Regeneration of Native Secondary and Tertiary Structures by Air Oxidation of Reduced Ribonuclease

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(Received for publication, October 31, 1960)

Although there is an abundance of evidence in support of the template hypothesis of protein biosynthesis (1), there appears to have been no satisfactory experimental basis for deciding whether the role of the template includes the coiling and folding of the protein chain to produce the secondary and tertiary structures or is restricted to the formation of amino acid sequence.

It has been reported (4) that fully reduced and enzymatically inactive bovine pancreatic ribonuclease can be oxidized in vitro by atmospheric oxygen to produce a soluble protein in high yield with a specific activity approaching that of native ribonuclease. The reduced molecule is apparently devoid of the native secondary and tertiary structures (5). Intramolecular oxidation of this form of ribonuclease to yield four disulfide bonds per mole from the eight sulfhydryl groups per mole would, with every conceivable combination of half-cystine residues, result in 105 isomers (6). However, the high regeneration of enzymatic activity suggests either that this oxidative process entails the preferential reformation of the disulfide bonds and other features of the secondary and tertiary structures that exist in native ribonuclease, or that there is more than one three-dimensional configuration of ribonuclease that would permit the expression of enzymatic activity.

The results of comparative studies on the main chromatographic components of native and fully reduced, air-reoxidized ribonucleases indicate that these enzymes possess identical secondary and tertiary structures. This evidence suggests that all of the information needed for establishing these structural features resides in the primary structure and therefore that the template may be concerned only with determination of amino acid sequence in the protein chain.

EXPERIMENTAL PROCEDURE

All measurements of optical density for determination of protein concentration were made in a Beckman model DU spectrophotometer at 280 nm.1 For spectral and optical rotation studies, RNase and its derivatives were routinely dissolved in 0.1 M KCl through which nitrogen had been passed for 15 minutes at room temperature. All dialyses were performed for 7 hours at 5° against distilled water through cellophane membrane that had been heated to reduce the pore size.2

Reduction and Air Oxidation of RNase—Bovine pancreatic RNase was obtained from the Armour Company (lot No. 381-050) and the Worthington Biochemical Corporation (lot No. R 573). Two hundred milligrams of this enzyme were dissolved to a concentration of 10 mg per ml in a solution that was 8 M with respect to urea and 0.285 M with respect to mercaptoethanol (Eastman Organic Chemicals) at pH 8.5 (previously adjusted with trimethylamine). Nitrogen was then passed through the reaction mixture for at least 15 minutes, and the reaction was allowed to proceed at room temperature (22-25°) in a stoppered tube for 4 hours. The protein was precipitated from the reaction mixture with a solution of acetone and 1 N HCl (39:1) at -5° and washed with this solvent and ice-cold ether as already described (8, 9). Reduction of the A4 and B components (100 mg each) obtained by chromatography of either native or Reox RNase3 on CM-cellulose columns, was performed as above after the pooled fractions comprising these peaks had been partially desalted by dialysis and lyophilized. For oxidation, the reduced RNase, reduced RNase A, or reduced RNase B was dissolved to a concentration of 2 mg per ml in distilled water, and an equal volume of 0.02 M Na2HPO4 was added. The solution was adjusted to pH 8.0 by addition of a few drops of 0.2 M NaOH. The oxidation vessel was a 250-ml graduated cylinder, and air, from a compressed air line, was introduced through a glass tube, extending to within 1 cm of the bottom, at a rate of one bubble per 2 to 5 seconds. After 20 to 24 hours of oxidation, the reactor, an average figure of 14.8% was used for correction of the weights of the A components of native and Reox RNases.

1 The dialysis tubing had been heated for 3 days at 80-85°, as was originally suggested by Dr. D. Kupke.

2 Hirs, Moore, and Stein (10) found two active components in beef pancreatic RNase by chromatography on columns of IRC-50 resin, which they designated as components A and B, for the major and minor peaks, respectively. This terminology is used in the present paper to distinguish between the major and minor peaks resulting from chromatography of RNase on CM-cellulose.

3 The abbreviations used are: Reox RNase, fully reduced, air-reoxidized ribonuclease; CM, carboxymethyl; CMC, S-carboxymethylcysteine; CAM, S-carboxyamidomethylcysteine; CAMC, S-carboxyamidomethylcysteimyl or S-carboxamidomethylcysteine.
tion mixture was lyophilized, redissolved in a few milliliters of water, and dialyzed. The dialyzed solution was lyophilized, and the dried protein was stored at -5° until its eventual fractionation on CM-cellulose.

