lysozyme. Polyacrylamide gel electrophoresis of F-II in urea shows the presence of a main component with characteristics similar to native lysozyme and other small components differing in charge and size. Chromatography on Bio-Rex 70 of whole irradiated lysozyme shows that a large part of F-II molecules are damaged since only 28% of the whole irradiated lysozyme elutes in a position comparable to native lysozyme, whereas the F-II fraction accounts for 61% of the whole irradiated lysozyme. The enzyme specific activity of F-II is 86% of native lysozyme. Difference spectra of the complex between F-II and glycol chitin show the same characteristics observed for the native lysozyme complex, but only 85% as much complex is formed. The loss of enzymatic activity of F-II must be caused by small alterations of the protein structure which do not cause significant alterations of the macromolecular properties.

Heavily damaged molecules of irradiated lysozyme tend to aggregate, especially at high ionic strength or high pH values. This aggregation is delayed by substrates, indicating an important role for tryptophyl groups in the aggregation. Perturbation difference spectra studies of irradiated lysozyme show that tryptophyl groups are removed from the contact with the solvent, supporting the suggestion that at high ionic strength or pH, aggregation involves some of the exposed tryptophyl residues.

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

Signed

Faculty member in charge of dissertation

ACKNOWLEDGEMENTS

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

The continual financial assistance of the United States Atomic Energy Commission is also gratefully acknowledged.

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CHAPTER I

INTRODUCTION

Biochemistry of Lysozyme

In 1922 Fleming discovered an enzyme which he called lysozyme. This enzyme has the power to dissolve certain bacteria, and as a class it is widely distributed in nature (1). Jolles has defined a lysozyme as an enzyme which possesses the following properties: it must be a basic protein of low molecular weight (~15,000) which is stable at acidic pH without significant loss of activity and unstable at alkaline pH; it must be active against suspensions of *Micrococcus lysodeikticus* and liberate, by its action on an appropriate substrate, compounds which can be detected by reagents for reducing sugars and for N-acetylhexosamines and which are derived from glucosamine and muramic acid (2). Lysozyme is called also β -glucosaminidase or muremidase or N-acetylmuramide glycanohydrolase.

The most common lysozyme is hen's egg white lysozyme. This enzyme forms about 3% of the hen's egg white and was first crystallized from egg white by Abraham and Robison in 1937. The crystallized preparations can be purified by ion exchange chromatography on Amberlite CG-50 as described by Tallan and Stein (3) or on carboxymethylcellulose as proposed by Jolles *et al.* (4). Recently Cherkasov *et al.* (5) have introduced a new method for the chromatography of lysozyme using chitin (poly- β -1 $\rightarrow$ 4-N-acetylglucosamine) as sorbent. Chitin is an insoluble substrate for lysozyme, and it may be

considered that the sorption process is due to specific enzyme-substrate interactions (6).

The primary structure of hen egg white lysozyme has been established independently by Canfield (7) and Jolles (8). Lysozyme is a protein comprised of a single chain of 129 amino acids that is cross-linked at four places by disulfide bonds located in positions 6-127, 30-115, 64-80, and 76-94 (9, 10, 11) and it has no free -SH groups.

The conformation of lysozyme has been determined by Blake *et al.* (12) using X-ray analysis at a $2 \text{ \AA}$ resolution that allows the location of 95% of the atoms within $0.25 \text{ \AA}$. The X-ray analysis shows that the molecule has a roughly ellipsoidal shape with dimensions of about $45 \times 30 \times 30 \text{ \AA}$ and has a marked cleft on one side. The helical structures in lysozyme are somewhat different from the standard structures, lying somewhere between an α -helix and a 3.0_{10} helix (13, 14). However, the axial translation per residue and the number of residues per turn fall close to the α -helix values given by Pauling and Corey (15). The percent α -helix in the molecule calculated from the X-ray analysis depends on the criteria chosen to characterize which amino acid residues are in an α -helix structure. If the α -helix is calculated on the basis of the number of amino acid residues that are within recognizable stretches of helix, a value of 19% to 21% is obtained. If the calculation is made on the basis of the number of residues falling within the α -helix of the dihedral angle plot, 55 points, each one representing an amino acid, a content of about 43% is obtained (13). Phillips (16) has made corrections on the number of residues obtained from the dihedral angle plot taking into account only those residues that are included in continuous sequences of 4

or more that contribute to recognizable helices. The percent of α -helix obtained from this value is about 36%, a number that is in agreement with the 35% obtained from optical rotatory dispersion measurements (17) and 35% minimum content of α -helix obtained with deuterium-hydrogen exchange as reported by Hvidt and Kanarek (18).

Another structure found in lysozyme is the antiparallel pleated sheet or β -structure that was predicted in 1964 by Hamaguchi and Imahori (19) from studies of the optical rotatory properties of lysozyme.

The X-ray analysis shows that almost all the hydrophobic amino acid residues are buried in the interior of the molecule with only a few exceptions. The tryptophyl residues deserve special mention because of their optical properties and their role in the active site. Three tryptophyl residues, 62, 63 and 108 are in the surface of the cleft or active site, and one tryptophyl residue, 123, is on the surface of the protein. Spectrophotometric studies carried out by Hayashi *et al.* (20) using perturbation techniques show that four tryptophyl residues are exposed if polyethylene glycol is used as a perturbant. Perturbation spectra of lysozyme with an artificial substrate, glycol chitin, shows a difference spectra which is caused by a red shift in the spectrum of the enzyme, indicating that new hydrophobic regions are formed around a tryptophyl residue (21).

The X-ray analysis has indicated that tryptophyl 62 is bound to the substrate and that the side chain of this residue moves 0.75 angstrom toward the position of the sugar. Studies carried out by Hayashi *et al.* (22) have shown that destruction of tryptophyl 62 causes total inactivation of the enzyme, a fact that is in perfect agreement with the data obtained from X-ray analysis. Imoto *et al.* (23) have shown

that destruction of Tryptophyl 108 renders an inactive product. However, the enzyme still can form a complex with the substrate, in this case glycol chitin. In the center of the cleft there are two hydrophilic groups, glutamyl-35 and aspartyl-52, both having an active role in the hydrolytic process.

Our detailed knowledge about the enzymatic activity of lysozyme is due mainly to the availability of small polymers of N-acetylglucosamine, dimers to tetramers, that can form stable complexes with the enzyme. These complexes, which are obtained by allowing the saccharides to diffuse inside lysozyme crystals, can be studied by X-ray analysis. The active site can be subdivided into six subsites denominated A, B, C, D, E, and F. In each subsite only one sugar residue can be located. This means that in the active site only six sugar residues can make contact with the enzyme (24). If longer polymers are available, the sugar residues above subsite A or below subsite F have no interactions with the enzyme. In subsite D, the pyranose ring of the sugar is distorted from the normal chair conformation toward a half chair conformation due to steric interactions of the enzyme with the CH_2OH group on carbon-6 of the sugar unit. It seems that this change in conformation of the sugar is involved in the activation of the substrate (25). The exact mechanism of hydrolysis is still not very clear. However, the work of Phillips and coworkers with X-ray analysis, Rupley *et al.* (26), and Parsons and Raftery (27), supports the following mechanism which has already been proposed (28): the unionized proton of glutamyl-35 attacks the linking oxygen between sugar residues D and E and weakens the $\text{C}_1\text{-O}$ bond. The C_1 forms a carbonium ion that is stabilized by the nearby charged aspartyl-52.

These actions, combined with the conformational distortion of the sugar, lower the activation barrier for the bond-breaking process. The glutamyl-35 proton is replaced by another from an ionizing water molecule, and the resultant hydroxyl ion then attacks the carbonium ion and completes the reaction. This model has strong support from the work of Lin and Koshland (29) which shows that glutamyl-35 and aspartyl-52 are involved in the catalytic process.

The correlation between the mechanism proposed from X-ray analysis on the crystalline lysozyme-inhibitor complex and the mechanism in solution depends on the conformation of the enzyme in solution and in crystalline state. Perturbation difference spectra (30) and optical rotatory dispersion (31) indicate that the conformation of lysozyme in solution is equal to the one in solid state. NMR studies indicate that there is a good correlation between the observed spectra and the theoretical one reconstructed from X-ray crystallographic coordinates (32). There is, however, one piece of information that does not support the similarity of conformations in solution and in crystalline state. Felsenfeld and Handschumacher (33) working with penicillin have found that penicillin can interact with the six tryptophan residues, whereas, in solid state the X-ray analysis shows that only one penicillin binds to the lysozyme molecule (34).

The physical chemical properties of lysozyme have been studied by Sophianopoulos (35, 36) who found aggregation of the protein to be a function of the pH. It seems likely that the association sites of lysozyme in solution are different from the contact points between molecules in the crystalline state (13). In solution the process of association involves the active site (37) which is not involved in

the contact areas in crystalline state. The availability, purity, and knowledge about this enzyme made it a very good model for the study of radiation effects on proteins.

Effects of Ionizing Radiations on Proteins

As a result of the interaction between ionizing radiations and proteins, an extensive degradation is observed indicating that the energy of ionizing radiations triggers changes in the protein structure. One of the most important effects of the ionizing radiation is the formation of ions upon absorption by the matter. In the case of X- and gamma rays, the transfer of energy from a photon to an atom results in the ejection of electrons which in turn constitute a new corpuscular ionizing radiation which can cause secondary ionizations and/or excitations (38). Bethe has postulated that an ionizing particle passing through a number of atoms can displace some electrons from one shell to another of higher energy causing an excitation without producing an ionization (39). The ionizations and excitations resultant from the interaction between radiation and matter lead to secondary processes, some of which are very important in the field of radiobiology.

Much of our knowledge about the secondary processes initiated by ions comes from gas phase studies done with mass spectrometer (40). An ionization will frequently turn a molecule into a free radical by one of the following mechanisms:

a- Neutralization of an ion producing excited state followed by dissociation into free radicals with neutralization

b- Dissociation of an excited ion into a radical-ion and a radical

The excited molecules resultant from a primary effect or from a secondary process of ionization can also give rise to free radicals by one of the following mechanisms:

a- An excited molecule resultant from interaction of radiation with matter dissociates into free radicals

b- An excited molecule undergoes abstraction reactions that generally involve reduction of the excited molecule through hydrogen abstraction from another molecule

Excited molecules can undergo a process called energy transfer which can occur by two mechanisms, excitation transfer and charge migration (41). In the excitation transfer process, the electrons involved in the excitation remain associated with the same molecular units. This process occurs by a nonradiative energy transfer where the conditions are that the acceptor unit has equal or less excitation potential than the donor unit and is appropriately orientated. Charge migration process can occur by semiconduction with energies much less than those required for ionization. In order to allow the conduction phenomenon to occur, the electrons involved must be associated with orbitals that stretch over extended domains of biological components such as the framework of hydrogen bonds in a pleated-sheet configuration found in

certain proteins. Definite mechanisms for the charge migration process are not known (42).

Dessauer in 1923 proposed a mechanism for the interaction of ionizing radiation with matter which is different from the excitation or ionization process already discussed and which he called "head point" (43). In the "head point" or "thermal spike" process as it is also called, localized regions of the absorbing medium are heated at high temperature, the same that can cause extensive changes in the matter, especially in organic compounds (44). Simmons (45) working with glycine crystals has found that the absorbed energy cannot be evenly distributed throughout the whole sample but tends to remain close to the point of deposition, resulting in the thermal spike effect postulated by Dessauer.

The chemical changes caused in the matter by the ionizing radiation can occur by two distinct mechanisms: (a) by direct action, in which case the initial process of energy deposition occurs in the molecule undergoing change itself; and (b) by indirect effect, in which the molecule studied does not absorb the energy but can be affected by the reactive moieties formed by deposition of the energy in the material surrounding the molecule under observation. In radiobiological studies, the material surrounding the molecules under study, proteins, nucleic acids, etc, is usually water. Alexander (46) has pointed out that the lesions that cause death of irradiated cells are due mainly to direct effect and that the indirect effect observed in dilute solutions is very unlikely to occur in the cellular milieu.

In 1944 Lea (47) proposed that biological material is inactivated primarily by ionizations rather than by excitations. On this basis,

he developed his well known "target theory" which states that a biologically active entity is inactivated as a result of at least one primary ionization (which is postulated to arise on an average for every 100 eV of absorbed energy) produced anywhere within the sensitive target volume associated with such entity (48). Study of the shape of survival curves for enzymes inactivation by ionizing radiation shows an exponential curve which is in agreement with the single ionization model. Application of probability theory to this situation demonstrates that when enough energy has been deposited on the sample to produce one ionization in each molecule, 37% of the original activity remains. This dose or amount of energy is denominated D_{37} , and it has been possible to make an approximate relation between this value and the molecular weight of the sensitive target (49). Augenstein (50) has analysed the molecular weights of different enzymes calculated from radiation studies (target theory) by Pollard *et al.* (51) and found that under certain experimental conditions, the molecular weights estimated by this method agree fairly well with those calculated with physico-chemical methods. However, for some enzymes, serious deviations from the correct molecular weights were apparent.

Working with catalase, Setlow (52) found that temperature decrease causes a concomitant decrease in the cross section of the target, and he proposed as an explanation, a transfer of energy through the molecule which is very temperature dependent. This energy transfer mechanism was not considered important in the original Lea's target theory; however, Augenstein *et al.* (53) concluded from the analysis of Brustad's work on trypsin (54) that nonionizing excitations and thermal facilitation of charge migration accompanying ionization are probably

of equal or greater importance than the ionization process in enzyme inactivation. Fluke (53), working with dry lysozyme and ribonuclease, has found that the activation energy for the denaturation of ribonuclease by ionizing radiation differs from the values for lysozyme inactivation and also from those given by Augenstein and Mason (56). Fluke concluded that the target theory of Lea is still interesting but is incomplete and needs to be extended to include the temperature effect.

Another factor against the "target theory" is the fact that the radiosensitivity of a biological entity is also dependent on the presence of foreign compounds in the sample and, in the case of proteins, on the pretreatment of the sample. Wilson (57) has shown that dry preparations of deoxyribonuclease irradiated in the presence of DNA show a radiosensitivity different from the pure enzyme. Tramer and Shugar (58) found a mutual protective effect in gamma-irradiated dry mixtures of ribonuclease and lysozyme. The radiosensitivity of trypsin-trypsin inhibitor has been studied by Pollard *et al.* (51) and Shore *et al.* (59), and the radiosensitivity of the enzyme in the complex is still uncertain. However, Shore *et al.* have shown that the sensitivity is different in the presence of the inhibitor and, moreover, that the target for esterase activity and protease activity are different in the trypsin from the complex. This effect of foreign substances on the sensitivity of enzymes is not limited to substrates of enzymes but to a wide variety of compounds. Butler and Robins (60) and Riesz (61) have shown that metal ions can protect solid enzymes in dry metal-enzyme complexes. All these experiments speak in favor of a mechanism or mechanisms different from the one hit-one target theory.

and support the energy migration mechanism and the role of non-ionizing excitations. The denaturing effect of radiations on proteins is also related to the physical state of the solid material. Wilson (62) observed that the sensitivity of dry DNase is a function of the pH before lyophilization. Henriksen (63) has reported differences in the radical yield of crystalline and non-crystalline trypsin that can be related to differences in inactivation of the two preparations. Ten Bosch (64) has found different G values (G value = number of reactions that have occurred for every 100 eV of energy absorbed) for different partially degraded collagen with nearly the same primary amino acid composition as the parent molecule. These different behaviors of an irradiated protein show that the structure of the protein at different levels, primary, secondary, tertiary and quaternary structure, has an important role in the response of the protein to radiation, suggesting that mechanisms like energy migration are important.

