Reports

Reductive Cleavage of Disulfide Bridges in Ribonuclease

Total cleavage of the four disulfide bridges in ribonuclease (RNase) may be achieved with complete loss of enzyme activity, as assayed (*1*) with ribonucleic acid as substrate. Bovine pancreatic ribonuclease (Armour) (12 mg/ml) was reacted with thioglycollic acid [400 moles per mole of enzyme, assuming 13,683 as the molecular weight of ribonuclease (*2*) and a water content of 9.38 percent in the air-dried preparation] in 8*M* urea at *p*H 8.5 (adjusted with aqueous trimethylamine) for 4.5 hours at room temperature. The reduced ribonuclease was precipitated with acetone and 1*M* hydrochloric acid (39/1) at −5°C, and the sample was washed three times with this solvent and twice with cold ether. The material so obtained was completely reduced, as indicated by the presence of eight sulfhydryl groups per mole of enzyme on spectrophotometric titration with *p*-chloromercuribenzoate (*3*).

An alternative method that was used for following the extent of reduction involved selective carboxymethylation of the sulfhydryl groups of reduced ribonuclease (14 mg/ml) by reaction, under nitrogen, with iodoacetic acid (700 moles per mole of protein) for 2 hours at room temperature in an autotitrator. Trimethylamine (5 percent aqueous solution) was added continuously to maintain the *p*H at 8.5. The reaction was stopped, and the carboxymethylated protein was precipitated and washed as described for the reduced material. (The carboxymethylated protein was used as a test for the completeness of this washing procedure by its treatment with thioglycollate as in the reduction procedure, with subsequent washing as described. Titration with *p*-chloromercuribenzoate of the protein so treated revealed no sulfhydryl groups, thus establishing the complete removal of thioglycollate from the reduced enzyme by the washing procedure.) The product was hydrolyzed for 16 to 18 hours in 6*N* hydrochloric acid at 100°C. S-Carboxymethylcysteine is known to be stable under these conditions of hydrolysis (*4*). The hydrolyzate was dinitrophenylated (*5*) and chromatographed in two dimensions. A mixture of toluene, pyridine, chloroethanol, and 0.8*N* ammonium hydroxide (10/3/6/6) was used in the first dimension (*5, 6*), and, in the second, tertiary amyl alcohol and 0.2*M* phthalate buffer at *p*H 5 (2/1) (*7*). The component which moved identically with an authentic sample of dinitrophenyl-S-carboxymethylcysteine was eluted, and its concentration was determined spectrophotometrically (*5*). The chromatographic analysis revealed 8 moles of dinitrophenyl-S-carboxymethylcysteine per mole of reduced, carboxymethylated ribonuclease, but no trace of bis-dinitrophenylcystine. This method of analysis thus confirmed the completeness of cleavage of disulfide bridges as determined by titration with *p*-chloromercuribenzoate.

Carrying out the reductions in 8*M* urea for shorter periods of time, with a smaller excess of thioglycollate, or at slightly lower *p*H values, yielded partially reduced materials with varying degrees of activity (see Fig. 1).

In the absence of urea, but with other conditions identical with those described for the reduction of ribonuclease, complete cleavage of disulfide bridges in the enzyme was not achieved. This finding parallels that of Lindley (*8*), who demonstrated that only one of the three disulfide bridges in insulin was reductively cleaved by thioglycollic acid in the absence of urea. In the present experiments, production of sulfhydryl groups was linear up to 30 minutes, reaching a value of 3.7. After 2 hours, 4.6 sulfhydryl groups were formed. It appears that two of the disulfide bridges in ribonuclease may be opened with relative ease, but that the remaining two are cleaved appreciably only in the presence of urea. In contrast to the results experienced with complete reduction, enzyme activity was diminished but not entirely lost during partial reduction. A correlation between the number of sulfhydryl groups per mole and enzyme activity is given in Fig. 1, which summarizes all reduction experiments performed. Carboxymethylation of the partially reduced material caused no further reduction in enzyme activity, nor did the conditions of carboxymethylation affect the activity of native ribonuclease.