**Alkylation of Reduced RNase**—Reduced RNase, after the washing with acetone-HCl and ether, was dissolved to a concentration of 10 mg per ml in an aqueous 3 M solution of urea, through which nitrogen had been passed for at least 15 minutes at room temperature. An amount of iodoacetic acid (twice recrystallized from petroleum ether, b.p. 35-70°, and diethyl ether), or iodoacetamide (twice recrystallized from petroleum ether, b.p. 35-70°, and ethanol) which produced a molar ratio of alkylating agent to protein of 70:1 was dissolved to 100 mg per ml in water, and the solution was adjusted to pH 8.5 with trimethylamine. This solution was added to the reduced RNase solution, and the mixture was allowed to stand at room temperature for 15 minutes with occasional addition of trimethylamine to maintain the pH at 8.5. The alkylated protein was precipitated with acetone-HCl at -5° and washed with this solvent solution, and the mixture was allowed to stand at room temperature through which nitrogen had been passed for at least 15 minutes, then lyophilized and redissolved in water and lyophilized. With reduced RNase as the starting material, use of either alkylating agent resulted in yields of 80 to 90% for the alkylated protein.

**RNase Assay**—Ribonucleic acid was prepared by the method of Crestfield, Smith, and Allen (11) and was used as the substrate for RNase assay by the method of Anfinsen et al. (12). For assay with either barium uridine-2',3'-cyclic phosphate or barium cytidine-2',3'-cyclic phosphate (Schwarz BioResearch) as substrate, 1.5 ml of the RNase solution (25 to 50 μg per ml) in 0.1 M sodium acetate buffer of pH 5.0 were mixed with 1.5 ml of the substrate solution (0.4 mg per ml, in the same kind of buffer), in a 3 ml quartz cuvette, and the optical density increase was followed in a Cary model 14 M recording spectrophotometer at 2800 A. Although the enzymatic hydrolysis of cyclic phosphates obeys first order kinetics (13), the increase of optical density was nearly linear within the first 2 minutes of reaction, and the slope during this period, for a given sample of RNase, could be compared directly with that for native RNase A, obtained under identical conditions, to determine the percentage of the specific activity of the native enzyme.

**Chromatography of RNase and Its Derivatives on CM-Cellulose**—CM-cellulose was prepared by the method of Peterson and Sober (14) and stored at about 5° as a slurry in 0.01 M sodium phosphate buffer of pH 6.0. Enough of this mixture was poured into a glass column (2.5 x 40 cm), fitted with a sintered glass disk at the bottom, to produce a height of 18 to 20 cm after its compression by air at 10 p.s.i. A sample of native RNase, reduced, alkylated RNase, or Reox RNase, not exceeding 200 mg and dissolved in 2 ml of 0.01 M sodium phosphate buffer of pH 6.0, was added to the column. After passage of 250 ml of the above buffer through the column, gradient elution with 0.1 M sodium phosphate buffer of pH 7.5 was started, with the concentrated buffer first passing into a reservoir containing 250 ml of the dilute buffer.

4. Typical yields are as follows: reduced RNase, 90%; soluble protein resulting from oxidation of reduced RNase, 52%, with reduced RNase as the starting material; Reox RNase A from fractionation on CM-cellulose, 40%, with soluble Reox RNase as the starting material. Over-all yield of Reox RNase A from native RNase = 19%.

7. Fully reduced RNase, at the concentration and pH presently reported for alkylation, has a tendency toward turbidity except in the presence of 3 M urea.

The entire operation was carried out at 5° at a flow rate of 1 to 6 ml per hour, and with fraction volumes of 3.5 to 5 ml. The fractions comprising the A peak (whether of native or Reox RNase) from three columns were pooled and lyophilized. Each sample was dissolved in a few milliliters of water, and most of the salt was removed by dialysis. The last traces of salt were removed by passing the protein solutions through mixed bed ion exchange columns of Dowex 50 and Nalcite SAR-10 according to the procedure of Åqvist and Anfinsen (15). The two products were then lyophilized in preparation for the studies involving proteolytic degradation and separation of peptides, spectra, rotation, viscosity, and immunological specificity.