In 1955 Gordy and coworkers (65) using ESR spectroscopy at room temperature observed two different kinds of radicals in dry proteins irradiated with ionizing radiations. These radicals were identified as $\text{-CO-}\dot{\text{C}}\text{H-NH-}$ and $\text{-CH}_2\text{-S}\cdot$ and were denominated glycine radical and sulphur radical respectively. Following Alexander (48), the free radicals present in irradiated proteins will be discussed in relation to the primary structure of the proteins. Chemical analysis and ESR studies of proteins and mixtures of amino acids in the same proportions as found in the proteins show striking differences, indicating that the alteration of the amino acid residues is dependent on the sequence in the protein (66).

Experiments at low temperatures (77°K) using ESR spectroscopy have shown a different spectra from the one observed at room temperature (67). On warming to higher temperatures, the characteristic "glycine" and "sulphur" type resonances were observed. This change in the spectra is an indication that the characteristic resonances observed at room temperature are secondary radicals ($R_2\cdot$). The nature of the primary radicals that give origin to carbon radicals or "glycine radicals" is not clear. Riesz and coworkers (68) have used tritiated hydrogen sulfide (HST) as a radical scavenger exposing the gamma-irradiated lyophilized proteins to HST in order to form carbon-tritium bonds in the protein. The secondary or "glycine" radicals presumably react with the HST to form the bond tritium-carbon.

Upon hydrolysis of the protein, it is possible to measure the content of tritium in each amino acid. The tritium was not found in great proportions on the glycine amino acid as was supposed, but it was distributed in almost all the amino acids. The relative distribution of tritium depended in part on the amino acid sequence in the protein. Drew and Gordy (69) explain the formation of glycine radicals by the loss of an α -hydrogen atom from a peptide bond

the calculated spectra of this radical coinciding with the observed spectra of the "glycine radicals" in irradiated proteins. The nature of the "sulfur radical" is better known. This radical is observed in

all sulphur containing proteins and in all mixtures of non-sulphur containing proteins and thiol-containing compounds (70). Two mechanisms have been proposed in order to explain the origin of the secondary sulphur radicals or "cystine radicals". Ormerod and Singh (71) concluded from studies with proteins containing sulphur that the "cystine radicals" are originated from cystine anions resultant from electrons captured or trapped on cystine residues. Obviously the trapped electrons are secondary electrons ejected during the ionization process caused by a high energy quantum. Henriksen (72) has proposed that the cystine radicals are formed as a result of an energy transfer in the protein, the sulphur groups acting as sinks for radiation energy. Henriksen *et al.* (73) do not postulate the cystine anion intermediate but instead a type of mechanism where the formation of a thioether would be expected according to the following reaction:

Burke and Augenstein (74) have compared the effects of gamma rays and 1 MeV electrons on trypsin and conclude that the patterns of destruction of the amino acid residues are different for both radiations, indicating that it is possible that the secondary electrons do not play as important a role as is believed. Hutchinson (75) proposed a model for inactivation of ribonuclease where the most important alteration was the breakage of disulfide bonds. However, further investigation by the same author (76) showed that there is no significant opening of disulfide bonds. Dose (77) found indications that some of the disulfide bonds of lysozyme were cleaved to thiol groups when the enzyme was irradiated with X-rays in the presence of nitrogen. Stevens *et al.* (78) did not find thiol groups in lysozyme irradiated with gamma rays *in vacuo*;

however, further work (79) with some inactivated fractions from irradiated lysozyme has shown that for some fractions, the number of disulfide bonds in mixed disulfides of cystine and inactivated fractions is different from the expected content of 8 bonds per molecule. Alexander and Hamilton (66) have found that irradiation of bovine serum albumin by 2 MeV electrons produces new very labile thiol groups. Garrison and coworkers (80) have studied the formation of carbonyl groups and amide groups in polypeptides and some proteins, this formation being enhanced by the presence of oxygen. The mechanism for production of these groups is still unclear and the proposed labile imino ester and dehydropeptide hydrolysis have been found unsatisfactory in explaining the G-values for the production of ammonia and carbonyl groups (81). Carbonyl and amide groups are formed in dry bovine serum albumin when it is irradiated with high energy electrons without evidence of degradation. Friedberg and Hayden (82), working with ATP-creatine phosphotransferase and gelatin, have found that the ATP-creatine phosphotransferase does not show any evidence of splitting into smaller fragments. Gelatin that is a fibrous protein shows degradation and fragmentation by the action of ionizing radiation, and the same degradation effect was shown in polyamino acids irradiated with gamma rays (83). Cooper and Russell (84) have studied the effect of gamma rays on lyophilized samples of soluble collagen, obtaining results similar to those reported by Friedberg and Hayden (82) for gelatin. Hayden *et al.* (83) have pointed out that the degradative effect of ionizing radiation is different for fibrous and globular proteins, and that this degradation can be an indication that cleavage of N-C and C-C linkages of the peptide has occurred. Garrison (81) proposed the following sequence of

reactions to explain the cleavage of the C-N linkage in a polypeptide chain,

and, as final products, amide, fatty acid and the "glycine radical" type are obtained. Friedberg and Riesz (85), using gas chromatographic methods to analyse the acid hydrolysates of irradiated proteins and polyamino acids, have been able to characterize acetic and propionic acid, compounds predicted by the Garrison scheme. However, there still is no explanation for the difference in behavior for globular and fibrous proteins.

As a result of the chemical modifications caused in the primary structure of a protein by the ionizing radiation, expected alterations of the secondary and tertiary structure are observed. Fricke and co-workers (86, 87), from studies on the thermal stability, serological properties and optical rotatory properties of gamma irradiated ovalbumin, concluded that the irradiated protein contains a spectrum of different protein molecules in which the secondary and tertiary structures were disrupted. Irradiation of crystalline bovine serum albumin produces aggregation as determined by light-scattering, and at doses low enough to avoid the aggregation process the protein shows an abnormal behavior when it is analysed by ultracentrifugation, indicating disorganization of the secondary and/or tertiary structure (88). Using infrared spectroscopic techniques to follow the deuterium exchange process in irradiated proteins, Stevens *et al.* (78), working with lysozyme

and α -chymotrypsin, and Hayden and Friedberg (89), working with ribonuclease, have been able to measure the destruction of the secondary structure of these proteins as a function of the doses of gamma rays. Marciani and Tolbert (90) have studied the loss of conformational integrity of irradiated lysozyme using tritium water exchange and gel filtration. Slobodian and Fleisher (91), using the same technique of the previous authors, have found that ribonuclease irradiated in solution by gamma rays shows a progressive unfolding of the α -helix and a loss of the intramolecular side chain hydrogen bonds. Viscosity studies with different irradiated proteins (82, 83) support the previous reports of disorganization of the secondary and tertiary structure by irradiation.

Application of chromatographic techniques for the fractionation of irradiated pure proteins shows the presence of different fractions or components at different stages of denaturation, confirming the findings of Frike and coworkers (87). Slobodian *et al.* (92) introduced the ion exchange chromatographic methods for the fractionation of ribonuclease irradiated in solution with X-rays isolating different inactive and partially active components. Independently Stevens *et al.* (93) and Kravchenko *et al.* (94) introduced ion exchange chromatography on weakly acidic resins for the fractionation of lysozyme irradiated with gamma rays and electrons respectively. Both authors were able to isolate several modified lysozyme products with different enzymatic specific activity, but dissolution of the irradiated protein in the buffer used as eluent caused a partial irreversible precipitation of the protein.

Jung and Schüssler have applied gel chromatography on Sephadex G-50 for the isolation of products formed from ribonuclease irradiated

in solution (95) and in solid state (96). In both cases it was possible to show the presence of polymers of ribonuclease. Haskill and Hunt (97) have used the gel chromatography method for the isolation of products from irradiated ribonuclease and have characterized some of the physical alterations of the products. Again aggregation is one of the alterations, and for a fraction called "denaturated", a partial unfolding has been characterized from spectroscopy studies. Maksimov *et al.* (98) have studied the gamma-irradiated ribonuclease by the method of gel filtration on Sephadex G-100, and they conclude that the tertiary structure is changed in practically all the molecules as a result of irradiation. Maksimov and coworkers (99, 100) extended their studies to irradiated lysozyme. They fractionated the irradiated lysozyme in two fractions by salting out and studied each fraction by gel filtration, finding that the salt insoluble fraction is the one that shows most marked changes. Stevens *et al.* (101) have studied the aggregation process of irradiated proteins in the presence and absence of H_2S . If H_2S is present during the irradiation process, aggregation decreases in ribonuclease and increases in lysozyme and trypsin. Haskill and Hunt (102) have designed a method to detect "sublethal" lesions in irradiated ribonuclease that consists of the reduction of disulfide bonds and reoxidation under the conditions indicated by White (103). Ribonuclease irradiated in vacuo does not show changes in the activity after treatment, whereas the ribonuclease irradiated in the presence of O_2 shows a decrease of activity upon reduction and reoxidation. Stevens *et al.* have applied a similar test to an inactive-insoluble fraction from irradiated lysozyme and found that some reactivation is obtained. These studies together with ESR and interceptor techniques

for free radicals support the possibility that disulfide bonds play a very important role in the inactivation process resulting from interaction between proteins and ionizing radiation.

Irradiated enzymes show a different enzymatic behavior from the native enzymes, reduction of the specific enzymatic activity being the most often noted. Haskill and Hunt (102) have measured the Michaelis constant (K_m) and the overall velocity (V_{max}) for irradiated solid ribonuclease. The K_m does not show change for the native or irradiated enzyme whereas the V_{max} is lower for the irradiated enzyme. Similar results have been observed by Riesz and White (104) for irradiated dry ribonuclease. Mee (105) has reported that the K_m and V_{max} of chymotrypsin irradiated in solution with X-rays are different from the native values and change with the dose administered. The optimum conditions for the enzymatic reaction can differ from the native enzyme upon irradiation. For solid irradiated lysozyme the pH dependence of the rates of hydrolysis of yeast RNA were different from native ribonuclease (104). Dose *et al.* (106) have reported that the optimum pH for lytic activity of lysozyme is shifted toward lower values in lysozyme irradiated with high doses, 100 Mrad, of X-rays. Maksimov *et al.* (107) using tetra-N-acetylchitotetraose as substrate have concluded that the pH dependence for the activity of irradiated lysozyme is more complex than that for native lysozyme. Dose (108) and Marciani *et al.* (109), following an approach similar to the one postulated by McLaren and Luse (110) for estimation of quantum yields for enzyme inactivation, have developed a theory to calculate the $G_{inactivation}$ value for enzymes like ribonuclease and lysozyme. It is based on an independent cumulative inactivation of the amino acid residues in some form related

to the enzymatic activity.

At the present stage of progress in the field of radiation chemistry of proteins, it is difficult to decide what mechanism or mechanisms are the most important. The one hit-one target theory (50) has been found to be inaccurate under many conditions, and it seems that the inactivation process is the resultant of a series of independent events some of which are more important. An attempt will be made here to try to relate these processes, especially the disruption of disulfide bonds to the inactivation caused by ionizing radiations.

CHAPTER II

EXPERIMENTAL PROCEDURES

Enzyme and Substrates: Egg white lysozyme (E. C. 3.2.1.17) was obtained from the Sigma Chemical Company and Seikagaku Fine Biochemicals. The 3x crystallized preparation from Sigma was purified by chromatography on the carboxylic cation exchange resin Bio-Rex 70 following the procedure of Stevens and Bergstrom (111). Lysozyme, 6x crystallized from Seikegaku, was chromatographically homogeneous and was used without further purification.

Micrococcus lysodeikticus (ATCC 4968), spray-dried powder, was obtained from Miles Laboratories and Worthington Biochemical Corporation. Water-soluble alcohol-insoluble chitodextrin was prepared by the method of Berger and Reynolds (112). Paper chromatography (113) of this compound indicated the absence of oligosaccharides smaller than the heptamer of N-acetyl glucosamine. Glycol chitin was synthesized from purified chitin by the method of Senju and Okimasu (114) and fractionated by the modification of Hayashi *et al.* (21).

Reagents: Commercial sodium dodecyl sulfate (SDS) was recrystallized from boiling ethanol, and urea, reagent grade, was deionized and recrystallized from ethanol. Trinitro benzene sulfonic acid (TNBS) was recrystallized from 1N HCl. Bio-Rex 70 and Bio-Gel of different porosities were obtained from Bio-Rad Laboratories. Tritiated water at a concentration of 1 curie/g was obtained from New England Nuclear, and all other chemicals were at least reagent grade.

Irradiation Conditions: Lyophilized lysozyme samples were placed in Pyrex glass tubes and dried overnight *in vacuo* at a pressure of 6-8 μ of Hg using an oil diffusion pump and a liquid nitrogen trap. The tubes were sealed *in vacuo* and irradiated in a Cs-137 source at 22°C and at a dose rate of 0.383 Mrad/hour. The dose rate was determined using the Fricke ferrous sulfate dosimeter recommended by Weiss *et al.* (115). A value of 15.7 was used for $G[Fe^{+3}]$ for ^{137}Cs gamma rays (116).

Protein Determination: The concentrations of native and irradiated lysozyme were determined using different methods, depending on the needs of accuracy and/or speed and on the concentration of protein in the samples. The absorbance at 280 nm was used in all chromatographic work. The concentration of very dilute solutions, 5 to 50 $\mu g/ml$ of unirradiated protein, was determined by the difference between the absorbances at 215 and 225 nm (117). This method is almost independent of the amino acid composition of the protein. For accurate determinations of protein concentration in the chemical studies of the fractions from irradiated lysozyme, two different micromethods, the microbiuret (118) and the Cu-capacity binding (119), have been used. Both methods are independent of the amino acid composition depending only on the number of peptide bonds available in the protein. In some studies the amount of protein was measured gravimetrically with a Mettler micro balance.

