SUMMARY

Chromatographically homogeneous egg white lysozyme has been subjected under reduced pressure to 0.67-m.e.v. $\gamma$-rays. At 37% destruction of enzymic activity, three inactive aggregates and one partially active fraction have been isolated by salt precipitation and chromatographic procedures. The aggregates, upon reduction with 2-mercaptoethanol and reaction with iodoacetic acid, give derivatives with molecular weights of 12,000 to 15,750 in comparison with one of 14,500 for reduced carboxymethylated lysozyme. One urea-insoluble aggregate becomes soluble upon reduction and, after air oxidation, in dilute solution gives active enzyme in 15% yield. Mixed disulfides of cystine and this or other inactive fractions also give significant (10 to 20%) yields of active enzyme upon incubation with cysteine. Disulfide analyses showed close to the expected number of disulfide bonds in two of the mixed disulfide derivatives if one assumes no fragmentation but simply aggregation of lysozyme monomer molecules. Amino acid and disulfide content of the active component from reactivation of one mixed disulfide derivative agreed well with that found for the active component from reactivation of the mixed disulfide of native lysozyme. Thus a significant portion (15 to 20%) of radiation inactivation in solid lysozyme can be explained by the rupture of disulfide bonds followed by formation of "incorrect" intermolecular disulfide bonds.

Damage at disulfide bond sites has been recently invoked as an explanation for part of the destruction of enzymic activity caused by $\gamma$-radiation in dry ribonuclease (1) and insulin (2). Hunt and Williams have estimated (3) that as much as 30% of the inactivation of RNase that occurs in the presence of oxygen might be a result of disulfide bond breakage. This estimate was based on the number of free sulfhydryl groups detectable after irradiation by titration with $p$-hydroxymercureibenzoate. With the same enzyme after $\gamma$-irradiation under reduced pressure, Friedberg and Hayden (4) could detect only small amounts of free sulfhydryl groups after doses large enough to inactivate about 40% of the sample. Similarly, we have not been able to detect production of sulfhydryl groups in egg white lysozyme (mucoprotein $N$-acetylmuramidase, EC 3.2.1.17) after radiation doses of 20 mrads when 37% of enzymic activity had been destroyed (5), nor have we, or other workers, been able to detect significant destruction of constituent amino acids during the irradiation of lysozyme (6) or R-Nase (4) in the solid state at doses at which loss of enzymic activity occurs.

However, none of these measurements on whole irradiated samples would be capable of reflecting such changes as disulfide interchange in the active portion of the sample. This difficulty has been eliminated by separating the inactive material from active material in samples of dry chromatographically homogeneous lysozyme irradiated with $\gamma$-rays under reduced pressure. Some of the inactive fractions obtained are fairly simple and some are surely very complex mixtures. However, from some of these inactive materials we have been able to regenerate enzymic activity by reduction with 2-mercaptoethanol followed by reoxidation or disulfide interchange in very dilute solution. Such are the results presented here, and we feel that they lend strong support to the notion that at least a part of radiation inactivation in solid lysozyme can be explained by disulfide bond damage followed by reformulation of disulfide bonds, possibly upon dissolving the irradiated sample, to give "incorrect" pairing of the cysteinyl residues and, consequently, inactive radiation products.

EXPERIMENTAL PROCEDURE

Materials—Lyophilized Micrococcus lysodeikticus cells were obtained from Worthington. Bio-Gel P-10, 50 to 150 mesh, and BioRex 70, 200 to 400 mesh and -400 mesh, were purchased from Bio-Rad. Sephadex G-25 and G-100 were obtained from Pharmacia. Materials for disc electrophoresis were supplied by Canal Industrial Corporation. Urea was recrystallized from absolute ethanol. Fluorescein mercuric acetate was prepared according to the procedure of Karush, Klinman, and Mark (7). All other reagents were of analytical grade and glass-distilled water was used for the preparation of solutions.

Egg white lysozyme (three times crystallized) was obtained from Pentex, Inc., and was chromatographically purified in all instances prior to use. The procedure of Tallan and Stein (8) was modified as follows: BioRex 70, 200 to 400 mesh, was used in place of XE-64; the pH was lowered to 7.09 and potassium...
replaced sodium as the cation in the 0.2 M phosphate buffer used for column equilibration and elution; elution rates were slower (1.0 ml per min) than for a comparable size XE-64 column; and a 2-fold higher column height to column diameter ratio was used. Under these conditions at room temperature, 92 to 94% of the crystalline lysozyme is eluted as a single peak. Pooled peak fractions were decanted by the procedure of Dixon (6).

