Selective Reduction and Mercuration of Cystine IV–V in Bovine Pancreatic Ribonuclease*

R. Sperling, Y. Burstein, and I. Z. Steinberg

ABSTRACT: Bovine pancreatic ribonuclease was found to react with the sulfhydryl reagents dithioerythritol or dithiothreitol in two steps: a fast step followed by a much slower one. The product at the end of the fast step was carboxymethylated and digested by pepsin, and the digest was analyzed by paper electrophoresis and chromatography. Only cystine bond IV–V was reduced during the fast reduction step. The partially reduced protein obtained at the end of the fast step reacts with either 1 or 2 moles of mercuric ions, yielding derivatives of the type –S–Hg–S– or –SHg$^+$, respectively. The monomercury derivative was crystallized in a form which is very nearly isomorphous with the monoclinic crystalline form of native ribonuclease.

The mono- and dimercury derivatives, as well as the carboxymethyl derivative of ribonuclease reduced at cystine IV–V, are enzymically as active as the native protein and are resistant to digestion by trypsin. Similarly, the mercury derivatives exhibit an optical rotatory dispersion which is identical with that of native ribonuclease. Three of their tyrosine side chains titrate abnormally as in native ribonuclease. The cystine bridge IV–V in bovine pancreatic ribonuclease thus seems to be completely unnecessary for enzymic activity or for maintaining the native macromolecular conformation of ribonuclease.

Disulfide bonds play a key role in stabilization of the native three-dimensional conformation of many proteins. Cleavage of all disulfide cross-links in a protein, by reduction or by oxidation, leads to complete loss of biological activity, in as diverse protein classes as the enzymes, trypsin inhibitors, hormones, and immunoglobulins (for a review, see Anfinsen, 1965–1966, and Anfinsen, 1964). Total rupture of these bonds brings about a marked change in the macromolecular conformation of the protein (Harrington and Schellmann, 1956; Harrington and Sela, 1959; Goldberger and Epstein, 1963).

While the importance of the disulfide linkages to the protein's native conformation has been well established, less is known concerning the stringency of the requirements regarding the number, length, or geometry of these linkages for the maintenance of biological activity and conformation of the protein. There is some evidence that partial modification of the disulfide bridges is sometimes possible without loss of biological activity. It has been suggested that during the course of reduction of the disulfide bonds in RNase by thioglycolic acid, partially reduced intermediates are produced which are enzymically active (Sela *et al.*, 1957). Similarly, disruption of one (Caputo and Zito, 1961) or two (Azari, 1966) disulfide bonds in egg-white lysozyme with no parallel loss of enzymic activity has been reported. It has also been found that disruption of one disulfide bond in trypsin, trypsinogen (Light and Sinha, 1967), and bovine trypsin inhibitor (Kress and Laskowski, 1967) does not lead to any loss in activity. Even in a multichain protein such as immunoglobulin G it is possible to break a few interchain disulfide bonds without change in conformation or biological activity (Fleischman *et al.*, 1962). Two specific cystine bridges (IV–V and III–VIII) in RNase have been cleaved with phosphorothioic acid (Neumann *et al.*, 1967); it was found that the modified enzyme was more active than RNase toward cytidine 2′,3′-cyclic phosphate and was digestible by trypsin, but resembled the native enzyme in some other properties. The effect of lengthening the disulfide bonds in a protein has been studied (Steinberg and Sperling, 1967); RNase was fully reduced and the sulfhydryl groups liberated were bridged in pairs by mercuric ions. The product, which contained four mercury atoms per molecule, showed some resemblance to the native protein in that it contained abnormal tyrosine residues; the enzymic and immunogenic reactivities were, however, markedly impaired.

In the present study it is shown that one of the disulfide bridges in bovine pancreatic RNase, *i.e.*, that of cystine IV–V, is completely unnecessary for enzymic activity or for maintaining the pertinent features of the macromolecular conformation of this protein. This bond has been perferentially reduced by dithioerythritol and carboxymethylated. The sulfhydryl groups liberated on reduction were also blocked by mercuric ions, according to the reaction (Edelhoch *et al.*, 1953; Cecil, 1963; Webb, 1966), $-S^- + Hg^{2+} \rightleftarrows -SHg^+$. This reaction was shown to have a very high association constant (about 10^{20}) (Stricks and Kolthoff, 1953; Simpson, 1961); therefore, mercuric ions bind preferentially to the sulfhydryl groups. The product obtained, contained two mercury atoms per protein molecule. In addition, the disulfide bridge IV–V was extended by about 3 Å by introducing a mercury atom between the sulfur atoms, according to the reaction (Edelhoch *et al.*, 1953; Cecil, 1963; Webb, 1966), $2(-SHg^+) \rightleftarrows -SHgS- + Hg^{2+}$, and all the products were almost indistinguishable from the native protein. The last derivative crystallizes isomorphously with the monoclinic form of native RNase.