Enzyme Assay: The enzymatic activity of the native and irradiated lysozyme and the different fractions from irradiated lysozyme were assayed on two different substrates: cells of *Micrococcus lysodeikticus* and on glycolchitin. Most enzyme activities were assayed on cells of

M. lysodeikticus using the method described by Shinitzky *et al.* (120). The wave length chosen for the assay was 645 nm. The course of lysis of *M. lysodeikticus* by the enzyme at 25°C was followed in a Gilford 2000 or a Cary 16K spectrophotometer. Temperature effects on the enzymatic activity were studied in the Gilford 2000 spectrophotometer equipped with thermospacers, and the temperature was measured with a copper-constantan thermocouple attached to one of the cuvettes. Composition of the buffers used in these measurements depended on the kind of experiment being performed.

The apparent affinity constants (K_a app.) of the different samples of enzyme for *M. lysodeikticus* were measured following the method of Locquet *et al.* (121). In order to obtain the optimum conditions of pH and ionic strength for the lytic activity of lysozyme, the buffer used was 0.01 M phosphate - 0.04 M NaCl ($I = 0.05$) (122). Following the nomenclature of the original paper, the substrate concentration is expressed in mg/l and the reaction velocity in mg/l/sec. All measurements were carried out at 25°C. The enzyme solutions were prepared with distilled water in order to avoid precipitation of the fractions from irradiated lysozyme by the salts of the buffer.

The spectroscopic characteristics of the enzyme-substrate complex between lysozyme and glycol chitin have been studied following the procedure described by Hayashi *et al.* (21). The buffer solution used was 0.1 M acetate, pH 4.0 and the final concentrations of lysozyme and glycol chitin were 0.1 and 0.2%, respectively. The difference spectra in the ultraviolet region (270-320 nm) were recorded in a Cary 16-K spectrophotometer within the range of 15-17 minutes after the substrate was added to the enzyme solution. The incubation and

recording of the spectra were done at 25°C. The optical densities in the difference spectra were converted into the difference molar extinction coefficients, $\Delta\epsilon$.

Tritium-hydrogen Exchange: The measurement of the hydrogen exchange kinetics in native and irradiated lysozyme was made following the method of Englander (123). Native lysozyme at a concentration of 10 mg/ml was warmed in tritiated water (HTO) at 65°C for 2 hours. This temperature is above the temperature of reversible configurational transition for lysozyme. The activity of the HTO was approximately 1 mc/ml and the pH was adjusted to 5.5 by addition of acetic acid and pyridine to a final concentration of 0.1 M and 0.3 M respectively. After cooling, the solution was centrifugated at 2000 x g. to remove insoluble protein formed during the process of equilibration of the exchangeable hydrogens with tritium. The clear supernatant was lyophilized, and the dry protein obtained was named T-lysozyme. T-lysozyme was irradiated following the procedure described before under "Irradiation conditions". Irradiated samples were dissolved in O₂-free 0.1 M acetate buffer, pH 5, and the back-exchange was measured according to the following procedure. Aliquotes of 0.4 ml of the protein solution (10 mg/ml) were subjected to gel filtration on 14 cm x 2.2 cm Bio-Gel P-2 (50 - 100 mesh) columns at 5°C and using a flow rate of 1.6 ml/cm²/min. The tritiated macromolecule and the tritiated solvent were separated during this process. The exchange was measured at different times of incubation of T-lysozyme in 0.1 M acetate buffer at 5°C. The effluent from the gel filtration process was collected in fractions of 1.4 ml, and the absorbance at 280 nm and the tritium activity were measured for each fraction. The first fraction of each

column was used as a blank for spectrophotometric measurements and to obtain the background for the liquid scintillation counting. Tritium counts per minute in each fraction were determined using a Beckman LS 250 Liquid Scintillation System. A 0.5 ml sample of each fraction was added to 15 ml of scintillation cocktail having 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 efficiency for tritium was determined by spiking the samples with a toluene-³H standard.

The parameter, hydrogen atoms/molecule of lysozyme was calculated by the equation given by Englander (124),

$$H/\text{molecule} = \frac{C/D}{C_0} \times 111 \times E_p$$

where C/D is the ratio between counts/min/ml of the fraction to absorbance at 280 nm of the fraction.

111 is the atom concentration of hydrogen in water.

C_0 = counts/min/ml of protein-H₂O equilibrium mixture.

E_p = molar extinction coefficient of lysozyme at 280 nm.

The determination of the total number of exchangeable hydrogens in lysozyme was made on a solution of T-lysozyme lyophilized at 40°C. It was necessary to subtract 18 hydrogens from the number of hydrogens obtained in this sample due to the presence of 9 molecules of strongly bound water which cannot be removed by the lyophilization process (18). The different kinetic classes of hydrogens were calculated using the mathematical procedure described by Englander (123).

Ultraviolet Difference Spectra: The difference spectra of H₂O-soluble

irradiated lysozyme and some of its components were measured against native lysozyme. The concentration of protein was 2 mg/ml and was determined gravimetrically in a Mettler microbalance. The protein samples were dried *in vacuo* over magnesium perchlorate for many days in order to minimize the error in weight due to water content of the sample. In order to achieve complete solubility of the protein and normalize the absorption properties of the chromophoric residues present in the same, 4M guanidine hydrochloride, pH 5.4, and 5.4 M guanidine, pH 0.30, were used as solvents. Clear solutions for spectrophotometrical work were obtained by filtration through a nylon Millipore filter with a pore size of 14μ . The difference spectra were measured between 240 to 360 nm using the Cary 16K spectrophotometer. The temperature was maintained at 25°C . In order to avoid the effect of light scattering due to aggregates on the spectra, the method recommended by Alexander and Hamilton (66) was followed. The change in absorbance, $\Delta\text{Abs.}$, was multiplied by the wave length raised to the fourth power (λ^4) and plotted against λ . This method allows the characterization of changes in absorption due to chemical alterations and/or conformational changes.

Difference Spectra Caused by Changes in Acetic Acid Concentrations:

The difference spectra of native lysozyme, irradiated lysozyme and its fractions caused by changes in the concentration of acetic acid were recorded directly in a Cary 16K spectrophotometer by the use of two matched 1-cm quartz cuvettes, each of which contained two 0.5 cm compartments in tandem which were separated by a quartz window. The cuvette in the reference beam contained the lysozyme, 2mg/ml, in 0.1 N acetic acid in one compartment and 1 N acetic acid in the tandem

compartment. The cuvette in the sample beam contained 0.1 N acetic acid in one compartment and lysozyme, 2 mg/ml, in 1 N acetic acid in the tandem compartment. The measurements were made after 12 hours in order to obtain equilibration of the changes in absorbance. The difference spectrum was recorded between 240 to 340 nm., at 25°C. The amount of protein in each sample was determined gravimetrically using a microbalance. The pH of the protein solutions in 0.1 N acetic acid was 2.98 and for the solution in 1 N acetic acid was 2.42. The optical densities in the difference spectrum were converted into the difference molar extinction coefficients, $\Delta\epsilon$.

Perturbation Difference Spectra: The perturbation difference spectrum of native and irradiated lysozyme was studied using the method of Williams *et al.* (124). The perturbant was 20% (v/v) polyethylene glycol (degree of polymerization: 6), and the buffer was 0.1 M acetate, pH 5. The protein was present at a concentration of about 1 mg/ml. As a standard for 100% exposure of tryptophyl residues, a model mixture of N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tryptophan ethyl ester was used. The proportions of tryptophan and tyrosine derivatives in the model mixture were the same as those found in native lysozyme (124). The degree of tryptophyl exposure was calculated following the method described by Herskovits and Laskowsky (125). The spectra were measured at 25°C.

Spectrophotometric Analysis of Tyrosine: Total tyrosine was determined by the increase in absorption near to 295 nm caused by the phenoxide ion. The method follows the procedure described by Inada (126), using 4 M guanidine·HCl as solvent. The blank was protein in 4 M guanidine·HCl, pH 7, and the sample was protein in 4 M guanidine·HCl-0.33 M KOH,

pH 12.80. The readings at 2 wave lengths, 295 and 300 nm, were measured after equilibration of the change in absorbance. The molar extinction coefficient for the phenoxide ion in lysozyme was calculated using a sample of native lysozyme. The values obtained were 2490 at 295 nm and 2805 at 300 nm. The value for ϵ_{295} agrees very well with the values of 2480 and 2500 reported by Edelhoch (127) and Kurihara *et al.* (128) respectively. The value for ϵ_{300} does not agree with the values of 2270 and 2616 reported by the previously mentioned authors. However, it is possible to observe that there is also a significant difference between these two values. These differences and the deviations reported by Edelhoch (127) for determinations of tyrosine in proteins using the 300 nm absorbance indicates that this measurement is not a function of the phenoxide ion concentration alone. The amount of protein in each sample was determined gravimetrically using a microbalance.

Polyacrylamide Gel Electrophoresis

(a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis:

Determinations of molecular weights were made by SDS-polyacrylamide electrophoresis as described by Dunker and Rueckert (129). The total concentration of acrylamide in the gels was 5%. Reduction of the disulfide bonds in the proteins used as "markers" as is described in the original methods (129) was omitted. The proteins used in the procedure as markers were:

Bovine serum albumin (BSA), tetramer.	M. W.: 264,000 (129)
BSA, trimer.	198,000 (129)
Bovine- γ -globuline (BG-II).	160,000 (129)
BSA, dimer.	132,000 (129)

Ovalbumin, dimer.	92,000	(129)
Bovine serum albumin, monomer.	66,000	(129)
Edestin, monomer.	52,000	(130)
Ovalbumin, monomer.	46,000	(129)
Pepsin.	35,500	(129)
Trypsin.	23,800	(129)
Lysozyme.	14,300	(7)

Native lysozyme and the products from irradiated lysozyme were studied with and without cleavage of the disulfide bonds. Samples without cleavage of the disulfide bonds were treated before electrophoresis by two different methods, as recommended by Dunker and Rueckert (129) and Maizel *et al.* (131) except that in both cases the 2-mercaptoethanol was omitted from the incubation mixture.

The reduction of the disulfide bonds was performed following the method of Shapiro *et al.* (132). The thiol groups formed during the reduction were alkylated with iodoacetamide. The excess of iodoacetamide and decomposition products of the reaction with 2-mercaptoethanol were removed by dialysis against 0.05 M phosphate buffer, pH 7.2, containing 0.2% SDS. The sample containing the same ionic components as the tray buffer plus 20% glycerol was applied by injection and layered between the tray buffer and the top of the gel. In some experiments the H₂O-soluble irradiated lysozyme was redissolved in 0.05 M phosphate buffer containing 1% SDS and 0.05 M iodoacetamide. The iodoacetamide was removed by dialysis and the sample, mixed with glycerol to a final concentration of 20%, was layered on the gel. The purpose of alkylation was to avoid the formation of polymers by reoxidation of the thiol groups to intermolecular disulfide bonds.

Estimation of protein in the polyacrylamide gels was made by densitometric scanning of the stained gels with Coomassie Blue at 570 nm in a Gilford spectrophotometer modified for the scanning of acrylamide gels. The concentration of protein was assumed to be proportional to the area under the peak obtained from the absorbance scan of the stained gel (133). The determination of molecular weights was made following the methods of Wolf *et al.* (134) and Dunker and Rueckert (129).

(b) Polyacrylamide gel electrophoresis with 2.5 and 8 M urea:

Disc gel electrophoresis on polyacrylamide in the presence of 2.5 M urea, pH 2.70 and 8 M urea, pH 4.28, was carried out as described by Panyim and Chalkley (135). The final concentration of acrylamide in the gels was 15%. The gels were fixed and stained with Coomassie Blue following the method of Chrambach *et al.* (136).

(c) Size and charge isomers resolution by disc gel electrophoresis:

Following the approach of Smithies (137) and Hedrick and Smith (138), a method was designed to characterize the nature of some of the isomers in the fractions from irradiated lysozyme. The electrophoresis procedure was carried out in the presence of 2.5 M urea in order to assure solubility of the damaged components. When the log of the component mobility relative to native lysozyme was plotted versus acrylamide concentration, the size isomeric components gave a family of nonparallel lines, and charge isomeric components gave a parallel family of lines. The isomers of different size were retarded by the "sieving action" of the gel, the same that is relatively greater at higher concentrations of acrylamide for bigger molecules. In the case of isomers with different charge but equal size, the relative sieving effect is the same for all the molecules at any concentration of

acrylamide, the mobility depending only on the charge of the molecule. The gel concentrations were varied between 7 to 15% by dilution of a concentrated acrylamide solution. The composition of the gels was very similar to that used by Panyim and Chalkley (135). In order to obtain a mechanically stronger gel, the N,N'-methylenebisacrylamide/acrylamide monomer weight ratio was raised to 1:100. The gels were fixed and stained with the same procedure used for disc gel electrophoresis with 2.5 M urea (136). Native lysozyme was used as a marker for the measurement of the relative mobilities, and the data obtained from the electrophoresis was treated following the method of Smithies (137).

Fractionation of Irradiated Lysozyme

(a) Ion exchange column chromatography:

The procedure described by Stevens *et al.* (93) was used for chromatography of irradiated lysozyme. In this procedure the irradiated lysozyme was dissolved in O₂-free water and after one hour at 0°C, an equal volume of 0.2 M potassium phosphate buffer, pH 7.12, was added to the protein solution and the sample was allowed to stand overnight at 4°C. The final concentration of protein was 20 mg/ml. The precipitate formed during the treatment with phosphate buffer was spun down by centrifugation and the clear supernatant was applied to a Bio-Rex 70 (200-400 mesh) column. As eluent 0.2 M K-phosphate buffer, pH 7.12, was used. The dimensions of the columns used were 2.5 cm x 56 cm for analytical purposes and 5 cm x 85 cm for preparative scale. The position of the protein being eluted was determined by its absorbance at 280 nm. In some experiments when the elution with 0.2 M phosphate

was finished, as determined by the absorbance of the eluent at 280 nm, a linear gradient of urea 0 - 6 M in 0.2 M K-phosphate buffer, pH 7.12, was applied to the column. The total volume of the gradient was 3 times the volume of the resin bed. A batchwise procedure was used on the Bio-Rex 70 loaded with protein: samples of resin plus protein were extracted with 6 or 8 M urea at pH values between 7 to 13. The amount of protein released by this treatment was evaluated from the absorbance at 280 nm of the clear solution obtained by brief centrifugation of the resin suspension.

