Radiation Produced Aggregation in Crystalline Preparations of Ribonuclease, Lysozyme, and Trypsin (34344)

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(Introduced by H. E. Sauberlich)

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Gamma irradiation of crystalline proteins produces a heterogeneous population of damaged and unaltered molecules. Most previous studies of radiation effects on activity and structure of enzymes have been made on the entire irradiated sample. Consequently, it has been difficult, if not impossible, to clearly show that an alteration in structure has occurred in an active or inactive molecule. Clearly, it is desirable to obtain a separation of unchanged molecules from the several altered components of an irradiated protein and then study the altered fraction in attempts to correlate a loss of activity with a particular structural change.

Difficulties have been encountered by workers in separating and recovering inactive fractions from irradiated ribonuclease using ion-exchange columns (1). Better success has been obtained by taking advantage of differences in solubility in dilute salt solutions in separating radiation products from egg-white lysozyme (2). However, such a procedure has not been entirely reproducible in this laboratory and must be supplemented by chromatographic steps to secure resolution of the numerous components present in salt soluble and salt-insoluble fractions. Thus a procedure based upon rapid chromatography of irradiated samples soon after their dissolution in water seemed desirable. Since aggregates appear to be the dominant radiation products in lysozyme (2) and ribonuclease (3), gel filtration on a loosely cross-linked polyacrylamide gel presented an approach towards effecting a rapid and mild separation which would not be expected to cause alteration in the products *per se*; would minimize postirradiation changes leading to further aggregation after sample dissolution; and would simultaneously give immediate information on the size of the aggregates produced during irradiation. Polyacrylamide gel was chosen in preference to Sephadex gels since lysozyme monomers are known to be bound to Sephadex gels giving rise to anomalous retention volumes (4). Such a separation procedure has been employed successfully with three crystalline enzymes after irradiation and these results are presented below.

Free radicals arising during the course of irradiation have been implicated as intermediates in aggregate formation. Haskill and Hunt (5) observed that aggregation in irradiated crystalline ribonuclease occurs through fairly slow free radical reactions which can be altered by the presence of H_2S decreasing aggregate formation even though the yield of denatured monomer molecules is enhanced by a factor of 2. It was deemed desirable to extend such findings to at least two other enzymes—lysozyme and trypsin—which have been the subject of intensive radiation studies. In this connection it is interesting to note the work of White *et al.* (6) who assessed free radical distribution in several dry gamma-irradiated proteins by intercepting the free radicals with tritiated hydrogen sulfide gas and subsequently measured the tritium residing in various amino acid residues after acid hydrolysis. These workers found that destruction of protein conformation prior to irradiation gave rise to tritium distributions distinctly different from those of corresponding native proteins. Therefore, conformation is a major determinant of free-radical distribution in irradiated native proteins. One might also predict that aggregation would be a variable quantitative reflection of radiation inactivation as one examines

several proteins though qualitatively aggregation may occur as a general rule. The effect of H_2S should also be variable in reducing aggregation from protein to protein not only because of variable distribution of free radicals in individual proteins with their unique native conformations but also because of variability between the proteins in accessibility of sites for reaction with H_2S.

Materials and Methods. Ribonuclease prepared according to Kunitz (17) was obtained from the Sigma Chemical Corp. (Type III-A, 90–95% homogeneous, lot 106B-8634). Trypsin prepared according to McDonand and Kunitz (18) was obtained from Mann Research Laboratories (1X Cryst., salt-free, lot N1965). Chromatographically homogeneous lysozyme was prepared from Pentex 3X crystallized material (19) as described previously (7). Other chemicals used were reagent grade unless specified otherwise. Glass-distilled water was used in preparing all solutions.

Irradiation procedures were the same as those previously used (8) except that a cobalt-60 radiation source delivering 1.77 Mrads/hr to the sample was employed. No temperature increase deleterious to enzymic activity occurred during irradiation. Hydrogen sulfide gas was placed in some ampoules at 0.8 atm prior to irradiation. The gas used was C. P. grade supplied by Matheson Company.