The solution was adjusted to 5% acetic acid by the addition of glacial acetic acid, and the protein was absorbed on BioRex 70 resin (400 mesh) previously equilibrated with 5% acetic acid. Salts were removed by washing with 5% acetic acid, and the protein was eluted from the resin with 50% acetic acid. Acetic acid and water were removed from the salt-free protein solution by lyophilization.

Irradiation and Fractionation Procedure—Samples were dried under reduced pressure and weighed into glass tubes which were then evacuated to a pressure of 20 μ, sealed, and irradiated in a kiloCurie 137Cs source at 22–24°, 0.44 mrad per hour, for a dose of 20 mrads. A total of 10.28 g of chromatographically homogeneous lysozyme was irradiated in these studies to permit isolation of several hundred milligrams of some radiation products, accounting for only 5 to 6% of the irradiated sample. After isolation of several hundred milligrams of some radiation products, the pH was adjusted to 8.6 by the drop-wise addition of 10 ml of 2-Mercaptoethanol, and the lyophilized reduced protein was then chromatographed on columns of BioGel P-10, columns, equilibrated and eluted with 0.1 M Tris-acetate buffer (50 ml) containing 2 M urea and 0.1 mM cysteine and maintained at 37°. Aliquots were removed periodically and assayed for enzymic activity. Suitable controls of the inactive disulfide derivative and untreated lysozyme were assayed during the course of an experiment.

In other experiments, 50-fold larger reactivations of the mixed disulfide derivatives of Fraction PRUR and of chromatographically homogeneous lysozyme were performed and the reactivated protein was removed from the incubation mixture after 3 hours with the use of the desalting procedure previously described for chromatographically purified lysozyme. The desalted reactivated protein was then chromatographed on columns of BioRex 70, 2.0 X 50.0 cm, 200 to 400 mesh, equilibrated and eluted with 0.2 M potassium phosphate buffer, pH 7.4, containing 2.8 X 10^-3 M cysteine and maintained at 37°. Aliquots were removed periodically and assayed for enzymic activity. Suitable controls of the inactive disulfide derivative and untreated lysozyme were assayed during the course of an experiment.

Analytical Procedures—Enzymic assays were performed with lyophilized M. lysodeikticus cells as substrate (15) at a temperature of 30° maintained in a Beckman model DU spectrophotometer fitted with a thermostopper assembly. Protein concentrations were determined from the absorption at 215 and 225 μm (16) and 280 μm (17). Results from these measurements agreed well with results obtained with the ninhydrin method of Moore.
and Stein (18). The efficacy of desalting procedures was established by a negative test for phosphate according to Fiske and SubbaRow (19).

Fractions I and II were examined for homogeneity in several ways. One consisted simply of rechromatography on Sephadex G-25 in the same manner as originally obtained, except for the use of a smaller analytical column, 2.0 X 50.0 cm. Appearance of a single symmetrical peak was taken as an indication of homogeneity. A second method was that of disc electrophoresis at several pH values, as described by Ornstein (20) and Reisfeld, Lewis, and Williams (21).

Molecular weight values were calculated from osmotic pressure measurements, and, in some cases, from retention volumes on columns of Sephadex G-100. Osmotic pressure measurements (\(\pi\)) were made on four to six concentrations (c) of a particular fraction ranging from 0.5 to 10.0 g/100 ml with the use of the Mechrolab model 503 low temperature (5\(^\circ\)) automatic membrane osmometer. The solvent used was 0.055 M sodium citrate-6.0 M urea, adjusted to pH 3.75 with 1.0 N hydrochloric acid. The ratio \(\pi/c\) was plotted against c and extrapolated to \(c = 0\). This extrapolated value of \(\pi/c\) was used in the equation \(\pi/c = RT/M_n\) to calculate the number average molecular weight (\(M_n\)) of a given fraction (22).

Retention volumes on Sephadex G-100 columns were determined by a modification of the method of Whitaker (23). The gel was swollen in a 0.0 M urea solution containing 0.3 M sodium acetate and 0.1 M sodium chloride, pH 7.01. Columns (4.0 X 120.0 cm) of this material were packed and washed with the same solution for 6 days at 1-m hydrostatic pressure. Ten to 30 mg of protein were dissolved in 2.0 ml of the solution, applied to the column, and eluted. Complete details on protein standards and calculations of molecular weights are given by Whitaker (29).

After 24 hours of acid hydrolysis, amino acid analyses were performed according to the procedure of Moore, Spackman, and Stein (24). The tryptophan content of samples was estimated from ultraviolet spectra in decinormal sodium hydroxide (25). The disulfide content of various mixed disulfide derivative preparations was determined from their ability to quench the fluorescence of fluorescein mercuric acetate (7).