(b) Salt fractionation of the irradiated lysozyme:

The method of Maksimov and Osipov (99) was followed with some modifications. One-half gram of lyophilized water-soluble irradiated lysozyme was dissolved in 100 ml of 0.1 M acetic acid. Then 5.85 gm of NaCl were added to this solution to give a concentration of 1 M. The mixture was stirred for one hour at room temperature during which time a flocculent precipitate formed. After one hour of stirring, the precipitate (Fraction I) was separated from the soluble fraction (Fraction II) by centrifugation at 30,000 to 40,000 g at 4°C for 15 to 20 minutes. The solid pellet obtained was redissolved in 60 ml of 0.1 M acetic acid and precipitated again by adding sufficient NaCl to give a concentration of 1 M. After 40 to 60 minutes the suspension was centrifuged under the same conditions as before and the supernatant was discarded and the pellet resuspended in 0.1 M acetic acid plus 1 M NaCl. The sample was again centrifuged and the supernatant discarded in order to remove traces of the soluble fraction (F-II) that may have been trapped in the solid material. The final precipitate (F-I) was redissolved in 50 ml of 0.1 M acetic acid. The solutions of F-I and F-II

were desalted by gel filtration on Bio-Gel P-2 in 0.1 M acetic acid. The pooled fractions containing the protein free of NaCl were concentrated to 15 ml in a rotary evaporator under vacuum at 25-30° and lyophilized to dryness. The lyophilized F-II was redissolved in 0.1 M acetic acid plus 1 M NaCl in order to precipitate any contamination of F-I. The F-II was again obtained as a lyophilized powder after desalting on Bio-Gel P-2 and concentration.

Gel Filtration Column Chromatography

Polyacrylamide gels, Bio-Gel P-30, P-60 and P-150, were swollen in 1 M acetic acid for 3 to 5 days, and the fine particles were repeatedly poured off. The columns were packed following the instructions of the manufacturer (Bio-Rad Laboratories). The initial separation was made by chromatography on Bio-Gel P-60 (100-200 mesh). For analytical purposes a 2.3 x 190 cm column was used and a 5.7 x 180 cm column was used for preparative scale. The flow rates were 0.072 and 0.038 ml/cm²/min respectively. Fifteen to 20 mg of protein were applied to the analytical column and up to 350 mg to the preparative column. A 5.0 x 85 cm Bio-Gel P-30 (100-200 mesh) was used for further purification of some of the components isolated by gel filtration on Bio-Gel P-60. For study of the fractions with a large hydrodynamic volume, a 3.8 x 140 cm Bio-Gel P-150 (100-200 mesh) column was used. In all cases the flow rate was kept constant by use of a Mariotte flask. The position of the protein in the eluent, 1 M acetic acid, was determined by measurements of the optical density at 280 nm. The fractions containing protein were pooled and concentrated in a rotary evaporator and lyophilized.

Chemical Studies

Ammonia determination in irradiated lysozyme at pH 5.50.

Lysozyme irradiated with 45.8 Mrad was dissolved in O_2 -free, NH_3 -free 0.05 M acetate buffer, pH 5.5, at a final concentration of 9 mg/ml. One ml of protein solution was applied to a Bio-Gel P-2 (50-100 mesh) column and eluted with 0.05 M acetate buffer. The flow rate was $3 \text{ ml/cm}^2/\text{min}$ and fractions of one ml were collected using a drop counter. The position of the protein in the eluent was determined by measurement of the absorbance at 280 nm in each fraction. The concentration of the ammonia was determined colorimetrically in each fraction with the phenolhypochlorite method (139). A similar Bio-Gel P-2 column was loaded with 1 ml of a solution containing 8 mg of native lysozyme and 20 μg of ammonia nitrogen in order to determine the elution position and recovery of each one.

Ammonia determination at pH 9.83. The determination of ammonia at pH 9.83 was carried out using a diffusion method (140). Standard Conway diffusion cells were washed with 1 N H_2SO_4 , rinsed with distilled water and dried in the oven. In the central compartment of each cell, 1 ml of 0.1 N H_2SO_4 was deposited, and in the external compartment the next solutions were added in the order mentioned: 1 ml of protein solution (2 mg/ml) in 5% SDS solution and 1 ml of 0.2 M $NaHCO_3/NaOH$ buffer, the final pH of the mixture being 9.82 to 9.85. A blank containing all the reagents except the protein was run with the protein samples. The determination of ammonia was made following the direct Nesslerization procedure as described by Folin

and Wu (140). In order to obtain a very high sensitivity, the absorbancies were measured at 420 nm using cuvettes with a path length of 2.5 cm.

Amino groups determination with 2,4,6-trinitrobenzenesulfonic acid (TNBS). The original method of Habeeb (141) for total amino groups in proteins was followed. The reaction was carried out in the presence of 2.5% SDS which kept the protein in solution and exposed the amino groups to the action of the TNBS. A pH of 9.83 was chosen because the possible SDS-amino group complex dissociates at pH values above 8.5 (141) in most proteins. Satake *et al.* (142) have shown that the reaction of TNBS with amino groups increases as the pH value rises, but at extremely alkaline conditions the TNBS begins to decompose, an observation verified by Scheele and Lauffer (143). At the pH used in these experiments, 9.83, no decomposition of TNBS was observed. The absorption spectra between 315 to 480 nm was measured in a spectrophotometer Cary 16K provided with cell holders for cells in tandem. In the blank holder, one cell contained the TNBS and the other cell the protein, both in the same buffer with SDS. In the sample holder one cell contained the protein plus the TNBS in buffer with SDS and the other cell only the buffer - SDS solution. This method was followed in order to avoid the possible interference due to new chromophores that can be present in the irradiated protein. The molar extinction coefficient for free amino groups by the TNBS method was calculated to be $1.08912 \pm 0.7\%$, a value that agrees very well with those given in the literature (141, 142). Between the

limits of error imposed by the method, no significant differences were observed in the amount of amino groups in native lysozyme reacting in the presence or absence of SDS.

Ninhydrin amino-nitrogen analysis. The method of Harding and MacLean (144) was used. The amount of amino-nitrogen was assumed to be proportional to the absorbance at 550 nm. During the heating process the samples developed some turbidity which made the method less reliable. The protein concentrations for the TNBS and ninhydrin methods were determined by the copper-binding capacity method (119).

Determination of sulfhydryl groups in irradiated lysozyme.

(a) Determination with the Ellman's reagent. The method described by Sedlak and Lindsay (145) using 5,5'-dithiobis-(2 nitro-benzoic acid) or Ellman's reagent (146) was followed without modifications. As a solvent for the reaction the 0.5% SDS solution was chosen. Protein concentrations were determined using the Cu-binding capacity method (119).

(b) Determination with mercury orange. The procedure described by Sakai (147) using 1-(4-chloromercurylphenylazo)-naphthol-2 or mercury orange was followed. The protein concentration was determined gravimetrically, and the molar extinction coefficient of 1.833×10^4 at 470 nm was used for the calculation of $\mu\text{moles -SH}$ as described in the original paper (147).

(c) Characterization of sulfhydryl groups in irradiated lysozyme by the Saville's method. The modification of the Saville's method as

described by Todd and Gronow (148) was applied to the irradiated lysozyme, but development of turbidity did not allow the use of the method for quantitative work. It was found that the addition of p-dioxane to the reaction slows the instantaneous precipitation observed in samples treated according to the original method. This allows the comparison of the spectra from the colored azo dye obtained with glutathione with the one obtained using irradiated lysozyme. This method permits only the detection of the presence of the sulfhydryl groups that are present in very small amounts in the irradiated protein.

Determination of carbonyl groups. The carbonyl content in irradiated lysozyme and in fractions from irradiated lysozyme was determined using the method described by Hamilton (149), the same that has been used for determination of carbonyl groups in proteins irradiated with 2 MeV electrons (66). The spectra of the chromophore produced showed two maximums, at 430 and 520 nm. The measurements at 520 nm were reproducible and independent of the presence of protein as could be evaluated from measurements made on protein samples containing pyruvic acid as an internal standard for carbonyl groups.

CHAPTER III

RESULTS

1. Fractionation of Gamma-Irradiated Lysozyme by Salt Precipitation Chromatography

In order to understand the mechanism of radiation inactivation of solid enzymes, a detailed knowledge of the altered products on both the physical and chemical level is necessary. However, the decrease in physical stability of irradiated proteins limits the fractionation procedures to those that cause a minimum of further denaturation in the molecule damaged by the radiation. In the present investigation, this fractionation has been made by a modification of the Maksimov method (99) which has been combined with a variety of gel chromatography procedures.

The salting-out process with NaCl of the irradiated lysozyme yielded two fractions, a salt insoluble fraction, F-I, and a salt soluble fraction, F-II. These have been found soluble in water and acid solutions with low ionic strength. In lysozyme irradiated *in vacuo* with 26 Mrad gamma radiation, the proportions of F-I and F-II were 39 and 61 per cent respectively. Analytical column chromatography on Bio-Gel P-60 in 1 M acetic acid whole irradiated protein and a mixture of F-I and F-II in the proportions found by the salting-out process showed similar elution profiles (Fig: 1). The only significant difference in the profiles was a slight increase in the amount of components eluting in the void volume for the reconstitute mixture accompanied by a corresponding decrease in the other peaks. This

FIGURE 1

Gel Column chromatography of whole irradiated lysozyme and a mixture of F-I and F-II.

quantitative difference indicates that the salting-out process caused a small increase in the formation of molecules with a large hydrodynamic volume component. However, the magnitude of this effect is not important. These results indicate that no significant alterations in the composition of the irradiated protein had been induced by the salting-out process.

Figure 2 shows the elution profile of the salt-insoluble fraction, F-I, resolved by gel chromatography on Bio-Gel P-60 using as eluent 1 M acetic acid. The data obtained confirms the previous results of Maksimov *et al.* (100) who used Sephadex columns. However, the use of a longer column and a better gel system allowed the resolution of more components, some with a hydrodynamic volume exceeding the corresponding volume of a globular protein with a molecular weight of 60,000 and eluting in the position of the void volume. For practical purposes F-I was subdivided into three components, F-IA, F-IB and F-IC, each of which was reanalyzed by gel chromatography on several different gels and by electrophoresis on polyacrylamide under different conditions. F-IA was analyzed by gel chromatography on Bio-Gel P-150 in an attempt to isolate the different species that form this component. Figure 3 shows a distribution of species from F-IA with some of the components eluting in a position near to that corresponding to the void volume. This indicates that those species have a hydrodynamic volume that corresponds approximately to the volume occupied by a globular protein of molecular weight 150,000. It is possible to observe in Figure 3 that the cross-contamination of the different peaks is very extensive, making the isolation of reasonably pure components impossible by the use of a one-cycle gel chromatography system. Fraction F-IB was

FIGURE 2

Gel column chromatography of salt insoluble fraction, F-I.

FIGURE 3.

Gel column chromatography of F-IA.

rechromatographed on Bio-Gel P-30, and about 90% of the material eluted in a single slightly asymmetric peak. The remaining 10% was eluted as a mixture of overlapping peaks. The main peak was labeled F-IB. The small components were not further studied. F-IC was not subjected to further gel chromatography because of the small amount.

The NaCl soluble material, F-II, was resolved by gel chromatography on Bio-Gel P-60 in 1M acetic acid to give two major components: the principle one accounts for 95 to 97 percent of F-II (Figure 4). The chromatographic pattern is the same for F-II subjected to one or three salting-out processes, indicating that the small components are not contaminants from the salt-insoluble fraction, but entities of F-II. The main component, denoted F-IIC, eluted in the position corresponding approximately to the elution volume of native lysozyme but in a very asymmetric peak that seems to be formed by at least two components. Further chromatography of F-IIC on Bio-Gel P-30 allowed the separation of one of the components which represented less than 5% of the original material applied to the column but failed to resolve the other components of F-IIC. The main component obtained from rechromatography on Bio-Gel P-30 was labeled F-IIC in order to avoid a more complex nomenclature.

The observed asymmetry of certain peaks from the gel chromatographic studies on irradiated lysozyme was not limited to the products from the irradiated protein, but was observed also in the gel chromatography of human gamma-globulin and native lysozyme under similar conditions as those used for the study of F-I and F-II components.

The interpretation of the Bio-Gel column chromatography results in terms of molecular weights of the various fractions is not possible.

FIGURE 4

Gel column chromatography of F-II.

The alteration of the hydrodynamic properties of these components, both by the irradiation and by the 1 M acetic acid solvent do not allow any reliable correlation of these results with molecular weights.

2. Electrophoretic Analysis of Gamma-Irradiated Lysozyme and Its Fractions Separated by Salt Precipitation and Gel Chromatography

(a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis: The reliability of molecular weight determinations by sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis has been extensively verified (129). With the help of this technique it has been possible to determine molecular weights of the various fractions isolated by salt precipitation and gel chromatography. Figure 5 shows a photograph of several typical SDS electrophoresis gels. A series of protein markers subjected to electrophoresis without the disruption of intramolecular disulfide bonds show an excellent linear relationship between the log of the molecular weight and the relative mobility with respect to lysozyme (Figure 6). These results agree with the observations of Dunker and Rueckert (129) that in the presence of the detergent, the chain unfolding of proteins has a very small effect on electrophoretic behavior. The data in Figure 6 also show molecular weights of components resolved by SDS electrophoresis of the whole irradiated lysozyme. The monomer and a series of aggregates of polymers of lysozyme with an apparent degree of polymerization between 2 to 7 were observed as well as a weak shadow at the correct location for an octamer (Figure 5). The mobilities of these resolved components also correspond to bands resolved by electrophoresis of the various F components separated

FIGURE 5

Sodium dodecyl sulfate polyacrylamide electrophoresis
of irradiated lysozyme and its various products.

FIGURE 6

Plot of the logarithm of the molecular
weights versus their relative mobilities.

as described above.

The molecular weights obtained from the corresponding calibration curve showed an error somewhat larger than that given in the literature (129). In order to eliminate the possibility of a charge effect due to the different amino acid composition of the markers used, the logarithm of the assumed aggregation number of the polymers was plotted against the relative mobilities of the polymers to give a good straight line relationship (Figure 7). The linear relationship is an indication that the molecular weights of the polymers are integral numbers of the monomeric lysozyme. While the data shows a good regression line drawn through the points, a slight downward curvature can be observed, especially at the concentration of gel used in this work. Wolf *et al.* (134) working with polymers of ribonuclease formed by reaction with diethyl pyrocarbonate have found the same deviation from linearity. In order to test the harmonic nature of radiation induced polymers by a different procedure, the method described by Wolf *et al.* (134) was used. The log of the molecular weight of the polymers was plotted as a function of the log of the assumed aggregation number, and the best straight line through the experimental points intercepted the ordinate at the monomeric value when the log n is zero (Figure 8). The value for the monomer was found to be 14,400, in good agreement with the value obtained from sequence studies, 14,307, (7,8), indicating that molecular weights of the aggregates are integral multiples of the monomeric value of lysozyme.

Figure 9 shows the relative amounts of the different aggregates present in the whole irradiated lysozyme as resolved by SDS polyacrylamide electrophoresis. At the dose used, 26 Mrad, the predominant

FIGURE 7

Plot of the assumed aggregation state of the polymeric forms of lysozyme versus their relative mobilities.