Enzymic assays on lysozyme samples were performed using lyophilized *Micrococcus lysodeikticus* cells as substrate (9). Tryptic assays were performed by the procedure of Kunitz (10) employing vitamin-free casein (Nutritional Biochemical Corp.) as substrate. Measurements of ribonuclease activity employed yeast ribonucleic acid (Pabst Laboratories) as substrate (11).

In these studies spectral measurements made directly at 280 mμ did not provide a valid means of estimating protein concentration since irradiation is known to increase absorbancy in this spectral region in ribonuclease (3). Therefore, protein estimations were based on Ninhydrin color yields of samples submitted to complete acid hydrolysis (12). This method is not invalidated by amino acid destruction during irradiation up to the maximum dose employed (8). Hydrolysis was accomplished by placing 1.0 ml of sample solution (0.1–4.0 mg of protein) in a Pyrex tube with 1.0 ml of conc HCl; then freezing, evacuating, and sealing the tube, and heating the sealed tube at 115° for 16 hr. Contents of the hydrolysis tube were neutralized with sodium hydroxide and an aliquot of the hydrolysate was reacted with Ninhydrin reagent. The Ninhydrin reagent contained 20 g of Ninhydrin and 1.5 g of hydrindantin dissolved in 650 ml of methyl Cellosolve and 350 ml of 4.0 *N* sodium acetate buffer, pH 5.5. The latter buffer was added to other components dissolved in methyl Cellosolve after bubbling nitrogen through the methyl cellosolve solution for 15 min. This reagent could be stored at 5° for 3 weeks without adversely affecting its quality for these assays. Color development was done by adding a neutral aliquot of sample hydrolysate to 3.0 ml of 4.0 *N* sodium acetate buffer, pH 5.5, and 2.0 ml of Ninhydrin reagent, heating 15 min at 93°, cooling the reaction mixture, adding 5.0 ml of ethanol: water (1:1) mixture and reading the absorbance at 570 mμ. If necessary dilutions were made of the reaction mixture with the ethanol:water mixture. Norleucine and crystalline enzyme standards were carried through the entire procedure with each set of samples. Color yield from unknown samples were expressed as norleucine equivalents. One mg of protein was experimentally shown to produce 9.2 $\pm$ 0.2, 8.7 $\pm$ 0.1, and 9.0 $\pm$ 0.3 norleucine equivalents of Ninhydrin color for ribonuclease, trypsin, and lysozyme, respectively.

Chromatography on cross-linked polyacrylamide columns (Bio Gel P-100, 50-150 mesh, Bio-Rad Laboratories) was performed as follows. The irradiated sample was dissolved as completely as possible in 25.0 ml of water without exposure to air. The water used had been bubbled with nitrogen for 15 min to remove dissolved oxygen. Insoluble material was removed by high speed centrifugation and 23.0 ml of the supernatant applied to a 2.6 $\times$ 95.0-cm gel column previously equilibrated with 0.1 *M* acetic acid. The columns were maintained at 49 $\pm$ 1°F. and

eluted with 0.1 *M* acetic acid using a Technicon variable speed proportioning pump to deliver elutant to the column at a rate of 1.1 ml/min. Fractions of 20.0 ml were collected in tubes residing in a 5° refrigerated bath and absorbancy at 280 mμ measured on their contents. Tubes comprising a peak in the elution profile were pooled and lyophilized to remove acetic acid and water. The lyophilized powders were dissolved in distilled water (ribonuclease and lysozyme) or 10^{-3} *N* HCl (trypsin) and aliquots of this solution used for enzymic assays or estimation of protein content.