RESULTS

The radiation dose level (26.0 mrad) at which 37% of enzymic activity is destroyed in dry lysozyme was chosen for large scale fractionation procedures. At higher dose levels which approach complete enzymic inactivation, one would expect multiple ionizations in some molecules (26). It would be difficult to ascertain whether enzymic activity loss occurred through a primary structural change resulting from a single, supposedly lethal, event (27) or a secondary change resulting from a second or third ionization within or near the enzyme molecule. At doses lower than 26.0 mrad, inactive material is not produced to the extent necessary for purification of products arising in small amounts.

Two (Fractions H\(_2\)OR and PRUR) of the four fractions isolated from irradiated lysozyme are not soluble in aqueous solutions and thus could not be assayed for enzymic activity. Molecular weight determinations or homogeneity tests could not be performed on these fractions. Most likely, the two fractions each contain more than one component. Their complete lack of activity makes them good subjects for reactivation studies described later. The chromatographic Fractions I and II (Fig. 1) yield only one symmetrical peak upon rechromatography on Sephadex G-25 columns. Also, they show only one band during disc electrophoresis at pH 4.3, 6.6, and 9.5. These components are, therefore, sufficiently well defined materials for characterization. Fraction I is completely inactive, while Fraction II has only minimal activity in comparison with unirradiated lysozyme. Fraction III is slightly active also, but accounts for only 0.3% of the irradiated sample, so no further attention has been devoted to this material in these studies.

Molecular weight estimations were performed on Fractions I and II and reduced carboxymethylated derivatives of all of the fractions isolated. These values are shown in Table I, along with the extent to which each fraction occurs in the irradiated sample. The five fractions listed total 35.7% of the sample, indicating a 96.5% recovery of the inactive material from a 37%
**TABLE II**

Reactivation* of reduced lysozyme and reduced Fraction PRUR from irradiated lysozyme

| Fraction                        | Reactivation time | Optical density | Reactivation
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PRUR</td>
<td>mins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.011</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>0.013</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>0.014</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>23*</td>
<td>0.015</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>Chromatographically homogeneous lysozyme</td>
<td>49</td>
<td>0.043</td>
<td>19.4</td>
</tr>
<tr>
<td>Blank</td>
<td>None</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Reduced PRUR</td>
<td>None</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Reduced chromatographically homogeneous lysozyme</td>
<td>None</td>
<td>0.003</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* Reactivation was accomplished by diluting the column eluate to 0.05 mg of protein per ml with 0.1 M Tris-acetate buffer, pH 8.5, final volume of 10.0 ml, and incubating at 28° in a 50-mI Erlenmeyer flask.

† Enzyme assay: 0.5 ml of reaction mixture was added to 3.0 ml of 0.03% suspension of lyophilized Micrococcus lyseolitikus cells in 1/15 phosphate buffer, pH 6.25, and incubating at 30°.

‡ Assayed aliquot of the reduced protein containing an amount equal to that assayed from reactivation mixtures.

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Since molecular weight values for the carboxymethylated derivatives all cluster rather closely around that of the carboxymethylated derivative of native lysozyme, we were encouraged to pursue the possibility of reactivating inactive fractions via reduction with 2-mercaptoethanol and reoxidation in dilute solution according to the procedure of Epstein and Goldberger (12). The results of these experiments with Fraction PRUR are given in Table II. Significant reactivation was observed, but we were able to observe only half as high a percentage reactivation with chromatographically homogeneous lysozyme as did Epstein and Goldberger under similar conditions. Further, there was a blank value for unreactivated control that was undesirably high in comparison with that for Fraction PRUR.

In view of the above experimental difficulties, it was decided to attempt reactivation of the mixed disulfide derivatives of cystine and the four major isolated fractions according to the procedure of Kanarek et al. (14). These workers had reported over 90% recovery of activity after 24-hour incubations of such mixed disulfides with cysteine. In Figs. 2 and 3 are depicted the elution patterns for the mixed disulfide derivatives of the various fractions. Clear separation of the first peak from urea was obtained; removal of this reagent is essential to "correct" formation of disulfide bonds during the reactivation step (29). A minor or second protein peak contaminated with urea and reagents is seen in each elution pattern. This minor peak may be a by-product of the reduction and mixed disulfide formation reactions. The major peak in the elution patterns accounted for 50 to 60% of the original sample submitted to the procedure.