Materials

Bovine pancreatic ribonuclease A (five-times crystallized

* From the Weizmann Institute of Science, Rehovot, Israel. *Received April 17, 1969.* A preliminary report was presented at the 38th Meeting of the Israel Chemical Society (Sperling *et al.*, 1968). This research was supported in part by U. S. Public Health Service Grants No. GM13637 and AM5098.

type 1A lot no. 95B-0330), cytidine 2′3′-cyclic monophosphate (lot no. 84B-0560), *p*-hydroxymercuribenzoate sodium salt (lot no. 64B-5170), and Tris, reagent grade (lot no. 14B-5300), were purchased from Sigma (St. Louis, Mo.). RNase A (phosphate free lot no. RAF-6JB), pepsin (twice crystallized, lot no. PM691), and trypsin (lyophilized, twice crystallized, lot no. TRL 6295) were purchased from Worthington Biochemical Corp. (Freehold, N. J.). Ribonucleic acid (yeast nucleic acid lot no. 6502) was obtained from Schwarz BioResearch, Inc. (Orangeburg, N. Y.). Dithioerythritol (lot no. K-5531) was a product of Cyclo Chemical Corp. (Los Angeles, Calif.). Dithiothreitol and [U-^{3}H]dithiothreitol were purchased from Calbiochem (Luzern, Switzerland). Mercuric chloride labeled with ^{203}Hg (Hg-203 cat. no. D-1) was purchased from the Israel Atomic Energy Commission (Yavne, Israel). [2-^{14}C]-Iodoacetic acid (lot no. CFA-269 batch 11) was obtained from the Radiochemical Centre (Amersham, England). Ethylenimine was obtained from Fluka (Buchs, Switzerland). 2-Methyl-2,4-pentanediol (White Label) was a product of Eastman Organic Chemicals (Rochester, N. Y.). Analytical reagent grade urea, obtained from BDH Ltd. (Poole, England), was recrystallized from 95% ethanol; solutions of this compound were prepared immediately before use. All other chemicals were of analytical grade.

Methods

Spectrophotometric measurements were made with a Zeiss Model PMQ II spectrophotometer. Quartz cells with 1-cm light path were used.

Fluorescence measurements were performed with an Aminco-Bowman spectrophotofluorometer, at 90° to the excitation beam, using 1-cm fluorescence quartz cells. The various RNase derivatives which were tested had the same optical density at 280 mμ, which was not higher than 0.1.

RNase concentrations were determined spectrophotometrically at 277.5 mμ using $\epsilon_{277.5\,m\mu}$ 9800 l. mole^{-1} cm^{-1} (Sela and Anfinsen, 1957).

Optical rotatory dispersion was measured in a Jasco spectrophotometer Model ORD–UV-5 using quartz cells of 10- and 1-mm path length with scale expansion of 0.1° full scale.

Ultracentrifuge. Sedimentation velocity measurements were performed in a Spinco Model E analytical ultracentrifuge at 59,780 rpm with a double-sector, capillary-type synthetic boundary cell (Richards and Schachman, 1959).

pH-Stat titrations were carried out in an all-glass thermostated reaction vessel under nitrogen atmosphere, using a Radiometer type TTTlc automatic titrator in combination with TTA3 titration assembly and SBR_2–SBU1 titrograph recorder. The electrodes were the G 2222B glass electrode and the K4112 calomel electrode (Radiometer, Copenhagen).

Spectrophotometric Titrations of Tyrosine Residues. About 0.07 μmole of protein was dissolved in 2.0 ml of 0.15 M aqueous NaCl in a titration vessel, equipped with a G2222B glass electrode, a K4112 calomel electrode (Radiometer), and a magnetic stirrer. A slow stream of nitrogen was introduced to exclude CO_2. The pH of the protein solution was adjusted by adding 1 M NaOH solution from a micrometric syringe and most of the solution was transferred to a flow cell (1-cm optical path, Helma 150-QS) by an automatic transferator (GME, Middleton, Wis.), and the absorbance at 295 mμ was measured. The protein solution was then forced back into the titration vessel by the transferator, the pH was raised again, and the procedure was repeated. Corrections were made for the increase in the volume of the protein solutions when the molar extinction coefficients were calculated.

Potentiometric titrations of sulfhydryl groups with mercuric chloride were carried out with a gold amalgam-saturated calomel electrode pair (Cecil, 1955).

Amino acid analyses were performed on the Beckman-Spinco automatic amino acid analyzer Model 120C, after hydrolysis in constant-boiling HCl for 22 hr at 110° (Spackman *et al.*, 1958; Raftery and Cole, 1966).

Radioactivity measurements were performed on Packard Model 3003 TriCarb liquid scintillation spectrometer, Vanguard Model 880 automatic chromatogram scanner, and Packard Model 320E TriCarb flow monitor, attached to the amino acid analyzer.