To facilitate comparison of elution profiles absorbancy readings at 280 mμ were normalized to obtain a value one would expect to see if 200 mg of protein had been present in the sample applied to the column. Amounts actually applied to a column ranged from 52.0 to 111.5 mg for ribonuclease; 46.1–87.4 mg for trypsin and 112.3–259.0 mg for lysozyme samples. It should be emphasized that the percentage distribution of various fractions in irradiated samples (Tables I and II) are based upon Ninhydrin color yields

TABLE I. Radiation Inactivation in Crystalline Lysozyme.[a]

Radiation dose (Mrads)	Radiation conditions	Inactivation (%)	Sp act[b]	(%) H_2O res.	(%) Soluble aggregate[c]	(%) Active monomer[c]
None	Heat 16 hr at 62°	36.9	0.98	36.1	6.4 (0.00)	58.1 (1.00)
None	Heat 16 hr at 62°	38.4	0.83	21.6	12.2 (0.03)	54.6 (0.74)
19.95	Vacuo	27.3	0.94	28.3	2.9 (0.10)	51.5 (0.90)
19.95	H_2S	16.7	0.95	16.0	15.2 (0.52)	55.0 (1.00)
45.00	Vacuo	59.0	0.57	28.5	7.4 (0.07)	49.1 (0.58)
45.00	H_2S	50.5	0.73	38.7	40.2 (0.91)	17.4 (0.90)
95.50	Vacuo	89.5	0.14	20.5	10.7 (0.00)	34.5 (0.17)
95.50	H_2S	71.1	0.33	12.3	45.2 (0.00)	31.3 (0.30)

[a] Each value is the average of duplicate determinations that agreed with each other within 5%.

[b] Measured on the soluble protein fraction before chromatography.

[c] Values given in parentheses are specific activity measured on the pooled peak fractions after chromatography.

TABLE II. Radiation Inactivation in Crystalline Trypsin.[a]

Radiation dose (Mrads)	Radiation conditions	Inactivation (%)	Sp act[c]	(%) H_2O res.	(%) Soluble aggregate	(%) Active monomer
None	Heat 16 hr at 62°	16.2	1.00	16.0	0	69.7
None	Heat 16 hr at 62°, H_2S	35.4	0.74	12.0	—	—
9.87	Vacuo	16.8	0.90	6.5	0	93.3
9.87	H_2S	34.0	0.78	15.8	55.9[b]	5.6
19.95	Vacuo	31.5	0.92	24.4	9.4	48.9
19.95	H_2S	71.6	0.44	11.4	40.8[b]	28.7
45.00	Vacuo	40.0	0.86	49.0	34.9[b]	0
40.20	H_2S	74.0	0.41	37.1	22.2	21.9

[a] Each value is the average of duplicate determinations that agreed with each other within 5%.

[b] These fractions contained demonstrable enzymic activity.

[c] Measured on the soluble protein fraction before chromatography.

after acid hydrolysis rather than the less reliable absorbancy readings at 280 mμ.

Results and Discussion. A series of proteins (bovine chymotrypsinogen A, egg albumin, bovine gamma-globulin and horse heart cytochrome *c*) have been chromatographed on P-100 columns to determine void volumes, V_0, (bovine gamma-globulin is excluded) and relating specific elution volume (V_e/V_0) to protein molecular weight. Including the values obtained for native ribonuclease, trypsin, and lysozyme, a good linear relationship was observed between the log of the molecular weight and the specific elution volume very similar to that reported by Haskill and Hunt (3) and Whitaker (13). We have thus adopted the practice of plotting the elution profile as a function of specific elution volume for a given fraction. An immediate indication of molecular weight (14) is then available for a peak entity.