When completely reduced and fully inactive enzyme (1 mg/ml) was subjected to oxidation by air bubbling at room temperature for 68 hours in 0.01*M* phosphate buffer at *p*H 7 to 8, ribonuclease activity reappeared to the extent of from 12 to 19 percent of the specific activity of native enzyme. The number of sulfhydryl groups per mole decreased during reoxidation. When partially reduced ribonuclease containing about four sulfhydryl groups per mole was reoxidized, there was no significant increase in activity or decrease in sulfhydryl groups. The results of all reoxidation experiments are summarized in Fig. 1. Native ribonuclease that was treated under these reoxidation conditions showed no change in activity, nor was activity regenerated in completely reduced, carboxymethylated ribonuclease under these conditions.

The disappearance of disulfide bridges from native ribonuclease during reduction, and their reappearance on oxidation of reduced enzyme, was followed

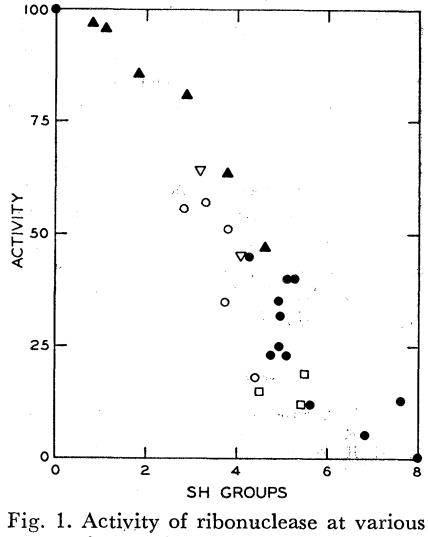

Fig. 1. Activity of ribonuclease at various stages of reduction (expressed as percentage of the specific activity of native ribonuclease) as a function of the number of moles of sulfhydryl per mole of enzyme. ▲, Reduction in absence of urea; ●, reduction in 8*M* urea; □, reoxidation of fully reduced, inactive ribonuclease; ○, reoxidation of samples containing more than six sulfhydryl groups per average molecule; ▽, reoxidation of samples containing about four sulfhydryl groups per average molecule.

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qualitatively by subtilisin digestion (*9*) of the carboxymethylated protein and paper electrophoresis (*10*) of the digest. Native ribonuclease treated in this way (*11*) yields several bands which contain disulfide bonds as shown by the cyanide-nitroprusside test (*12*). The stepwise disappearance of these bands on reduction and their reappearance on oxidation of reduced ribonuclease have been observed. Identification of the disulfide-containing bands resulting from the digestion of reoxidized preparations with those produced from native ribonuclease, as well as elucidation of the order of cleavage of the disulfide bonds during the reduction of the native molecule, must await further investigation.

The S-shaped distribution of the solid points in Fig. 1 suggests the formation of enzymatically active products at intermediate stages of reduction. Further, this figure indicates that, with as many as four sulfhydryl groups per average molecule, activity remains high. With the appearance of more than this number of sulfhydryl groups, activity drops rapidly. Positive confirmation of the existence of enzymatically active reduction intermediates will depend on their separation and characterization.

Various covalent (*9, 13*), secondary (*14, 15*) and tertiary (*15*) bonds can be broken in ribonuclease without loss of enzymatic activity, thus suggesting that the "active center" of the enzyme might comprise only a relatively small part of the molecule (*14, 16*). It appears from the present work that not all of the disulfide bridges in ribonuclease are essential for enzyme activity. A further study of the intermediate stages of ribonuclease reduction may reveal specific disulfide bridges associated with the "active center" of this enzyme.

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1 February 1957

Skin-Sensitizing Activity of Globulin Fractions from Rabbit Immune Serums

The intradermal introduction of certain rabbit immune serums into human beings, followed by challenge with antigen several days later, produces wheal and erythema reactions that are indistinguishable from those produced by challenge of sites prepared with human reaginic serum. It has been repeatedly found that skin-sensitizing activity and precipitin content of a given serum vary independently (*1*), and it has been suggested that skin-sensitizing antibodies might be nonprecipitating (*2*). From absorption experiments with an ovalbumin-rabbit antiovalbumin system, Vaughan and Kabat (*3*) have concluded that the antibody against the major antigenic component does not cause sensitization, but that antibodies against trace antigenic components are responsible.