FIGURE 8

Procedure for molecular weight determination of the monomeric unit present in the polymers from irradiated lysozyme.

species was the monomeric form, and the amount of the different aggregates decreases as the aggregation number increases. The distribution of monomeric and polymeric forms of lysozyme in the salt insoluble fraction, F-I, is shown in Figure 10. It is possible to observe a large decrease in the monomer content due to the salting-out fractionation process. Figure 11 shows the profile for the pherogram of F-IA. The almost complete absence of monomeric form indicates that this fraction contains mostly polymeric forms. Figure 5 shows that components of F-IB are similar in molecular weight to those in the salt soluble fraction, F-II. However, the monomeric form of F-IB shows a salt solubility and elution volume in gel chromatography that does not correspond to the native lysozyme or to F-II isolated from irradiated lysozyme.

Figure 12 shows that the whole irradiated lysozyme after reduction of the disulfide bonds with 2-mercaptoethanol and alkylation of the thiol groups formed with iodoacetamide has only one band in the SDS polyacrylamide gel electrophoresis which corresponds to the monomeric form. In the same figure, the SDS electrophoretic pattern of products of whole irradiated lysozyme that has been treated with iodoacetamide but not reduced is shown. The similarity between this the non-reduced pattern and that for water soluble irradiated lysozyme without iodoacetamide treatment indicates that the aggregates are not formed during the incubation process with urea and sodium dodecyl sulfate that is part of this electrophoresis procedure, but that they are present in the dissolved irradiated lysozyme.

An estimation of protein from the densitometric scanning was made by working with very low concentrations of protein samples (less than

FIGURE 9

Gilford densitometer scan of polyacrylamide
gel phoregram of whole irradiated lysozyme.

FIGURE 10

Gilford densitometer scan of salt insoluble Fraction F-I.

FIGURE 11

Gilford densitometer scan of salt insoluble Fraction F-IA.

FIGURE 12

SDS polyacrylamide electrophoresis of

- 1 - Native lysozyme
- 2 - Reduced and alkylated native lysozyme
- 3 - Irradiated lysozyme
- 4 - Irradiated and alkylated lysozyme
- 5 - Irradiated, reduced and alkylated
lysozyme

20 μ g per gel) in order to avoid the saturation effect described by Wieme (150), and by assuming a linear dye-uptake, similar for all the products. The relative amount of protein in each band corresponding to an aggregate, calculated from the area under each individual peak, and the aggregation number have an exponential relationship which is characteristic of random aggregation processes (151).

(b) Polyacrylamide gel electrophoresis in 2.5 and 8 M urea: Electrophoresis on polyacrylamide gels can be used for analysis of fractions from gamma-irradiated lysozyme. Urea in the electrophoretic system maintains the protein in a soluble form, thus avoiding precipitation of irradiated fractions commonly observed when electrical current is applied to the system. This procedure thus yields reliable information on the unstable samples being analyzed. Native lysozyme is very resistant to denaturation by urea (152), but the damaged products in irradiated lysozyme should have a greater susceptibility to denaturation caused by urea at different concentrations. The resolution of the different components will then be the result of the electric charge carried by the molecules and the size of the molecules (137). Both characteristics can be altered by urea denaturation.

Polyacrylamide electrophoresis was carried out in the presence of 2.5 M urea, pH 2.7 and 8 M urea, pH 3.7. The pH values for the gels containing 0.9 N acetic acid were calculated from the dissociation constants for acetic acid and activity coefficients of protons in urea solutions given by Bull *et al.* (153). Figure 13 shows electrophoretic patterns of native lysozyme, salt insoluble fraction F-I and its component F-IB, and salt soluble fraction F-II, in the presence of 2.5

FIGURE 13

Patterns of phoregrams for native lysozyme,
F-I, F-IB and F-II in the presence of urea.

2.5 M Urea

8 M Urea

and 8 M urea, Native lysozyme is homogeneous and presents the highest electrophoretic mobility. Fraction F-I shows four bands with lower electrophoretic mobilities than native lysozyme. The increase in urea concentration to 8 M causes a decrease in the relative mobilities of the slower components that may be caused by an increase in size by further denaturation. The relative mobilities of the two fastest components are little affected by changes in urea concentration. The pattern of F-IB shows that this component is formed by the two bands of F-I with higher electrophoretic mobilities. The salt soluble fraction, F-II, shows somewhat different patterns for the two concentrations of urea. The main band of F-II in 2.5 and 8 M urea presents identical electrophoretic mobility as native lysozyme.

Electrophoresis of F-I and F-II in the presence of 2.5 M urea and in gels with different concentrations of acrylamide allows a distinction between components different in charge and those that are different in size. Figure 14 shows plots of the relative migration (R_m) with respect to native lysozyme versus the reciprocal of the acrylamide concentration for the bands of F-I and F-II. The two fastest moving bands of F-I which are also present in F-IB show the same relative mobilities at all the acrylamide concentrations, indicating that the difference between this lysozyme fractions and native lysozyme is only in charge (see ref. 137). Determinations of the molecular weights for the F-IB components with SDS-polyacrylamide electrophoresis has shown that this fraction is a monomer of lysozyme. The slower bands of F-I show a significant progressive retardation as the acrylamide concentration increases which must be caused by differences in size. Determinations of molecular weights for components of F-I have shown the

FIGURE 14

Plot of the relative migration (R_m) of F-I and F-II versus the reciprocal of acrylamide concentration.

presence of polymers of lysozyme, a finding that agrees with the results of electrophoresis in 2.5 M urea.

The main band of F-II pherogram has an electrophoretic mobility that is equal to the mobility of native lysozyme; therefore, it is possible to assume that this fraction is very similar to native lysozyme in charge and size. The slower bands from F-II show a small retardation effect at increased acrylamide concentrations. These data indicate the presence of a small fraction of monomeric forms in F-II that differ from each other and from native lysozyme in charge and to a lesser degree in size. These electrophoretic results agree with the gel column chromatography of the F-II fraction which shows an assymmetric peak eluting at the column corresponding to native lysozyme that cannot be resolved by use of Bio-Gels with different ranges of porosities.

3. Spectrophotometric Studies of Irradiated Lysozyme and Fractions from Irradiated Lysozyme

The ultraviolet spectroscopic properties of irradiated lysozyme are different from those of native lysozyme. The differences can be due to conformational and/or chemical changes in the structure of the native protein caused by the ionizing radiation. By the use of appropriate denaturing agents and solvent systems, it is possible to obtain difference spectra that can be interpreted in terms of conformational or chemical changes.

(a) Difference spectroscopy in the presence of guanidine hydrochloride (GuCl): The difference spectra of irradiated lysozyme and one of its components, F-IB, relative to native lysozyme, were studied in the presence of 5.4 M GuCl, 0.07 N HCl, pH 0.3. Under the conditions used the lysozyme structure is completely denaturated and any interactions between chromophore groups and other parts of the protein are eliminated (154). As a result of the extensive unfolding, all the chromophores are exposed to the action of the solvent and then intrinsic absorbances are normalized. Thus, any differences in the spectra can be assumed a result of formation of new chromophores or destruction of the original chromophores present in the native lysozyme. Figure 15 shows the difference spectrum of F-IB, the monomeric salt insoluble component, versus native lysozyme. A broad peak is observed between 293 and 450 nm. This peak is preceded by a trough between 265 and 293 nm. The peak which showed absorption in the visible region is undoubtedly caused by new chromophores that are absent in native lysozyme. From the spectral characteristics of tryptophan and tyrosine in acid

FIGURE 15

Difference spectra in the presence of 5.4 M guanidine hydrochloride, pH 0.30, of whole irradiated lysozyme and F-IB against native lysozyme.

solutions (155) it seems that the trough is an indication of extensive destruction of tryptophan and to some extent of tyrosine. In the difference spectra of whole irradiated lysozyme, the trough or negative peak and the positive peak due to the chromophore show a reduction (Figure 15), due mainly to the presence in the sample of a significant fraction of products which do not have extensive damage in their original amino acid composition.

The difference spectra of the irradiated lysozyme and some of its components were also measured in 4 M GuCl at pH 5.4. Under this condition native lysozyme is not completely denatured and the irradiated lysozyme and its components retain some of their "normal" structure. Figure 16 shows the difference spectra obtained the mentioned conditions. The same data were also plotted as recommended by Alexander and Hamilton (66) (Figure 17). The difference spectrum for F-IIC, the least damaged component, shows a negative peak around 293 nm which is probably due to a differential exposure of buried tryptophyl residues by the action of the solvent (154). The broad peak in F-IIC observed around 300 nm seems to be an artifact due to an increase in the scattering intensity caused by the presence of some aggregates. The modified plot after Alexander and Hamilton (66), shows an increase in the base line without a definite maxima (Figure 17), which supports the role of scattering as the source of the increase of the absorbance around 300 nm.

The different spectra for the salt insoluble fractions, F-IA or F-IB, are very different from those observed for the salt soluble material, F-IIC. In the salt insoluble fraction, the negative peak is quite different. It is shifted toward shorter wave lengths, a

FIGURE 16

Difference spectra in presence of 4 M
guanidine hydrochloride, pH 5.4 of whole
irradiated lysozyme and some of its com-
ponents.

FIGURE 17

Difference spectra in presence of 4 M guanidine hydrochloride, pH 5.4 of whole irradiated lysozyme and some of its components. Plot $\lambda^4 \Delta \text{abs.}$ versus the wave length.

characteristic that seems to be a result of destruction of chromophores that absorb around 280 nm combined with perturbation caused by the exposure of the chromophores to the solvent (154). Two positive peaks are observed in those samples located around 250 nm and 300 nm respectively. A peak around 250 nm was observed by Alexander and Hamilton (66) in bovine serum albumin irradiated with electrons. They concluded that this peak is an indication of a new chromophore. The broad peak observed around 300 nm could be the resultant of the chromophore previously observed by studies in 5.4 M GuCl plus the perturbation caused by the exposure to the solvent of the tryptophyl residues.

(b) Difference spectra caused by differences in acetic acid concentration: Maksimov *et al.* (100) have shown that the hydrodynamic properties of irradiated lysozyme in 1 N acetic acid are different from lysozyme in 0.1 N acetic acid. Application of difference spectroscopy to this situation gives information about environmental changes of some chromophores caused by alteration of the protein structure.

The difference spectrum for native lysozyme in 1 N acetic acid relative to a solution in 0.1 N acetic acid, Figure 18, shows positive peaks at 275, 283.5 and 292 nm. Such positive peaks can be developed by two different mechanisms (156). Chromophores on the surface of the lysozyme molecule, especially tryptophyl residues, are exposed to the solvent and as a result, changes in the solvent composition cause spectral perturbations. Another way to develop a positive difference spectrum is by burying the exposed chromophores inside the protein via conformational changes or an aggregation. Under the conditions used here, burying processes are unlikely to occur. The positive peaks

FIGURE 18

Difference spectra of lysozyme, irradiated lysozyme, F-IA, F-IB and F-II caused by changes in acetic acid concentration. (Reference = 0.1 N acetic acid, Sample = 1 N acetic acid.)

observed above 270 nm are probably the result of solvent perturbations around the four exposed tryptophyl residues caused by the increase in concentration of acetic acid and not the result of major structural alterations in the protein molecule.

The difference spectrum for the salt soluble fraction, F-II, under the same conditions shows a pattern very similar to that obtained for the native enzyme. These spectral characteristics indicate that the structure of F-II is very similar to the native enzyme and that no appreciable weakening of the forces responsible for positioning of the chromophores has occurred. The acetic acid difference spectra for F-IA and F-IB, show a pattern that is almost opposite to that for the native lysozyme. Troughs are present around 275, 284 and 292 nm which are presumably caused by a change in the environment of buried tryptophyl residues now exposed to the solvent (154). These spectroscopic characteristics indicate that 1 M acetic acid causes extensive unfolding of these damaged fractions.

The $\Delta\epsilon$ at 292 nm has been postulated to be a measure of the extent of the unfolding or denaturation of lysozyme (157). Comparison of this value for the polymer fraction F-IA with the salt insoluble monomers F-IB suggests that F-IB is more unfolded or that the chromophore groups responsible for this peak are less hindered from the contact with the solvent than in F-IA. As a result a small degree of unfolding of F-IB can cause an extensive exposure of the chromophore groups which are not well hindered.

The acetic acid difference spectrum of the whole irradiated lysozyme seems to be the result of an addition of partial contribu-

tions of F-I and F-II components' difference spectra as would be expected.

(c) Perturbation difference spectroscopy with polyethylene glycol

(PEG): Perturbation difference spectra in the presence of polyethylene glycol (d.p. = 6) allowed the measurement of the degree of tryptophyl exposure in the native and irradiated lysozyme (Fig. 19). The exposure of tryptophyls in native lysozyme is pH independent, indicating that the lysozyme conformation is very rigid and that the same fraction of tryptophyls is exposed both in the monomer and in the aggregates (124). In order to avoid any interference in the measurements due to the aggregation, all the experiments were made at pH 5.0 which is the upper limit for the pH region where native lysozyme is monodisperse (36).

Table I shows the degree of tryptophyls exposure $[\Delta\epsilon/\epsilon]_{\text{rel}}$, and the relative change with respect to the native lysozyme values for different doses of gamma rays. The value for the relative exposure in native lysozyme, 0.50, agrees very well with the data reported by Williams *et al.* (124); 0.49, working under similar conditions. The data shows a decrease in the exposure of tryptophyls to the perturbant, PEG, which is exponentially related to the doses. These changes indicate the removal of tryptophyls from contact with the solvent which can be caused by an aggregation process which restricts tryptophyls exposure.

(d) Spectrophotometric analysis of tyrosine residues: Ionization of the phenolic group present in tyrosine residues into phenoxide ion by alkali causes a shift in the absorption band of the tyrosine residues

FIGURE 19

Perturbation spectra of irradiated lysozyme with
PEG as perturbant.

TABLE I

Relative Exposure of Tryptophyl Residues, $[\Delta E/E]_{rel}$,
and Ratio of the Exposures in Native and Gamma-irra-
diated Lysozyme with Polyethylene Glycol as perturbant

Dose Mrad	$[\Delta E/E]_{rel}^*$	$\frac{[\Delta E/E]_{rel} \text{ Irrad.}}{[\Delta E/E]_{rel} \text{ Nat.}}$
0	0.500	1.000
5	0.463	0.927
10	0.454	0.907
15	0.440	0.880
20	0.423	0.854
30	0.393	0.785
50	0.356	0.712

*Exposure estimated relative to the model mixture
analogue: N-acetyl-L-tryptophan ethyl ester = 4.0×10^{-4} M,
N-acetyl-L-tyrosine ethyl ester = 2.0×10^{-4} M.

from 275 nm to wave lengths around 295 nm (126). In lysozyme one of the tyrosine residues is bound and can be ionized only under very alkaline conditions, $\text{pH} > 13$. The process can be accelerated by the denaturing action of guanidine. It is impossible to measure the number of free and bound tyrosine groups in irradiated lysozyme because of the rapid precipitation in alkaline solutions. In order to dissolve the protein at $\text{pH} 12-13$ guanidine hydrochloride at a final concentration of 4 M is needed.