**Digestion of Native and Reox RNases with Nagarse and Subtilisin**—Reox RNase (5 mg) was dissolved in 1 ml of 0.1 M ammonium bicarbonate, and the temperature was brought to 37°. Then 0.05 ml of Nagarse or subtilisin (1 mg per ml in water) was added. Phenol red (0.005 ml of a 0.1% solution in 20% ethanol) was included as a control on the pH. The reaction was allowed to proceed for 2 hours, and the digest was lyophilized for 16 to 18 hours.

**Two-Dimensional Separation of Peptides from Nagarse and Subtilisin Digests of Native and Reox RNases**—The preparatory procedures used are essentially those of Katz, Dreher, and Anfinsen (18). Two milligrams of the lyophilized Nagarse or subtilisin digest were dissolved in 0.02 ml of water and dried on a sheet of Whatman No. 3MM paper (46 x 57 cm) in an area of approximately 1 cm² This paper was then subjected to a descending chromatography for 16 to 18 hours in the organic phase of a butanol, glacial acetic acid, and water mixture (4:1:5). The paper was dried in air at room temperature for 2 hours and then subjected to electrophoresis for 1 hour at a gradient of 35 volts per cm under Varooll, with a pyridine, acetic acid, water buffer of either pH 3.6 (1:10:289) or pH 6.5 (25:1:475). The paper was dried in a circulating oven at 80° for 15 minutes. The peptide spots were developed by dipping the paper in a 0.5% solution of ninhydrin in absolute ethanol and oven drying at 150°. To locate those peptides containing disulfide bonds, a duplicate paper was stained by dipping in a mixture of cyanide and nitroprusside by the procedure of Tocnies and Koib (19).

**Ultraviolet Spectrum**—The desalted, lyophilized A components of native and Reox RNases, as well as reduced, lyophilized RNase A, were dissolved in oxygen-free 0.1 M KC1 to a concentration of 1.0 mg per ml, determined spectrophotometrically. The ultraviolet spectra of these solutions were then obtained in a Cary recording spectrophotometer, model 14-M.

**Optical Rotation**—The desalted, lyophilized A components of native and Reox RNases were dissolved in oxygen-free 0.1 M KC1 to 10 mg per ml. Reduced, lyophilized RNase A was similarly dissolved to a concentration of 7 mg per ml. The concentrations of these solutions were found spectrophotometrically after the appropriate dilution. The optical rotation values were determined in a Rudolph precision ultraviolet polarimeter, model 80, equipped with the Rudolph photoelectric

8. Subtilisin is an extracellular proteinase from Bacillus subtilis, originally reported by Linderstrom-Lang and Ottesen (16) and was made available through the generosity of Dr. M. Ottesen, Carlsberg Laboratories, Copenhagen. Nagarse is a crystalline proteinase, isolated from a different strain of B. subtilis and first reported by Okunuki et al. (17). It was prepared by Nagase and Company, Japan, and imported by the Biddle Sawyer Corporation, New York.
RESULTS

Reduction and Alkylation of RNase—The eight sulfhydryl groups that are produced by reduction of the four disulfide bonds of the cystine residues in native RNase will react with iodoacetate and iodoacetamide to produce CMC and CAMC residues, respectively. The CAMC is converted to CMC by acid hydrolysis. Therefore, the CMC content of acid hydrolysates of reduced RNase, which has been alkylated with either of these reagents, may be taken as an indication of the extent of reduction.

Preliminary analyses (4) indicated essentially full reduction of RNase with sulfhydryl-containing reagents, as well as complete alkylation of the reduced enzyme with iodoacetate. Table 1 summarizes the results of analyses for CMC and cystine in acid hydrolysates of reduced, alkylated RNase. The averages of 7.94 and 7.83, for the moles of CMC per mole of RNase, show that reduction with mercuricethanol, as well as alkylation with either iodoacetate or iodoacetamide, were essentially complete. The small amounts of cystine may have been produced by decomposition of CMC during hydrolysis, since it has been observed (9) that about 5% of the CMC may undergo this conversion under the same conditions of hydrolysis.

Chromatographic Comparisons—Fractionation on CM-cellulose columns (Fig. 1) indicates that Reox RNase contains two main components which are chromatographically identical to native RNases A and B. Two other enzymatically active peaks are also discernible for Reox RNase, but these occurred as minor components and have not been investigated further. It is noteworthy that there is more B component for Reox RNase, and less A, than for the native enzyme. The possibility of interconversion of the A and B components during reduction and reoxidation was investigated by reducing native RNases A and B separately, air oxidizing each, and chromatographing the products on CM-cellulose columns. By this method, no Reox RNase A was detected in Reox RNase B, nor was any Reox RNase B found in the Reox RNase A. However, the yield of A component was 74% of that of B, and this difference occurred during oxidation, since nearly equal weights resulted from the reduction.
reduction of RNases A and B. Therefore, the increase in B component is the result of its higher yield on oxidation.