In the case of ribonuclease an aggregate peak is formed (Fig. 1). Aggregate formation increases with increased radiation dose at a rate already observed and reported by Haskill and Hunt (5). Also in agreement with their results we find that the presence of

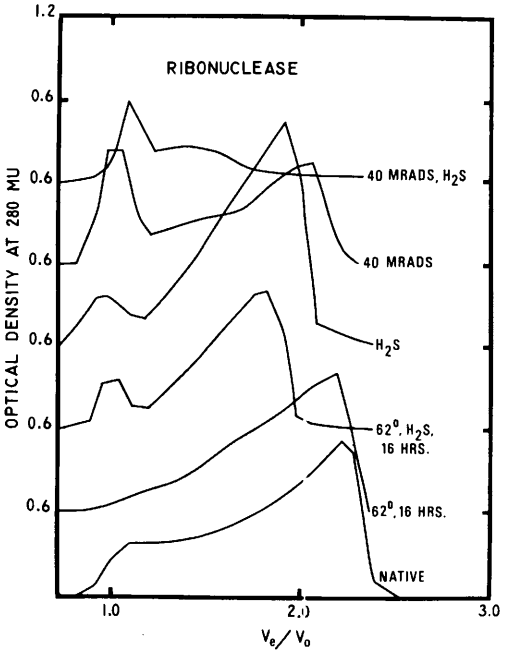

FIG. 1. Gel filtration of irradiated ribonuclease on P-100.

H_2S decreases soluble aggregate formation at the same time that the extent of inactivation is increased by about 40%. Apparently the increased concentration of inactive material appears as water-insoluble material which most likely is an aggregate held together by covalent bonds since we have observed this material to be insoluble in concentrated urea or detergent solutions. In contrast with the results of Haskill and Hunt we did not observe a "denatured" peak being clearly separated from the "native" material. However, it is apparent in Fig. 1 that the peak fraction of the second component in irradiated samples is eluted at a lower specific elution volume than the major peak in unirradiated ribonuclease that has received no H_2S treatment. Most likely these peaks contain some denatured monomer material that has not been separated from unaffected molecules.

It should be pointed out that the results presented in Fig. 1 were obtained at a dose level about twice the highest used by Haskill and Hunt. We obtained similar results at lower dose levels (9.98 and 23.15 Mrads) and confirm their conclusions that H_2S does enhance inactivation and decreases soluble aggregate formation at those dose levels. That overall aggregate formation does increase with increasing dose during our studies may be a reflection of our having used a larger sample than did Haskill and Hunt during irradiation. Dissolution then would have yielded a more concentrated solution in which aggregates would have a better opportunity to form if there were latent free radicals still existing in the dry irradiated sample.

It is interesting to note that simple exposure to H_2S was sufficient to completely alter the elution profile of ribonuclease samples. Soluble aggregate is formed and the second component elutes at a lower V_e/V_0. Exposure to both heat and H_2S produces the same effect accompanied by some inactivation and the formation of water-insoluble material.

Results obtained upon irradiation of lysozyme are in direct contrast to those seen with ribonuclease. The H_2S has the opposite effect of enhancing soluble aggregate formation (Fig. 2) but has no enhancing effect on

inactivation (Table I). If anything, H_2S affords some protection to lysozyme during irradiation. Most surprising of all is the high level of activity observed in the soluble aggregate fraction after 19.95 and 45.00 Mrads in the presence of H_2S. These aggregates elute at a V_e/V_o of slightly more than 1.0 indicating the formation of an aggregate larger than a trimer. The exact size and characterization of this material is the subject of further study. Apparently the aggregate has formed in such a manner that the active region of most of the original monomers is affected very little insofar as substrate binding and cleavage is concerned.

The presence of H_2S during heat treatment of crystalline lysozyme also enhances the formation of soluble aggregates. This aggregated material elutes at the same position as