Figure 20 shows the difference spectra between alkaline and neutral solutions of native lysozyme, irradiated lysozyme and some of its fractions. The results obtained can be calculated into terms of total tyrosine per molecule of lysozyme. The number of tyrosine residues in native lysozyme was calculated using the $\Delta\epsilon$ reported by Kurihara (128) and Eldhoch (127) is in perfect agreement with the three residues found by amino acid sequence studies (7,8). Irradiated lysozyme (26 Mrad) and the component F-IIC showed 3.10 and 3.00 tyrosine residues per molecule respectively. The difference spectra for these samples show a progressive disappearance of the peak around 287 nm and an increase in absorption around 275 nm. The salt insoluble components F-IA and F-IB showed an apparent increase in tyrosine content, the calculated value being 3.65 for F-IA and 3.51 for F-IB. Examination of the curves show that the F-I components give alkaline absorption spectras somewhat different from the ones for native lysozyme, irradiated lysozyme or the F-IIC component.

These spectra indicate the formation of new chromophores by radiation which absorb strongly under alkaline conditions. These abnormal

FIGURE 20

Difference spectra of lysozyme, irradiated lysozyme, F-IA, F-IB and F-IIC, caused by change in pH. (Reference pH 7.00, Sample pH $\approx$ 13.)

difference spectra can explain the apparent increase in tryptophan reported for irradiated lysozyme when this amino acid residue was evaluated from spectrophotometric measurements in strong alkaline conditions (78).

4. Enzymatic and Chemical Studies of Irradiated Lysozyme and Fractions from Irradiated Lysozyme

(1) Enzymatic studies: One of the most studied effects of ionizing radiation on enzymes is the change in enzymatic specific activity. The decrease in the catalytic properties of enzymes caused by irradiation are undoubtedly the result of many kinds of alterations in the protein structure. In many molecules, the alterations do not cause significant changes in the chemical or physical chemical properties of the protein, but the enzymatic properties which may be very sensitive will show that the enzyme has been damaged.

(a) Apparent affinity constant (K_a app.) and maximum velocity (V_{max}): The K_a app. for native lysozyme, irradiated lysozyme and fractions from irradiated lysozyme were determined by the method of Locquet *et al.* (121). The conditions recommended by Davies *et al.* (122) were followed in order to obtain the maximum lytic activity from the enzyme. The pH for the enzyme assay was 6.2 and the ionic strength (I) was near to 0.05.

Figure 21 shows that native lysozyme, irradiated lysozyme and the salt soluble fraction, F-II, from irradiated lysozyme have the same K_a app., 159 mg/l of *M. lysodeikticus* cells. However, an inhibition of the lysis by an excess of substrate was observed under the conditions

FIGURE 21

Variation of $1/v$ vs. $1/[S]$ for lysozyme, irradiated
lysozyme and F-II.

FIGURE 22

Variation of $1/v$ vs. $1/[S]$ for salt insoluble
fraction F-I.

used for the assay. This inhibitory effect by the substrate was not apparent under the conditions used in the original method (121), but Sharon (158) had reported an inhibitory effect of the substrate similar to the one described here. From the data plotted in Figure 21 it is apparent that whole irradiated lysozyme is more susceptible to the substrate inhibition than fraction F-II. The native lysozyme is the least susceptible of the three samples to inhibition. Substrate inhibition is presumably caused by nonspecific adsorption of the positively charged enzyme to the negatively charged cell surface (158). The maximum velocity ($V_{\max}$) and the percent enzymatic activity relative to native lysozyme for irradiated lysozyme and F-II are shown in Table II.

It was impossible to obtain meaningful data for the salt insoluble fraction, F-I, and its components. Figure 22 shows the relationship between $1/V$ and $1/[S]$ for F-I. The inhibitory effect caused by high concentrations of substrate and the flocculation effect described by Friedberg and Avigad (159) for assays of highly concentrated lysozyme solutions gave curves that cannot be used to calculate $V_{\max}$ and K_a app. Flocculation (159) was present even at very low concentrations of enzyme, 10 $\mu\text{g/ml}$, and the flocculation as measured by the increase in turbidity showed different patterns depending on the component from F-I.

(b) Spectrophotometric study of the enzyme-substrate complex of lysozyme with glycol chitin: Application of difference spectrophotometric methods to the reaction between lysozyme and the artificial substrate, glycol chitin, allowed the determination of some of the features of the enzyme-substrate complex for irradiated lysozyme and its components

FIGURE 23

Difference spectra of the enzyme-substrate complexes of glycol chitin with lysozyme, irradiated lysozyme, F-IA, F-IB and F-II.

(See Figure 23). Difference spectra were measured under conditions which permit the "saturation" of the enzyme by the substrate (21). The difference spectrum for the complex corresponding to native lysozyme presents the pattern described by Hayashi *et al.* (21) in which it is possible to see three peaks located at 276, 284 and 292.5 nm (Figure 23). Irradiated lysozyme and the F-II enzyme-substrate complexes show the same spectroscopic characteristics that are in the native lysozyme complex. However, there are quantitative differences.

In contrast, the difference spectra for the complexes corresponding to F-IA and F-IB present patterns very different from the one observed with native lysozyme (Figure 23). In the F-IA difference spectrum there is a shift of the peaks toward shorter wave lengths with a concurrent disappearance of the peak at 276 nm. The new positions are 283 and 292 nm, and both peaks present almost equal magnitude. In the pattern corresponding to F-IB, all the characteristics observed with the enzyme substrate complex for native lysozyme have disappeared. There are two peaks at 282.5 and 290.8 nm, and the peak at 276 is absent. The ratio of the peaks is approximately opposite to the ratio observed in the native lysozyme complex.

Hayashi *et al.* have shown that the difference intensity at 292.5 nm is proportional to the enzyme activity measured under various circumstances. In Table II the $\Delta\epsilon_{292.5}$ and the relative enzyme activities with respect to native lysozyme are shown. In the same Table, these results are compared with those obtained from assays of the lytic activity. There is a surprisingly good correlation.

(2) Chemical studies: The role of disulfide bonds in radiation inactivation of enzymes has been discussed by many authors (41,72). Only

TABLE II
Enzymatic Characteristics for Native Lysozyme, Irradiated Lysozyme and F-II
from Irradiated Lysozyme (Dose = 26 Mrad)

Enzyme	<i>M. lysodeikticus</i> *		Glycol chitin**		%E.A.(lysis)
	V _{max} . mg/l/sec.	% Enzymic activity	$\Delta\epsilon_{292.5}$	% Enzymic activity	%E.A.(diff. spect.)
Native lysozyme	2.00	100.0	1100	100.0	1.000
Irradiated lysozyme	1.39	69.5	814	74.0	0.940
F-II frac.	1.72	86.0	930	84.5	1.018

*Suspension of *M. lysodeikticus* in 0.01 M Na phosphate - 0.04 M NaCl buffer, pH 6.2, Ionic strength $\approx$ 0.05.

**Glycol chitin 0.2% in 0.1 M Na acetate buffer, pH 4.0, Ionic strength $\approx$ 0.018.

two papers (78, 160) deal with this subject relative to lysozyme, and they present quite different data about the formation of thiol groups. Under these circumstances further data on the formation of thiol groups is very desirable.

The Garrison theory of radiation damage to peptides suggests chain breaks at the peptide bond level with the corresponding formation of ammonia and carbonyl groups in the protein. There is only one report on this subject for a globular protein irradiated in the dry state (66). In the present investigation assays for these groups are presented.

(a) Sulfhydryl groups in irradiated lysozyme: Stevens *et al.* (78) have reported that no thiol groups were detected in solid gamma irradiated lysozyme. In this study, three methods, two quantitative and one qualitative have been used to measure free -SH groups in the irradiated protein. The modified Salville's method (148), a qualitative method for sulfhydryl groups, indicated the formation of a chromophore in irradiated lysozyme which has the same spectral characteristics as the chromophore obtained with reduced glutathione. Taking into consideration the specificity of this reaction, it is concluded that irradiated lysozyme possesses sulfhydryl groups.

Determination with the Ellman reagent of irradiated lysozyme freshly dissolved in O_2 -free water showed at 26 Mrad that there are 0.52 thiol groups per molecule (based on a mol. wt. for lysozyme of 14,300); G_{-SH} , 1.35. In contrast the number of sulfhydryl groups per molecule in fractions F-I and F-II is 0.12 and 0.05 respectively. Sulfhydryl groups in the irradiated lysozyme measured with mercury orange yielded different values from that obtained with the Ellman's

reagent. The number of sulfhydryl groups in a freshly dissolved solution of irradiated lysozyme was 0.22 per molecule. For a solution that was allowed to stand at 4° for 5-6 hours, that content was 0.164. This decrease indicates that these sulfhydryl groups are labile, an effect similar to that described by Alexander and Hamilton (66) in bovine serum albumin irradiated with electrons. The G value for the formation of sulfhydryl groups calculated from analysis of the fresh solution of irradiated lysozyme with the mercury orange reagent was 0.57. The lower G value obtained with the mercury orange could arise from steric factors as suggested by Bennett and Watts (161), or by interference of non-reacting sulfhydryls as was shown by Cannefax and Freedman (162). The G values for the formation of sulfhydryl groups reported in the literature show a spread distribution ranging from 1.41, reported by Hunt and Williams (163) working with ribonuclease, to 0.5, reported by Kay and Hutchinson (76) for the same enzyme. Dose (160) has reported a G value for the formation of sulfhydryl groups in solid lysozyme irradiated with X-rays of 0.9. However, the solid irradiated protein was dissolved in H₂O containing oxygen which can reoxidize some of the sulfhydryl groups. It is probable that the higher G value reported here, 1.35, obtained with the Ellman procedure is the more reliable.

(b) Amino group determinations and formation of ammonia: Table III shows the results of assay for free amino groups in native lysozyme, irradiated lysozyme and fractions of irradiated lysozyme. The groups were determined by two different methods, the method of Habeeb (141) using trinitrobenzenesulfonic acid (TNBS), which gave the most reliable figures, and the ninhydrin method of Harding and MacLean (141), which

TABLE III

Distribution of Free Amino Groups and Free Ammonia in Native Lysozyme, Irradiated Lysozyme and Fractions from Irradiated Lysozyme (Dose = 26 Mrad)

Sample	TNBS method			Ninhydrin method		NH ₃ /mole
	$\lambda_{\text{max.}}$ (nm)	-NH ₂ /mole	% -NH ₂ of Native	-NH ₂ /mole	% - NH ₂ of Native	
Native lysozyme	340	7.00	100	---	---	0
Irrad. lysozyme	340	7.15	102	---	---	0
F-I	340	7.07	101	7.0	100	---
F-IA	328	14.10	202	13.7	195	11.2
F-IB	340	6.80	97	6.8	97	2.6
F-IC	330	10.20	146	10.8	155	8.2
F-II	340	6.90	98	---	---	---
F-IIB	332	10.20	146	---	---	3.3
F-IIC	340	7.10	101	---	---	---

could be used only on F-I and its components. There is fairly good agreement between the results obtained with both methods. With the TNBS method, the wave length for the maximum in the chromophore is different for the reaction products of amino groups and free ammonia, the maximum being 340 nm and around 330 nm respectively. This difference is not observed when ninhydrin is used as the reagent in the method of Harding and MacLean. The native lysozyme, whole irradiated lysozyme and some components do not show significant differences in the total number of free amino groups, 7 per molecule, and the maximum absorption was 340 nm. In contrast the values for the apparent content of amino groups in the components of F-I and one component of F-II showed a definite increase, and the maximum for the spectra was shifted to values around 330 nm (See Table III) indicating possible free ammonia in the samples. Determination of free ammonia at pH 5.5 by the gel filtration method described under Experimental Procedures gives negative results and no indication for free ammonia was found with lysozyme irradiated up to 46 Mrad. Determinations of ammonia by the diffusion method with conditions similar to those used for the TNBS method of amino groups yielded different results (Table III). The ammonia increase was significant for samples that gave apparent higher content of amino groups than the normal value. It is interesting to note that the ammonia was present only in samples subjected to multiple fractionation steps.

(c) Carbonyl groups: The determination of carbonyl groups in irradiated lysozyme and its components were made using dinitrophenylhydrazine and spectrophotometric readings at 520 nm (149). In the whole irradiated lysozyme, dose = 26 Mrad, 0.28 carbonyl groups per molecule

TABLE IV

Distribution of Carbonyl Groups in Solid Lysozyme
Irradiated with 26 Mrad of Gamma-rays and in Some
of its Components

Sample	Carbonyl groups [*] per molecule	
Native	0.00	
Irradiated	0.28	
F-IA	0.45	
F-IB	0.42	F-I _{average} ^{**} = 0.45
F-IC	0.49	
F-IIC	0.17	F-II $\cong$ F-IIC = 0.17

*Average of duplicates with and without internal standard.

**Average calculated from F-IA, F-IB and F-IC values.

were measured. From this figure a G value of 0.72 for the formation of carbonyl groups was obtained, a value that is very close to the G value of approximately 0.8 reported by Alexander and Hamilton (66) for the formation of carbonyl groups in electron-irradiated solid bovine serum albumin. The components from F-I showed a higher number of carbonyl groups per molecule than the main component, F-IIC, of the salt-soluble fraction, Table IV. The number of carbonyl groups in irradiated lysozyme, 0.28, is equal to the number of carbonyl groups calculated on the basis of the partial contributions of F-I and F-II, 0.28. The number of carbonyl groups in F-I was the average of its three components, and the number of carbonyl groups in F-II is almost exclusively due to the component F-IIC. This balance indicates that the carbonyl groups are not produced during the fractionation of the irradiated lysozyme.

5. Fractionation of Irradiated Lysozyme by Ion Exchange Chromatography

The ion exchange chromatographic methods have been used for the fractionation of irradiated enzymes (92). However, a reevaluation of the method used by Stevens *et al.* (93) and Kravchenko *et al.* (94) for the ion exchange chromatography of the water-soluble products from irradiated lysozyme indicates that the fractionation made with phosphate buffer and ion exchange chromatography on resin Bio-Rex 70 causes further changes in the products. In this study, a material for modified substances from the solid irradiated lysozyme obtained with Stevens' fractionation procedure is as follows,

- I. Water-soluble irradiated lysozyme (26 Mrad): 100%
- A- Phosphate insoluble lysozyme: 24%

B- Phosphate soluble lysozyme:	76%
1. Irreversible bound to resin:	37%
2. Eluted from resin:	39%
a- Eluted near native lysozyme:	28%
b- Other components:	11%

A significant amount of material was irreversibly precipitated during the fractionation procedure with phosphate buffer indicating that the most damaged fractions have suffered further changes. The solubility properties of this fraction are different from the ones observed for the fraction F-I or NaCl-insoluble fraction obtained by the method of Maksimov *et al.* (99).