Chromatography of reduced RNase was not considered feasible because of the possibility of oxidation on the column. Reduced CAM RNase was eluted from the column as shown in Fig. 1. Two maxima were produced which appeared to coincide with those of the A and B components of native and Reox RNases, although a considerable spreading occurred. Reduced CM RNase, not shown in the figure, came off of the column as a single peak near the front, before gradient elution was started.

Preliminary experiments (23) revealed the activity of Reox RNase A to be 80 to 100% of the specific activity of native RNase A. In a further investigation, the activities of Reox RNases A and B were compared with that of RNase A, derived from an oxidation control experiment which was performed by subjecting native RNase to the same conditions of air oxidation, desalting, and chromatography as for reduced RNase. With ribonucleic acid, uridine-2',3'-cyclic phosphate, and cytidine-2', 3'-cyclic phosphate as substrates, the activities of the Reox RNases were the same as that of the control RNase A within an experimental error of ±10%.

The isoionic points of these peaks were determined by measurement of the pH values of their solutions after deionization on mixed bed columns of Dowex 50 and Nalcite SAR-10. For the A and B peaks, these values were 9.60 and 9.18, respectively, whether these components were of native or Reox RNase.

Disulfide Bonds of Native RNase A and Reox RNase A—The proteolytic enzymes, Nagarse and subtilisin, digest RNase with broad specificities to yield numerous small peptides, some of which are positive to cyanide and nitroprusside and therefore contain disulfide bonds. Differences in combinations of half cystine residues in native and Reox RNases should result in differences in the peptide patterns when the proteolytic digests of these enzymes are subjected to chromatography and electrophoresis. The results of typical experiments are shown in Fig. 2. It can be seen that the peptide patterns of native and Reox RNases are very nearly identical when either subtilisin or Nagarse is used for proteolysis. Since, on repeated separations of these digests by this method, no consistent differences in patterns could be found, no evidence was obtained for any differences in the locations of disulfide bonds in native and Reox RNases.

Spectral Comparisons—Reduction of RNase A causes the shift in ultraviolet spectrum shown in Fig. 3. This effect has been observed for several other reactions, all of which involve the denaturation of RNase (24). There is a considerable body of evidence which indicates that this shift is caused by the rupture of hydrogen bonds involving the hydroxyl groups of three tyrosine residues (24). Oxidation of reduced RNase causes the spectrum to return to the native position, except for an unexplained deviation in the vicinity of 2500 Å. These results indicate that the tyrosine residues become involved in hydrogen bonding, on oxidation of the reduced enzyme, to the same extent as those of the native enzyme.

Optical Rotatory Comparisons—As shown in Fig. 4 and Table

![Fig. 2. Two-dimensional separation of peptides resulting from proteolytic digestion of RNase. A, native RNase A digested with Nagarse; B, Reox RNase A digested with Nagarse; C, native RNase A digested with subtilisin; D, Reox RNase A digested with subtilisin. The first dimension is chromatography in butanol, acetic acid, and water. The second dimension is electrophoresis at pH 6.5 as described in the text. The encircled peptides are positive to cyanide and nitroprusside and therefore contain disulfide bonds.](image-url)
Fig. 4 (top, left). Optical rotatory dispersion of: \( \bullet \), native RNase A; \( \square \), reduced RNase A; \( \triangle \), Reox RNase A, plotted by the method of Yang and Doty (25). The rotatory dispersion constant, \( \lambda_c \), was found by taking the square root of the slope \( \times 10^6 \). \( [\alpha]_0^\lambda \) is the specific rotation, obtained at wave length \( \lambda \), expressed in m. 

II, the rotatory dispersion constant, \( \lambda_c \), drops from 236 m. to 226 m. with reduction. A similar change has been observed on oxidation of RNase with performic acid (5) and apparently reflects destruction of the secondary structure. Air oxidation of reduced RNase causes this constant to return to the native value, indicating that helical coiling has been re-formed to the same extent as it exists in native RNase A. These changes are also suggested by the decrease of specific rotation from \(-73.3^\circ\) to \(-106^\circ\) at the sodium D line (589 m.) with reduction and return to the native value on oxidation (Table II).