The purpose of this investigation (*4*) was to determine whether skin sensitizing activity of rabbit immune serums is associated with the same globulin component as precipitating antibody, or whether, analogous to skin-sensitizing activity of human serums (*5*), the skin-sensitizing activity of rabbit immune serums might not also be associated with other than gamma globulin components. Our experiments were not designed to ascertain the antigenic component against which skin-sensitizing activity was directed—that is, whether it was directed against the major antigenic component or against trace antigenic contaminants.

Rabbit antiserums were prepared against the following antigens: three-times-recrystallized chicken ovalbumin (Ea); crystalline bovine plasma albumin (BPA), Armour; crystalline hog chymotrypsinogen, Armour Lot No. 128–214; hemocyanin from keyhole limpet (*Megathura crenulata*); and three-times-crystallized bovine beta lactoglobulin. The rabbits received about 10 mg of antigen four times each week for 3 weeks and were bled 8 to 10 days after the last injection. All antiserums and normal rabbit serum were divided into a number of aliquots and stored at −20°C until used. The amount of precipitating antibody in each serum was determined by quantitative precipitin analyses using Nessler's reagent. Starch electrophoresis was performed in barbital buffer at *p*H 8.6, $\mu = 0.1$. The starch blocks were cut into 1-cm segments, and each was eluted with saline buffered at *p*H 7.1. Total protein analyses were performed by the quantitative biuret reaction (*6*).

The skin-sensitizing capacity of starch eluates and of controls consisting of diluted antiserum, normal rabbit serum, and starch electrophoresis eluates of normal rabbit serum was determined by passive transfer into human volunteers who had not been exposed previously to rabbit serum. Volunteers were carefully screened by preliminary intradermal tests in order to select only those who gave negative reactions to rabbit serum and antigen. Proper dietary instructions were given to all subjects and were reported to have been followed. Three or four days after the introduction of antiserum and antiserum fractions in 0.05-ml amounts, and in concentrations indicated in the Figs. 1, 2, and 3, the sites were challenged with 0.01 to 0.02 ml of 1-percent antigen in saline. Tracings on Scotch tape or paper were taken of most significant reactions, and in some cases reactions were photographed. The reactions were graded by appearance and average wheal diameter, according to the following scheme; negative, less than 5 mm; 1 plus, 5 to 7 mm, no pseudopods; 2 plus, 8 to 10 mm, small pseudopods; 3 plus, 11 to 14 mm, pseudopods; 4 plus, 15 mm or over, large pseudopods. In general, the diameter of the flare was twice that of the wheal.

Figures 1 to 3 are representative examples of the results obtained. The skin-test data represent the average reactions observed in three recipients. Data on the serum of one rabbit after 3 weeks of immunization with Ea, adminstered intravenously without adjuvant, are presented in Fig. 1, and on serum obtained after another 3 weeks of immunization of the

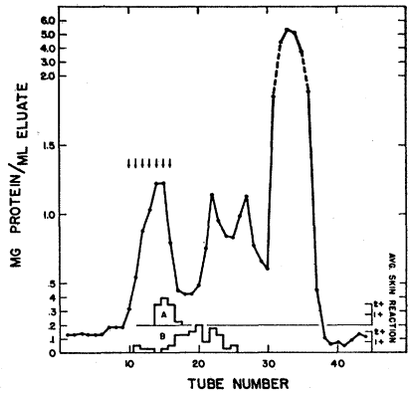

Fig. 1. Starch electrophoretic pattern of rabbit anti-Ea serum 261-1. Arrows indicate presence of precipitating antibodies. Block diagram *A* indicates skin reactions following intradermal introduction of eluates. Block diagram *B* indicates skin reactions 3 to 4 days later, following challenge of the prepared sites with antigen.