When the mixed disulfide derivative of chromatographically inactivated sample. It was necessary to conduct both osmotic pressure and retention volume measurements in concentrated urea solutions since the materials were not soluble in the usual dilute aqueous buffer solutions. The low pH of 3.75 was used in osmotic pressure measurements since it was found that aggregation occurred at more neutral pH values, particularly above pH 5 in the case of Fraction II. This observation probably explains the difference between the values obtained from osmotic pressure measurements with that from retention volume measurements since the latter were done at a more nearly neutral pH. The value of 63,900 obtained from retention volume measurements for Fraction I agrees well with that of 58,000 from osmotic pressure experiments. This value, taken with the one of 12,100 for the carboxymethylated derivative, indicates that Fraction I is a tetramer or pentamer of the original lysozyme molecule. Some, but not much, fragmentation of the molecule may have taken place during irradiation. Molecular weight values of 13,500 and 14,800, respectively, for the carboxymethylated derivatives of Fractions H2OR and PRUR indicate these materials to be high molecular weight aggregates of original lysozyme molecules that have suffered little fragmentation. M₄ values of 14,500 and 15,100 for chromatographically homogeneous lysozyme and its carboxymethylated derivatives, respectively, agree well with the expected values of 14,307 and 14,779 (28).
Fig. 3. Gel filtration of the mixed disulfide of cystine and radiation products from lysozyme. PRUS-I, Fraction I; PRUS-II, Fraction II. See Fig. 2 for legend.

TABLE III

Reactivation of mixed disulfides of cystine and inactive fractions from irradiated lysozyme

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Optical density&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Reactivation&lt;sub&gt;1&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;OR</td>
<td>0.030</td>
<td>17.2</td>
</tr>
<tr>
<td>PRUR</td>
<td>0.029</td>
<td>15.1</td>
</tr>
<tr>
<td>I</td>
<td>0.017</td>
<td>9.8</td>
</tr>
<tr>
<td>II</td>
<td>0.035</td>
<td>19.3</td>
</tr>
<tr>
<td>Chromatographically homogeneous lysozyme</td>
<td>0.155</td>
<td>84.5</td>
</tr>
<tr>
<td>Chromatographically homogeneous lysozyme standard, no reactivation</td>
<td>0.162</td>
<td>100</td>
</tr>
<tr>
<td>Blank&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.183</td>
<td>0</td>
</tr>
<tr>
<td>Chromatographically homogeneous lysozyme, no reactivation&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.001</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reactivation was accomplished by diluting the column eluate to 0.05 mg per ml with 0.1 M Tris-acetate, pH 7.4, containing 2.8 × 10<sup>-3</sup> M cysteine, in a final volume of 20.0 ml. This mixture was incubated at 37<sup.o</sup> for 3 hours in a 100-ml Erlenmeyer flask.

<sup>b</sup> Enzymic assay: 0.05 ml of reactivation mixture was added to 3.0 ml of 0.03% suspension of lyophilized Micrococcus lysoleikticus cells in m/15 phosphate buffer, pH 6.24, 30<sup.o</sup>.

<sup>c</sup> In amounts of 0.05 mg per ml of reactivation buffer. Untreated for disulfide preparation.

<sup>d</sup> Reactivation buffer.

<sup>e</sup> Assayed an aliquot of the unreactivated mixed disulfide of lysozyme containing protein equal to the amount assayed from reactivation mixtures.

TABLE IV

Disulfide content of mixed disulfides of cystine and inactivated fractions from irradiated lysozyme

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Eluate peak&lt;sub&gt;1&lt;/sub&gt; from F-10 column</th>
<th>Disulfide content by fluorescein merccuric acetate quenching&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;OR</td>
<td>First</td>
<td>5.0</td>
</tr>
<tr>
<td>PRUR</td>
<td>First</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>6.6</td>
</tr>
<tr>
<td>I</td>
<td>First</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>7.0</td>
</tr>
<tr>
<td>II</td>
<td>First</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>9.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> The second eluate peak (Fig. 2) in each instance is contaminated with urea and excess cystine.

Values are disulfide groups per mole weight of lysozyme and are averages of duplicate determinations in which each value agreed with the average within 1.0%; 8.0 disulfide groups per mole weight are assumed for the standard, the first eluate peak of the mixed disulfide of cystine, and chromatographically homogeneous lysozyme.