Viscometric Comparisons—Fig. 5 shows the results of experiments performed to determine the intrinsic viscosities of native, Reox, and reduced, alkylated RNases. Iodoacetate, rather than iodoacetamide, was used as the alkylating agent for reduced RNase, since alkylation with the latter resulted in a product, after desalting on the ion exchange column and lyophilization, was extremely difficult to solubilize. As shown in Fig. 5, the intrinsic viscosity of RNase increases from 0.033 to 0.186 when the enzyme is reduced. This change is of the same order as that reported by Harrington and Seila (5) and indicates an increase in molecular asymmetry, suggestive of the formation of a randomly coiled chain. They found a similar change in viscosity from cleavage of disulfide bonds by oxidation of RNase with performic acid. On air oxidation of reduced RNase, the intrinsic viscosity returns to the native value, indicating that the same degree of symmetry has been re-established as exists in the native molecule.

Immunological Comparisons—Results of the complement fixation test,\(^\text{10}\) presented in Fig. 6, demonstrate that Reox RNase is antigenic.

![Fig. 5 (top, right). Reduced viscosities of native RNase A (\( \bullet \)), reduced CM RNase A (\( \square \)), and Reox RNase A (\( \triangle \)), as functions of concentration. The intrinsic viscosities were determined by extrapolation of the reduced viscosities to zero concentration.](image)

**Fig. 6 (bottom). Complement fixation tests on native RNase A (\( \square \)) and Reox RNase A (\( \bullet \)).**

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<th>Physical constants for native, reduced, reduced CM, and Reox RNases</th>
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<td>Reox RNase A</td>
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\( ^a \) Maximal molar extinction, which occurs at 2775 Å and 2700 Å, for native and reduced RNases, respectively, shown graphically in Fig. 3.

\( ^b \) Intrinsic viscosity, determined from Fig. 5.

\( ^c \) Rotatory dispersion constant, determined from Fig. 4.

\( ^d \) Specific rotation at 589 m. 

\( ^{10} \) The complement fixation and quantitative precipitin tests were kindly performed by Dr. R. K. Brown, of the New York State Department of Health, by the methods of Mayer et al. (26) and Heidelberger and Kendall (27), respectively.
A is immunologically identical to native RNase A. A comparison of these proteins by the quantitative precipitin test\textsuperscript{10} has led to the same conclusion. Reduced RNase, on the other hand, is inert toward RNase antibody (28).

**DISCUSSION**

Many proteins have been partially denatured to an intermediate reversible stage by such means as heating, raising or lowering the pH, and exposure to urea, guanidine hydrochloride, or organic solvents (29-31), with no return to the native properties after further denaturation. More recently, studies on the reversibility of protein denaturation have included the reduction and reoxidation of disulfide bonds. For example, Audrain and Clauser (32) have reduced the one disulfide bond of oxytocin with borohydride but reports not more than 2\% of the specific enzymatic activity of native RNase restored on oxidation of the reduced form. On the other hand, full reduction of RNase with either thioglycolate or mercaptoethanol has yielded a product that can be oxidized to regenerate a specific activity similar in magnitude to that of the native enzyme (4). It remained to be shown, however, that the reduced, air-oxidized molecule was identical to native RNase.

The main chromatographic components of native and Reox RNases have now been compared by several chemical and physical techniques, by which any differences in secondary or tertiary structures should have been detected. The spectral comparisons showed a deviation of Reox RNase from native in the vicinity of 2500 A, which cannot be explained at present. Otherwise, there has been no evidence to indicate any differences in the coiling, folding, or locations of disulfide bonds of native and Reox RNases. Presently the evidence appears strong that they possess the same three-dimensional configuration.

The possibility has never been excluded that some of the secondary or tertiary structure, or some of both, remains intact after reduction of the four disulfide bonds of RNase and that such residual structure is responsible for the reorientation of the molecule to its original configuration. However, the presently reported changes in physical properties on reduction indicate that at least an extensive destruction of these structures has occurred. The fact that Harrington and Sela (5) have observed similar changes after cleavage of disulfide bonds of RNase by oxidation with performic acid adds weight to the tentative conclusion that the elimination of the native coiling and folding is complete by the present method of reduction.