On chromatography on Bio-Rex 70 resin, part of the phosphate-soluble irradiated lysozyme was bound to the resin and could not be removed by a linear gradient of urea at pH 7.12. The irreversible binding of proteins to the resin has been described for some labile plasma proteins (164). Extraction of the bound protein by a batch-wise procedure indicated that the release occurred with urea at pH 10-11. These results agree with the findings of Stevens *et al.* (165) who observed that the bound protein can be removed at pH 13 or more, in the absence of urea. The conditions used in this method will certainly cause significant alterations to the protein. The product is a result of a sequence of denaturation events: (1) action of the gamma rays, (2) phosphate buffer effects, (3) interactions between the protein and the resin, and (4) denaturation caused by high pH. Because of these problems and the poor yield of product material, this procedure was not further studied.

6. Conformational Studies of Irradiated Lysozyme with Tritium-Hydrogen Exchange

The destruction of enzymatic activity in irradiated lysozyme indicates the alteration of the protein structure at the conformational level. Whether conformation changes are direct effects or mediated only through chemical changes is an interesting question. Linderstrøm-Lang (166) postulated that measurements of the rate and extent of hydrogen exchange by deuterium or tritium in proteins provide information about their conformational and chemical structure. If this assumption is correct, determination of exchange curves at different doses of irradiation will provide information about structural changes in lysozyme caused by the ionizing radiation.

The determinations were made using Englander's technique as described before under Experimental Procedures. Tritiated lysozyme (lysozyme-T) was prepared, 0.1815 $\mu\text{c}/\text{mg}$. The possible damage caused by the β -particles from tritium is negligible in this experiment since this radioactivity level gives a dose of only 53 rad/day, and the experimental radiation levels used were in the megarad range. Considering the time needed for the irradiation and analysis of the samples, the damage would not be significant.

The total number of theoretically exchangeable hydrogens, H_e , in native lysozyme was computed from the amino acid composition of the enzyme (7). The experimentally determined number of total exchangeable hydrogens in native lysozyme, 272, agrees very well with the theoretical number of 274 exchangeable hydrogens, assuming that the pH is low enough to keep all the acid groups protonated.

Figure 24 shows the exchange curves for native lysozyme and lysozyme irradiated with 10 and 30 Mrad. The data shows that radiation increases the lability of the exchangeable hydrogens. Application of the mathematical analysis described by Englander (123) to these curves allows the calculation of the different kinetic classes of hydrogens, which have been related to structures in the protein molecule. The results are shown in Table V and are averages of at least two determinations. In native and irradiated lysozyme, the half-times of exchange for the different classes are very similar. For both materials the class I hydrogens exchange in a few seconds, and they are supposedly related to groups in the protein that are intrinsically fast exchangers and are not inhibited by local structures. The class II hydrogens exchange with a half-time of 7 to 9 minutes, and from experiments made with oxidized ribonuclease (123) this class seems to represent relatively unhindered peptide groups. Class III hydrogens exchange with a half-time of 2 to 2.5 hours. Englander (123) proposed that class III hydrogens are exchangeable hydrogens in the α -helical structure. Class IV hydrogens in lysozyme, as in ribonuclease, exchange very slowly, and measurements of lysozyme exchange at different pH values (123) indicating that they are associated with masked hydrogens of amino side chains buried in the interior of the protein. By comparison with the results of X-ray analysis of proteins, it is possible to postulate that this class represents an approximate measure of the hydrophobic regions in proteins. Evaluation of the apparent α -helix content from the size of class III hydrogens yields 34%, a value that is in good agreement with the 35% calculated from optical rotatory dispersion (17). The size of class I, 76 hydrogens, is higher than the corresponding

FIGURE 24

Rate of tritium hydrogen exchange in lysozyme
and irradiated lysozyme.

TABLE V
Size of the Kinetic Classes of Hydrogens and Ratio of the Classes in Native
and Irradiated Lysozyme

Sample	Class I*		Class II		Class III		Class IV	
	Size	Ratio	Size	Ratio	Size	Ratio	Size	Ratio
Native	127	1.00	21	1.00	43	1.00	76	1.00
Irrad. 10 Mrad	136	1.07	31	1.48	40	0.93	60	0.80
Irrad. 30 Mrad	132	1.04	60	2.86	28	0.65	47	0.62

*The data related to the exchange of class I hydrogens were calculated assuming that the total number of exchangeable hydrogens, H_e , and the number of protons dissociated are not altered by the irradiation.

value obtained with the Linderstrøm-Lang (18) method that involves the use of cryosublimation. Hydrogens of class I do not show significant alterations as a function of the radiation dose, however, in these calculations it has been assumed that the total number of exchangeable hydrogens, H_e , and the number of dissociated protons in the protein are not altered by the irradiation. As will be discussed later, these assumptions seem to be incorrect. The class II hydrogens show an increase in the number which is a function of the radiation doses. Class III and IV show an almost equal decrease that is exponentially related to the dose. The decrease of class III means disruption of the secondary structure, especially α -helix, information that is qualitatively similar to that reported by Stevens *et al.* for the deuterium exchange properties of irradiated lysozyme.

7. Dependence of the D_{37} Value on the Enzymatic Assay Conditions

It has been shown for many irradiated enzymes that the relationship between dose and change in enzymatic activity is semi-logarithmic over a limited range. The sensitivity of the enzyme to radiation can be expressed also in terms of the D_{37} value. This relationship has been used as an experimental basis to calculate inactivation cross sections of enzymes or a "sensitive molecular weight" as proposed by Lea (47). Many of the molecular weights calculated with this method from experiments made at room temperature are in fair agreement with the molecular weights measured by physical-chemical methods (51).

A typical inactivation curve for lysozyme irradiated *in vacuo* with gamma rays is shown in Figure 25. In the same figure "sensitive molecular weights", using Hutchinsons constant (49), are given as a

FIGURE 25

Inactivation of lysozyme by action of the
gamma rays. (Determination of enzyme activity
at pH 7.00.)

function of the pH used in the assay. The calculated molecular weight is in all cases larger than the true molecular weight of lysozyme.

A comparison of the D_{37} values for solid lysozyme reported in the literature and those reported here (Table VI) shows a spread in the results even when the enzyme has been irradiated with radiations of similar LET. Figure 26 shows that if the enzyme is dissolved in water there is less radiation loss than if it is dissolved in buffer. This effect can be most easily interpreted as a substrate protective effect combined with a delayed precipitation effect. Irradiated lysozyme is nearly completely soluble in water and is stable in solution. Irradiated lysozyme dissolved in the buffer is not so stable and begins to precipitate. Thus when the water solution is added to buffer and substrate, it only then begins to precipitate and the precipitation is delayed by the substrate. This protective effect by the substrate was at a minimum at pH 5.0, the same pH at which irradiated lysozyme dissolved in water had its maximum sensitivity (Figure 25). The inactivation curve for irradiated lysozyme dissolved in buffer (Figure 26) shows a very good qualitative correlation with the solubility curve for gamma-irradiated lysozyme as a function of pH as reported by Maksimov *et al.* (100).

The data presented here suggest that part of the irradiated enzyme is not precipitated in the presence of a substrate or substrate analogue and is able to hydrolyse cell walls of *M. lysodeikticus*. Solubility studies confirm this assumption. Products with low polar character like dioxane, glucose, and ethyl alcohol do not have significant effects on the precipitation process (Figs. 27, 28). However, the precipitation of the irradiated lysozyme was slowed down by the presence of compounds

TABLE VI
Values of D_{37} for Dry, O_2 -free Irradiated Lysozyme*

Radiation	pH	D_{37} (Mrad)	Reference
γ	3 to 8 ^a	31-46	This study
γ	7.1 ^b	65	Stevens <i>et al.</i> (78)
γ	7.1 ^c	50	Dose <i>et al.</i> (106)
d^+	7.1 ^b	50	Brustad (182)
γ	not reported	41	Shalek <i>et al.</i> (183)
e^-	7.1 ^b	44	Fluke (55)

*Assayed using *M. lysodeikticus* cells.

a- 0.06 M citric acid, 0.12 M Na_2HPO_4 , 0.05 M NaCl.

b- M/15 Na-phosphate buffer.

c- 0.15 M phosphate-citrate buffer.

FIGURE 26

Effect of the pretreatment of irradiated
lysozyme on enzymatic activity.

FIGURE 27

Effects of different chemical compounds on
the precipitation rate of irradiated lysozyme
(pH $\sim$ 5.0).

FIGURE 28

Effects of substrate-like compounds on
precipitation of irradiated lysozyme,
pH $\sim$ 5.0.

FIGURE 29

Effects of substrate-like compounds on
precipitation of irradiated lysozyme,
pH $\sim$ 6.4.

that can act as substrates or as inhibitors to the function of lysozyme (Figure 29). This effect was more significant at pH 6.30 than at 5.40. In relation with this effect, Sophianopoulos (37) has found that inhibitors of lysozyme interfere with the normal aggregation process observed for native lysozyme at pH values higher than 5.00.

The variation of D_{37} value as a function of the pH used for assay would indicate a possible shift of the optimum pH for the lysis of *M. lysodeikticus*. Under the experimental conditions used for the assays, the optimum pH for lysis with native lysozyme was around 6.0. This value is in very good agreement with the value reported by Shinitzky *et al.* (120) working under similar conditions. It did not show significant changes for lysozyme irradiated with doses up to 60 Mrad gamma rays (Figure 30).

In the presence of higher ionic strength solutions, the optimum pH is displaced toward lower values as shown in Figure 31. The increase in ionic strength also causes an inhibitory effect on the lytic activity of lysozyme (122). This effect is observed also with irradiated lysozyme (Figure 31), except that the irradiated lysozyme appeared to be more sensitive. Thus, for the conditions given in Figure 31, the irradiated lysozyme (46 Mrad) showed 52% inhibition, whereas native lysozyme showed 30% inhibition when the assays at high and low ionic strength are compared.

8. Apparent Activation Energy for Lysis of *M. lysodeikticus* Cells by Gamma-Irradiated Lysozyme

Studies of reactions catalyzed by two or more enzymes show that in many instances a different activation energy is found for each

FIGURE 30

Influence of pH on the activity of irradiated lysozyme.
Buffers: Mixtures of 0.12 M Na_2HPO_4 and 0.06 M citric acid, containing 0.05 M NaCl.

FIGURE 31

Influence of pH and ionic strength on the activity of native and irradiated lysozyme.

Buffers: A) 0.12 M Na_2HPO_4 - 0.06 M citric acid
0.05 M NaCl

B) 0.24 M Na_2HPO_4 - 0.12 M citric acid
0.10 M NaCl

enzyme (167). Ionizing radiation causes chemical and structural alterations that result in products with different enzymatic properties such as changes in the Michaelis constant (105), or unusual increase in the enzymatic specific activity (94). If the enzymatic properties of irradiated lysozyme are very different from the native lysozyme, an alteration of the heat of activation might be expected. However, the activation energy for lysis of cells cannot be interpreted in a mechanistic sense due to the complexity of the lytic process, the absence of corrections for effects of temperature on the ionization of groups in the enzyme or the cell surface, and other factors. Nevertheless, activation energy can give an indication of alteration in any of the variables.

The apparent activation energy for the lytic activity of gamma-irradiated lysozyme dissolved in water has been measured at three pHs with a very short exposure to the heat action, less than 150 sec. The values are given in Table VII. Figure 32 shows a characteristic Arrhenius plot for the lysis of *M. lysodeikticus* by irradiated and nonirradiated lysozyme. There are no significant changes in the values for the apparent activation energies with increasing doses of gamma radiation.

FIGURE 32

Arrhenius plot for the lysis of *M. lysodeikticus* by
.irradiated and nonirradiated lysozyme.

TABLE VII
Apparent Activation Energies for Lytic
Activity of Gamma-irradiated Lysozyme

Dose Mrad	E _a app. cal/mole*		
	pH 4.62	5.54	6.51
0	7300	9360	11,550
10	7290	9340	11,490
20	7290	9280	11,390
30	7380	9400	11,650

*Calculated from lytic activity measurements between 25° and 45°C. Enzyme concentration: 6.6 µg/ml.
M. lysodeikticus cells: 0.3 mg/ml in 0.12 M Na₂HPO₄,
0.06 M citric acid, 0.05 M NaCl buffers.

CHAPTER IV

DISCUSSION

The effects of ionizing radiations on enzymes have been partially explained by several mechanisms, all more or less independent from each other. These mechanisms can be classified in groups, depending on whether the inactivation results from a propose random or specific chemical or conformational change. Changes in nonspecific covalent bonds will result in nonspecific denaturation or random conformational changes. Following the primary interaction of radiation with matter, this nonspecific damage can originate in any of several ways. Thus, at the site of primary ionization or excitation, covalent bond changes may occur. Pollard *et al.* (51) have suggested that a volume of high excitation, perhaps a positively charge region, can migrate from bond to bond along the polypeptide chain and occasionally permit a valence bond to open. Platzman and Frank (50) postulated that the ionization process would give rise to a polarization wave which can cause disruption or many weak polar bonds. In both mechanisms the breakage of bonds by the excess of energy may be a random process or it may be fairly specific.

Some authors postulate that the enzyme inactivation is caused by rather specific lesions in certain groups of the protein. According to Augenstine's hypothesis (50), enzyme inactivation by physical methods such as heat or ultrasound or radiation is the result of a sequence of specific modifications of the protein conformation. Such a

mechanism implies a nonrandom disruption of bonds which are weak but essential for the maintenance of protein structure and, specifically, topography of the active site. Augenstine emphasizes the importance of disruption of disulfide bonds in this model because these bonds may be broken by energy absorbed in different parts of the protein and then localized at the disulfide links by an energy transfer process.

The formation of free radicals is considered one of the important mechanisms by which the enzyme structure is damaged. The work of Gordy (65), Riesz *et al.* (68) and other authors has shown that irradiated solid proteins have a large number of stable free radicals. These radicals have been classified into two groups, the glycine-like radicals and cystine radicals (65). The glycine-like radicals seem to be distributed among most of the amino acid residues (68). The cystine radicals are fairly distinct. Henriksen (72) proposes and presents data to show that the cystine radicals are the product of energy transfer processes. Ormerod and Singh (71) propose that they are created by the capture of secondary electrons by the cystine residues. In both cases, cystine radicals arise from primary processes occurring in sites different from the cystine residues. Both theories complement Augenstine's theory. However, the cystine residue role is absent in proteins that do not contain cystine, but they still are inactivated by radiation at comparable sensitivities.

From these and other studies on protein inactivation by radiation, it seems likely that most inactivation mechanisms occur concurrently and are interrelated. The disruption of secondary bonds like hydrogen bonds, Coulombic forces, and hydrophobic interactions, may or may not

occur independently of alterations of the primary structure including changes in disulfide bonds. At present purely conformational effects cannot be separated from the amino acid residues alterations caused by the radiation.