A chromatographically homogeneous lysozyme was reactivated with cysteine according to the best conditions used by Kanarek et al. (14), good activity recoveries of 70 to 90% were obtained within several hours (Table III). Also, no significant reactivation occurred when acidic solutions of the disulfide derivatives were assayed in the enzymic assay system so that no blank values needed to be subtracted from the results of enzymic assays on aliquots of reactivation mixtures. Reactivation of the disulfide derivatives of the inactive fractions (Fractions H<sub>2</sub>OR, PRUR, and I) ranged from 9.8 to 18.2%. Almost 20% reactivation was obtained with Fraction II. This fraction was 13.4% as active as a corresponding concentration of unirradiated lysozyme, so reactivation of the disulfide derivative of this material may not be significant above the level of activity already present. It is gratifying to note that certainly no activity was lost in the reactivation procedure with this fraction.

Disulfide analyses according to the fluorescence quenching procedure of Karush (7) were performed on both major and minor peaks obtained from chromatography of the mixed disulfide derivatives (Table IV). This method proved to be extremely sensitive, requiring only microgram quantities of sample. Urea did not interfere with the quenching reaction. Even the presence of cystine in a sample was not a serious difficulty since the disulfide bonds in this compound quenched fluorescence of fluorescein mercuric acetate only to the extent of 0.1% of that observed with equivalent amounts of mixed disulfides of lysozyme. In the case of only one derivative, Fraction I, did the major chromatographic component have the expected disulfide content of 8.0 per molecular weight of unirradiated lysozyme. The major component in the disulfide of Fraction H<sub>2</sub>OR was much lower than 8.0, and the one in the disulfide of Fraction PRUR was significantly higher than 8.0. The significance of these results must await further fractionation of the disulfide derivatives.
DISCUSSION

These results indicate that the active component from reactivated Fraction PRUR is very similar to, if not identical with, unirradiated lysozyme. Such results taken with the extent of reactivation obtained in the three inactive fractions clearly indicate that 10 to 20% of radiation inactivation in solid lysozyme can be conclusively attributed to disulfide bond damage. In our experiments, such damage has been reflected in the formation of disulfide bonds from "incorrect" pairs of cysteinyl residues in the irradiated sample, probably after dissolving the sample in degassed water. It seems unlikely that such bond formation would occur in the solid state. Also, it must be recalled that Fraction PRUR was initially soluble in water, but after phosphate precipitation of this material solubility in water was no longer possible. This observation would support the contention that incorrect disulfide bonds were formed during or after sample dissolution in water.

It probably is not completely appropriate to compare radiation effects in solid preparations of RNase and lysozyme. However, the two enzymes are very similar in molecular weight, basic nature, and chromatographic behavior. Also, their covalent structure is well established. Hunt and Williams, in particular, have presented considerable evidence (3) supporting the contention that disulfide bond rupture is a significant mode of radiation inactivation. The rate of radiation production of sulfur type radicals (electron spin resonance measurements), taken with the yield of free sulfhydryl groups measured in the whole irradiated sample, indicates that as much as 30% of radiation inactivation in ribonuclease may be due to disulfide bond rupture. These workers also observed that irradiated ribonuclease forms a gel when left standing in solution. Further, this gel formed a "plug" of inactive material on the top of their carboxymethyl cellulose columns used for chromatography of irradiated fractions and was invoked to explain failure to elute inactive materials that might well be subjected to the types of studies reported here for solid irradiated lysozyme, with particular reference to reduction followed by reactivation experiments. Friedberg and Hayden (4) report that reduction and reoxidation of irradiated ribonuclease do not effectively restore enzymic activity. However, this was observed with the whole irradiated sample at an unspecified dose. Considering the low yields that we obtained upon reoxidizing reduced inactive fractions from irradiated lysozyme, it seems likely that significant reactivation might not be apparent in a whole partially inactivated RNase sample. Use of the isolated inactive fraction and reactivation of the mixed disulfide derivative might yield success in solid irradiated RNase. Friedberg and Hayden (4) did find that solid ribonuclease reacts with cystamine during irradiation to form disulfide bonds. This was reflected in the detection of 0.20 mole of taurine per mole of ribonuclease after performic acid oxidation of the irradiated sample that had been dialyzed extensively to remove unbound cystamine. Although these workers attached little importance to this result, it is consistent with Hunt's contention that at least a portion of radiation damage in solid ribonuclease occurs through disulfide bond rupture.

In both ribonuclease and lysozyme, the major portion of radiation damage still requires explanation. In the case of lysozyme, the mixed disulfide of cystine and the inactive radiation products afford an opportunity for further fractionation and characterization of the materials obtained. Such derivatives are stable to treatments at acidic pH values and have been obtained through relatively mild procedures that would not be expected to further alter radiation products. Studies centered around partial enzymic hydrolysis to yield peptides for detailed characterization have been initiated in this laboratory.

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