The preferential return of the reduced RNase chain to the native configuration suggests a dependence of secondary and tertiary structures upon primary structure rather than upon a template. The function of such a template would then be restricted to determination of the sequence of amino acids in the protein chain. The features of primary structure that would govern the coiling and folding of the chain are not presently known. Karush (34) has suggested that attractive interactions among side chains in the vicinities of the cysteine residues may bring specific sulphydryl groups into position for reaction. However, it remains to be established whether these bonds are determined directly by the amino acid sequence, or whether some part of the secondary and tertiary structures is so determined which then governs the formation of specific disulfide bonds.

A search is presently being conducted for other simple proteins whose native secondary and tertiary structures may be preferentially regenerated on oxidation after full reduction. By comparisons of the amino acid sequences and disulfide bond locations in such proteins with those of RNase, it is hoped that progress may be made toward a precise delineation of the features of primary structure that direct the formation of secondary and tertiary structures. Such information may eventually make possible a detailed prediction, from a knowledge of amino acid sequence, of the coiling and folding of a protein molecule.

**NOTE ON AN X-RAY DIFFRACTION INVESTIGATION OF REDUCED-REOXIDIZED RIBONUCLEASE**

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The recovery of enzymic, spectral, and chromatographic properties after oxidation of reduced ribonuclease strongly suggests that the original disulfide bonds and conformation have been recovered. It appeared desirable to attempt to crystallize the reduced-reoxidized RNase (Reox-RNase) and compare its x-ray diffraction pattern with that of crystalline native RNase. We here report x-ray diffraction data that provide geometrical evidence for recovery of the native molecular conformation in Reox RNase.

Of the 14 crystalline forms of RNase that have been discovered we chose to attempt to crystallize Reox-RNase in Form II, because of its ease of crystallization and good crystallographic properties. (Forms I to VII have been described by King, Magdoff, Adelman, and Harker (35), Forms VIII to XIV will be described soon.) Form II is prepared from solution in a mixture of water and 2 methyl 2,4 pentanediol at about pH 5.

Samples of native RNase A and Reox-RNase A were supplied by Dr. Frederick White of the National Institute of Health. These had been prepared from the Worthington lot No. R 573. The standard crystals of this laboratory had been prepared from Armour lot No. 381-059, a mixture predominating in RNase A, but containing some RNase B.\textsuperscript{1} The x-ray diffraction data were obtained with a single crystal counter diffractometer equipped with an Eulerian cradle (36).

A solution of Reox-RNase in the crystallizing solvent deposited either Form II or Form XI crystals depending on whether the pH had been adjusted to 5 (Form II) or not adjusted at all (Form XI). Form XI is normally obtained when crystallization is carried out near the isoelectric point (about pH 9), either by

\textsuperscript{1} Crystals were prepared by Mrs. H. Bello.
using deionized RNase or by adjusting the pH with alkali or amine buffers. Form XI was obtained (in very high yield) with both the Reox-RNase A and the native RNase A, because these had been deionized by Dr. White. The XI crystals were imperfect, compared with those that had been obtained previously, probably as a result of one or more of the following causes: (a) very rapid crystallization, (b) pH lower than optimum, (c) (less likely) lack of B component.

The Form II crystals obtained from Reox-RNase were of as high quality as our usual crystals, and had the same lattice constants within the normal range of variation. The diffraction patterns of the CuKα doublet from the principal crystallographic planes were identical (within normal variation) both as to the positions and intensities of the reflections out to the largest angles measured, $2\theta = 60^\circ$, which corresponds to an interplanar spacing of 1.5 Å.

The Form XI crystals made from Dr. White's RNase gave such poor diffraction patterns that it was not possible to determine their lattice constants with any precision. We have not been able to obtain Dr. White's native RNase A in Form II. In our experience, small differences from standard preparations of RNase may lead to difficulties in crystallization. It seems likely that the process of reduction and reoxidation results in the elimination of impurities that inhibit formation of Form II. These impurities are apparently not removed by chromatography. Formation of XI crystals is so easy that the impurities do not hinder.

The lattice constants for the various crystals are given in Table I. From the essential identity of the Form II crystals of our standard RNase with those of the Form II Reox-RNase A, we conclude that the molecules of these two substances are geometrically very similar, perhaps identical, and pack identically in forming the crystals.

### CONCLUSION

Reduced-reoxidized ribonuclease has been obtained in two crystalline modifications one of which is similar to and the other identical to standard crystal preparations in x-ray diffraction patterns along the principal crystal axes. This reflects substantially identical molecular geometry for native and reduced-reoxidized ribonuclease.

### REFERENCES

Regeneration of Native Secondary and Tertiary Structures by Air Oxidation of Reduced Ribonuclease
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