The results presented in this thesis indicate that on irradiation of lysozyme, a series of molecules showing markedly different degrees of damage are produced. Initial salting out of whole irradiated lysozyme (26 Mrad) yields a NaCl insoluble fraction, F-I. Because of the salt insolubility, this fraction presumably contains molecules with extensive chemical and/or conformational damage. Gel chromatography of F-I gave evidence of significant increases in the hydrodynamic volume of the components which is an indication of extensive conformational damage in this fraction. The distribution of hydrodynamic volumes was very wide. Peaks were observed at elution volumes ranging from some near to native lysozyme to volumes corresponding to globular proteins of molecular weights 10 times larger than native lysozyme. The presence in F-I of the large molecules indicates aggregates, polymers and unfolded molecules.

Radiation damage to proteins is potentially so severe that aggregation of damaged molecules seems likely. Non-covalent aggregates have been reported for several proteins that under normal circumstances are in the monomeric state. Thus, ribonuclease associates upon freeze drying from acetic acid solutions (168) and serum albumin after thermal denaturation (169). The aggregation process seems to be a cooperative phenomenon in which hydrogen bonds, Coulombic forces, and hydrophobic interactions are involved (170). If the F-I macromolecules are the

result of association involving hydrophobic interactions, there must be hydrophobic contact regions on the surface of the molecules that form the aggregates (171). This process should involve removal of amino acid residues with hydrophobic side chains from contact with the solvent. Difference spectroscopy would indicate if any nonburied tryptophans which are highly hydrophobic have been removed from contact with the solvent (156). In 1 N acetic acid, Maksimov *et al.* (100) observed many molecules with a large hydrodynamic volume that were absent in 0.1 N acetic acid. The difference spectra of F-IA and F-IB, which are components of F-I, in 1.0 N versus 0.1 N acetic acid, indicate that the tryptophyl residues in these fractions are exposed to the solvent in 1 N acetic acid. This means that the large hydrodynamic volume molecules are mostly unfolded molecules. This exposure indicates that it is very unlikely that hydrophobic regions are involved in the formation of aggregates in the 1 N acetic acid used for gel chromatography in this thesis. Lewin (172) has indicated that arginine (guanidinium) groups can form ionic linkages with carboxylate groups from other molecules and thereby enhance aggregation. These ionic linkages are more stable in solutions with low ionic strength, and the low ionic strength of 1 N acetic acid, 0.004, would favor aggregation by ionic linkages. However, the high hydrodynamic volume components are not present when gel chromatography is carried out with 0.1 N acetic acid (100) which has a still lower ionic strength of 0.001. Moreover, Tanford (170) has pointed out that the addition of inorganic salts would favor dissociation if the exposed groups on the surface of the protein are charged but would favor aggregation if the groups are hydrophobic. The insolubility of F-I in 1 M NaCl suggests that the exposed groups are hydrophobic groups and that the ionic groups

are partially shielded from the surface (170).

The possibility of aggregates maintained solely by hydrogen bonds is very unlikely in an aqueous solution due to the fact that water molecules compete in a very effective form with proton donors and acceptors to form hydrogen bonds (130). Therefore, the formation of aggregates between the damaged molecules from F-I seems very unlikely.

Another possible source for fractions of high hydrodynamic volume is polymers and unfolded molecules. Molecular weight determinations of fractions from F-I with sodium dodecyl sulfate polyacrylamide electrophoresis (129) show the presence of covalent linked polymers with degrees of polymerization from 2 to 8. Treatment of the electrophoresis data by two different methods indicate that the polymers are formed only by integral numbers of the monomer of lysozyme. However, the polymers do not account for all the fractions with high hydrodynamic volume obtained from gel chromatography. Some of these components such as F-IB are formed almost exclusively by a monomer. These results show that many of the peaks observed in gel chromatography are molecules differing in size and shape from the combined effects of radiation and 1 N acetic acid.

Maksimov *et al.* (100), working with a Sephadex gel chromatography system, have postulated that the increase in hydrodynamic volume of the molecules of F-I takes place mainly because of intramolecular electrostatic repulsion. However, it is well known that native lysozyme is very resistant to structural alterations caused by changes in pH (154) or even by strong denaturing agents like urea and guanidine (154).

Tanford *et al.* (173) have shown for several proteins in 6 M GuCl that physical restrictions are imposed by the disulfide bonds on the unfolding

process. Jolles (174) has shown that it is possible to relate the content of cystine and tryptophyl residues from lysozymes of different origins with their heat stability which decreases as the cystine and tryptophyl content drop. It seems more likely that the conformational changes of F-I components are not the result of electrostatic repulsion but of damage in the primary structure.

Destruction or exchange of disulfide bonds in the irradiated protein should be capable of causing changes in size and/or shape of irradiated lysozyme. Augenstein (41), Henriksen (72), and others have proposed that the cystine residue is a trap for energy of excitation in the protein. Unfortunately studies made to show breakage of disulfide bonds do not give conclusive evidence that this is an important mechanism for radiation damage. The data of Dose (160) and that presented here show some formation of free thiol groups in irradiated lysozyme with a $G_{\text{value}} \approx 1$. However, the number of thiol groups per molecule hardly accounts for the enzyme inactivation. Moreover, determinations of cystine residues in different irradiated proteins (78) showed no significant changes in their original content. Riesz and White (175) have proposed that broken disulfide bridges may rejoin in aqueous solution, but they did not present experimental evidence for this hypothesis.

The results presented here show that irradiated lysozyme after reduction and alkylation is formed almost exclusively by monomeric forms. This clearly demonstrates that the polymers are formed by monomer units linked by disulfide bonds. These results are very different from those obtained by Alexander *et al.* (176) for solid bovine serum albumin irradiated with 2 MeV electrons. Reduction of disulfide bonds in this

material did not significantly change the molecular weight of the polymers. However, Maksimov *et al.* (177) have shown that irradiated lysozyme, treated with mercaptoethylamine, exhibits a reduction in the protein aggregative ability. Stevens *et al.* (79) obtained an increase in the enzymatic activity of irradiated lysozyme by reduction and controlled reoxidation of the disulfide bonds. The difference in results obtained for lysozyme and bovine serum albumin is not clear. Different radiations were used and the studies were made with different analytical procedure. In particular, light scattering was used to observe the polymers of BSA, and this is not easy to interpret as the SDS polyacrylamide gel electrophoresis studies.

The distribution of polymers in F-IA shows a nearly exponential relationship between the amount of each polymer and its degree of polymerization. This relationship is characteristic of a random polymerization process. An important question arises about the origin of the polymers. Are these products formed in the solid state during and after the irradiation or are they formed after the irradiated protein is dissolved in water? Disulfide bonds in native lysozyme are not available for reduction in the absence of powerful denaturing agents (176), and X-ray analysis shows clearly that the disulfide linkages are buried inside the molecule. The position of these bonds in the molecule and the restriction in movement of atoms in the solid state make it seem unlikely that the crosslinking occurs in the solid. However, it cannot be ruled out.

A better explanation for the results presented here is that crosslinking between heavily damaged molecules occurs when the solid

irradiated lysozyme is dissolved in O_2 -free water. Cystine radicals $-CH_2S\cdot$ can be easily demonstrated in irradiated solid protein, and these radicals have a long life even at room temperature. On solution in water, part of the damaged molecules may unfold exposing some of their disulfide bonds. These unfolded molecules can undergo a free radical polymerization initiated by the $-CH_2S\cdot$ radicals. The proposed mechanism is shown in the following scheme.

As a result of the radiation damage to the primary structure, scrambling of intramolecular disulfide bonds could occur during radiation and on solution or even during crosslinking. The mechanism would be the same as proposed above. This exchange of disulfide links would result in rather stable different forms of monomers and polymers such as have been observed in the gel electrophoresis studies in the presence of urea. Electrophoretically, fraction F-I is formed almost exclusively by four components. Two of these have been identified as monomers and form the peak F-IA in gel chromatography.

Dunnill (171) has analyzed the three dimensional structures of several proteins obtained from X-ray diffraction studies and has concluded that nonpolar residues play a critical role in protein folding. Yutani *et al.* (157) have shown that two tryptophyl residues are involved in the initial partial refolding of the reduced random coil form of lysozyme. On this basis one can suggest that the scrambling of disulfide bonds is caused in part by alteration of amino acid residues that are essential for the protein refolding. Tryptophan analysis should be an important aspect of radiation damage in proteins. Unfortunately, difficulties in tryptophan determinations (178) have resulted in little reliable data about the content of this amino acid in irradiated proteins. Stevens *et al.* (78) have shown that lysozyme irradiated with gamma rays at doses comparable to those used here does not show a significant destruction of tyrosine or phenylalanine but gives an unlikely increase in tryptophan content as determined spectrophotometrically.

Good evidence for tryptophan destruction by radiation was obtained

and is presented in this thesis. Difference spectra studies of F-I components compared with native lysozyme, both in the presence of GuCl, reveal the presence of a new chromophore or chromophores with absorption between 300 to 450 nm. Concurrent with this new peak in the difference spectra there is a trough which extends from 265 to 293 nm. In native proteins the absorption between 265 and 293 nm is almost exclusively due to tryptophyl and tyrosyl residues. The results from Stevens *et al.* (78) and those reported here for spectrophotometric determination of tyrosine show no change for the content of this amino acid. A comparison of the difference spectra in GuCl for F-I products versus native lysozyme with the difference spectra of photooxidized lysozyme versus native lysozyme given by Kravchenko and Lapuk (179), shows common characteristics. Destruction of tryptophyl residues by photooxidation causes a trough in the difference spectra between 260 to 297 nm which is comparable to the 265 - 293 trough observed in irradiated lysozyme. It is quite likely that tryptophyl residues are destroyed by the ionizing radiation, perhaps via a secondary radical reaction involving capture of hydrogen atoms released by ionizing radiation (180).

Garrison and coworkers have suggested that protein damage results in radiolysis of peptide bonds by a mechanism that gives ammonia or amides and carbonyl groups as final products. Gel chromatography and polyacrylamide electrophoresis studies of the whole irradiated protein and F-I components do not show any evidence for fragmentation of the main chain. However, F-I components show approximately one carbonyl group molecule. The gradual increase in observable ammonia that can be released at pH 9 in F-IA and F-IC indicates a progressive

deterioration of these damaged molecules resulting in groups that can be hydrolyzed. Oxidation with O_2 may play an important role here.

The gel chromatographic methods and difference spectrophotometric methods give general information about conformational changes. Hydrogen exchange studies with whole irradiated lysozyme allow a more discriminatory study of damage at different structural levels. The data presented in this thesis show that lysozyme irradiated at different doses has an exponential loss of Class III and IV exchangeable hydrogens related to hydrophobic or α -helical and β -structures. The rate of destruction for the hydrophobic regions and α -helical or β -structures was approximately the same, indicating a concurrent destruction of secondary and tertiary structure. At the same time there is an increase in Class II hydrogens related to structures that in the opinion of Englander (123) are in a more or less random-chain conformation. These results support the concept that damaged molecules with large hydrodynamic volumes are the result of the loss of their secondary and tertiary structure.

Gel electrophoresis and SDS polyacrylamide electrophoresis studies show that the NaCl soluble fraction, F-II, from irradiated lysozyme (26 Mrad) is formed almost exclusively by monomeric lysozyme with a hydrodynamic volume similar to native lysozyme. Polyacrylamide gel electrophoresis in the presence of urea shows a main component whose size and electrical charge are similar to native lysozyme. In addition to the main component there are three small components which have less positive charge and slightly larger volume than native lysozyme. However, the amount of these products is not significant. Spectrophotometric characteristics of F-II are very similar to those

for native lysozyme and there are no indications of new chromophores or significant conformational changes. The only observed chemical damage is the presence of carbonyl groups, one for every six molecules of lysozyme. This fraction constitutes 60% of the material which is far more than the 28% of native-like material that can be separated by Bio-Rex 70 chromatography. In addition, this fraction does not show full enzyme specific activity. One concludes that there are many damaged molecules in this fraction but that they show most macromolecular properties identical to native lysozyme.

Studies of the enzyme specific activity of F-I and F-II give results that correlate very well with chemical and conformational data. F-I, the heavily damaged fraction is almost inactive. The least damaged fraction, F-II, has a lower activity than native lysozyme, 86%. K_a app. for *M. lysodeikticus* cells is the same for F-II and native lysozyme. F-II has a slight increase in the substrate inhibition phenomena which may be interpreted as a result of alterations in the electric charge of the protein molecules (158). The difference spectra of the F-II enzyme substrate complex with glycol chitin has the same general pattern as that observed with the complex of native lysozyme. However, the magnitude of the peaks is smaller, indicating less formation of enzyme substrate complex. An explanation of this effect is that radiation has damaged the side chain of one or more amino acids located in the active site cleft. As a result the substrate does not bind with tryptophyl 62, responsible for the observed difference spectra (22). The correlation between the results for enzyme activity obtained from lysis of cells and difference spectrophotometry of the enzyme-substrate complex (21), indicates that there

is little formation of nonproductive complexes (23). The lytic activity of F-I is less than 10% of native lysozyme, and there are nonspecific interactions between protein and cell surface at low and high concentrations of *M. lysodeikticus*. The difference spectra of the glycol chitin complexes for F-IA and F-IB show atypical characteristics. The position of the peaks and the profile of the spectra, especially for F-IB, indicate that there is less interaction between the substrate and tryptophyl 62. Hayashi *et al.* (22) have shown that the difference spectra of lysozyme-substrate complex is caused by very specific interactions between glycol chitin and tryptophyl 62 residues in lysozyme. Thus a major defect of F-IA and F-IB is that they don't bind the substrate, but the enzyme activity is even more decreased. Therefore, there must be many molecules that make non-productive complexes. Additional support for the formation of complexes between substrates and damaged molecules came from studies with whole irradiated lysozyme. Perturbation difference spectrophotometry with polyethylene glycol shows that irradiated lysozyme in solutions of high ionic strength and/or high pH undergoes an aggregation process which involves tryptophyl residues or products with properties similar to tryptophan. The aggregation process eventually results in the formation of a precipitate. The presence of substrates like chitodextrin or inhibitors like N-acetyl glucosamine causes a delay in the precipitation and, therefore, in the aggregation process. Native lysozyme also aggregates in solution, and Sophianopoulos (37) has demonstrated that substrates interfere with the aggregation process, indicating involvement of the active site in the aggregation. These results agree with the conclusion of Kratochvill *et al.* (181) that

in the aggregation processes of denaturated proteins, the entire molecular surfaces are not involved in aggregation but only certain parts, the aggregation sites.

The behavior of F-I with respect to substrate, ionic strength and pH can explain in part the difference in D_{37} values reported for irradiated lysozyme. However, the consistency of the different values indicate that activity of irradiated lysozyme is mostly due to slightly damaged molecules which have enzymatic characteristics similar to native lysozyme. The constancy of the apparent energy of activation for lysis of *M. lysodeikticus* with lysozyme irradiated at different doses indicates that irradiation does not create damaged molecules with significant activity which possess a very different configuration in the active site from the one observed in native lysozyme.

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