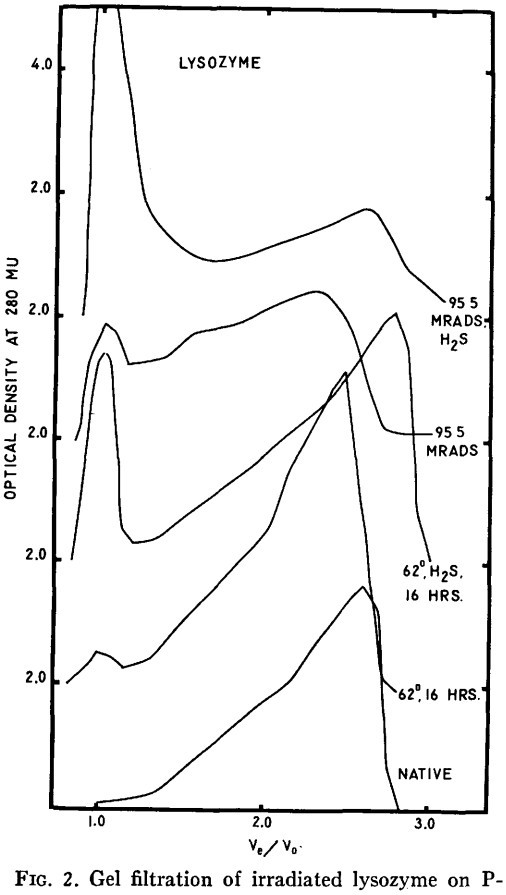

FIG. 2. Gel filtration of irradiated lysozyme on P-100.

aggregates from irradiated lysozyme but are completely devoid of lytic activity. Inactivation under the influence of heat is affected little by the presence of H_2S. Apparently monomer molecules are inactivated prior to or during the aggregation step promoted by H_2S.

Finally, the behavior of crystalline trypsin during irradiation with or without H_2S differs from both lysozyme and ribonuclease (Fig. 3). Both aggregate formation and inactivation during irradiation are enhanced in this enzyme by the presence of H_2S. The extent of inactivation is increased by a factor of about 2.0 (Table II) which is somewhat more than the factor for the enhancement of inactivation seen in ribonuclease. Aggregate formation is increased markedly. However, it should be pointed out that the same number of intermolecular interactions in trypsin as in lysozyme would lead to the formation of about twice as much aggregate on a percentage weight basis since the molecular weight of trypsin is about twice that of lysozyme. Soluble aggregates formed from trypsin in the presence of H_2S during irradiation do retain some activity whereas those formed in the absence of H_2S are inactive. The activity levels observed have not been reproducible in these experiments and therefore no value is included in Table II. Insoluble aggregate formation is enhanced in irradiated trypsin samples and accounts for much of the enhanced inactivation yield seen in the presence of H_2S.

The results presented here indicate that attempts to rationalize the mode of radiation inactivation in enzymes on a general basis is dangerous. At best, it appears that aggregation via some unknown mechanism accounts generally for a portion of the inactivation seen in the three enzymes studied in this work. That aggregation is not necessarily deleterious to enzymic activity is proven by the isolation of active soluble aggregates from both lysozyme and trypsin irradiated in an atmosphere of H_2S. An earlier result consistent with this observation is the observation that regeneration of enzymic activity in inactive lysozyme radiation aggregates is possible by reduction of disulfide bridges followed by

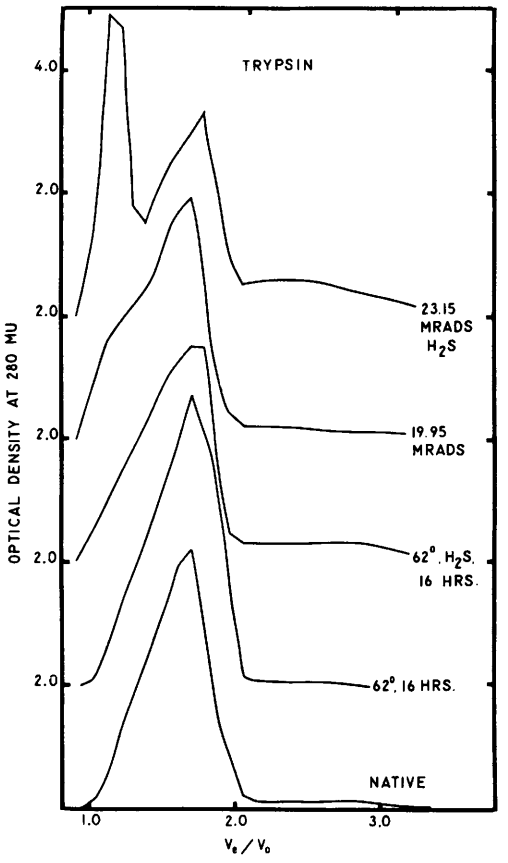

FIG. 3. Gel filtration of irradiated trypsin on P-100.

reoxidation in air in dilute solution (2). Similar regeneration experiments have not been possible in the case of ribonuclease (15). Thus, the formation of "incorrect" disulfide bridges by rearrangement appears to occur in lysozyme but not in ribonuclease at least in a manner which can be reversed by reduction and reoxidation. Main chain cleavage has not been ruled out as a mode of inactivation in lysozyme. Cleavage of the main chain does accompany and probably accounts for inactivation during irradiation of ribonuclease (16). This has been shown by gel filtration studies of irradiated ribonuclease samples reduced with mercaptoethanol to cleave disulfide bridges. These studies reveal protein fragments which elute from gel columns after the reduced ribonuclease monomer and, therefore, are smaller in molecular weight. Similar results have been obtained with isolated lysozyme radiation products (2).

Summary. Soluble and insoluble aggregates are major inactive radiation products *in vacuo* from crystalline lysozyme, ribonuclease, and trypsin. Soluble aggregates can be cleanly separated from active monomer materials by gel filtration on P-100 columns. This separation also permits quantitation of aggregate formation. Such products increase with increasing dose. If H_2S is present during radiation, aggregate yield is decreased in ribonuclease and increased in lysozyme and trypsin. In the case of the latter two enzymes aggregates produced in the presence of H_2S retain significant activity levels. These results indicate the free radical scavenger, H_2S, promotes aggregate formation from active monomers in which free radicals have been produced by irradiation.

1. Hunt, J. W. and Williams, J. F., Radiation Res. **23**, 26 (1964).

2. Stevens, C. O., Sauberlich, H. E., and Bergstrom, G. R., J. Biol. Chem. **242**, 1821 (1967).

3. Haskill, J. S. and Hunt, J. W., Radiation Res. **31**, 327 (1967).

4. Gelotte, B., J. Chromatog. **3**, 330 (1960).

5. Haskill, J. S. and Hunt, J. W., Radiation Res. **32**, 606 (1967).

6. White, F. H., Jr., Riesz, P., and Kon, H., Radiation Res. **32**, 744 (1967)

7. Stevens, C. O. and Bergstrom, G. R., Proc. Soc. Exptl. Biol. Med. **124**, 187 (1967).

8. Stevens, C. O., Tolbert, B. M., and Reese, F. E., Arch. Biochem Biophys. **102**, 423 (1963).

9. Shugar, D., Biochim. Biophys. Acta **8**, 302 (1952).

10. Kunitz, M., J. Gen. Physiol. **30**, 291 (1947).

11. Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page, J., and Carroll, W R., J. Biol. Chem. **207**, 201 (1954).

12. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem. **30**, 1190 (1958).

13. Whitaker, J. R., Anal. Chem. **35**, 1950 (1963).

14. Crestfield, A. M., Stein, W. H., and Moore, S., Arch. Biochem. Biophys., Suppl. **1**, 217 (1962).

15. Haskill, J. S. and Hunt, J. W., Biochim. Biophys. Acta **105**, 333 (1965).

16. Haskill, J. S., and Hunt, J. W., Radiation Res. **32**, 827 (1967).

17. Kunitz, M., Biochem. Prepn **3**, 9 (1953).

18. McDonald, M. R. and Kunitz, M., J. Gen. Physiol. **29**, 155 (1946).

19. Fevold, H. L. and Alderton, G., Biochem. Prepn **1**, 67 (1949).

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