Acrylamide disc electrophoresis was performed on a Shandon apparatus, using 15% gels, pH 4.3, for 90 min according to Reisfeld *et al.* (1962).

Preparative acrylamide electrophoresis was performed on a Shandon apparatus at pH 4.3 using 15% acrylamide gels (Reisfeld *et al.*, 1962). Riboflavin was used as initiator instead of persulfate, and β-alanine acetate was used as the buffer and the eluent; 50 mg of protein was dissolved in 1 ml of 40% sucrose and applied to the top of the gel. Electrophoresis was carried out at 30 mA for the first 30 min, after which the current was raised to 80 mA. Elution velocity was approximately 12 ml/hr; 10-min fractions were collected and checked for absorption at 280 mμ, radioactivity, enzymic activity, and were hydrolyzed and subjected to amino acid analysis.

Trypsin Digestion. RNase derivatives (2.5 mg) were dissolved in 1 ml of 0.05 M NaCl adjusted to pH 8.0 with 10^{-2} M NaOH. To this solution 50 μl of trypsin (1 mg/ml; in 0.05 M NaCl adjusted to pH 8.0) was added. The digestion was performed at 30° with continuous stirring under nitrogen atmosphere. The reaction was followed by means of the pH-Stat as described above.

Immunological Procedure. Quantitative precipitin tests with the γ-globulin fraction (precipitated with 40% saturated $(NH_4)_2SO_4$ at 4°) of rabbit antisera to bovine pancreatic ribonuclease and the various RNase derivatives were performed as described by Bauminger *et al.* (1967).

Ribonuclease activity was assayed both with RNA and cytidine 2′,3′-cyclic phosphate as substrate. Activity on RNA was measured spectrophotometrically according to the procedure of Kunitz (1946), at 25° in 0.1 M acetate buffer (pH 5.0). The assay using cytidine 2′,3′-cyclic phosphate as substrate was performed in the Zeiss spectrophotometer with scale expansion, at 290 mμ, using quartz cells with inserts to shorten the optical path length to 0.5 mm. Substrate concentration was 4 mg/ml at pH 7.0, 0.1 M Tris-chloride buffer (Frensdorff and Sela, 1967).

Dialysis experiments were performed with preheated (80°, 3 days) Visking seamless cellulose tubing (Union Carbide Inc.).

Alkylation of Partially Reduced RNase. CARBOXYMETHYLATION. [2-^{14}C]Iodoacetic acid (tenfold excess over each sulfhydryl group) was dissolved in 1 M Tris-chloride buffer (pH 8.0) having the same volume as the protein solution and added, under nitrogen, to the stirred reaction mixture. After 30 min the whole mixture was subjected to gel filtration on a Sephadex G-25 column (2 × 80 cm) and eluted with 0.2 M acetic

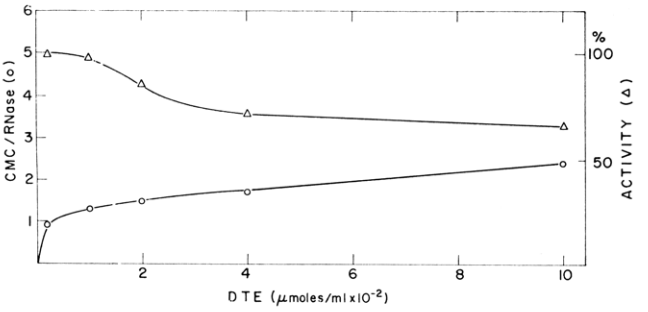

FIGURE 1: Extent of formation of CMC residues in RNase upon reduction with increasing concentrations of DTE and subsequent treatment with iodoacetic acid (○). Reduction was carried out at RNase concentration of 1 μmole/ml in 1 M Tris buffer (pH 8.0) for 60 min at room temperature. The enzymic activity of the corresponding CM-RNase derivatives of RNA (Kunitz, 1946) is included (△).

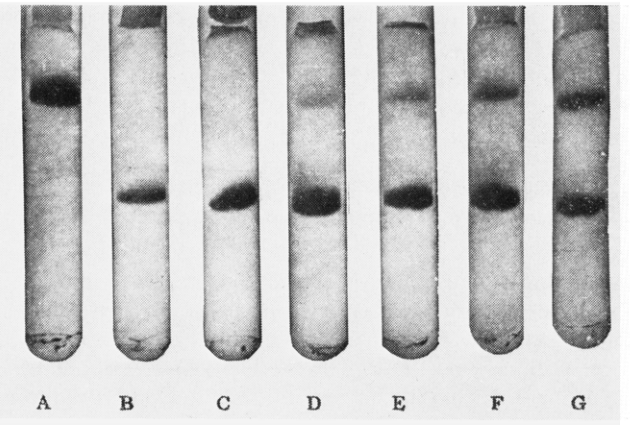

FIGURE 2: Acrylamide gel electrophoresis of carboxymethylated derivatives of RNase, obtained upon reduction of RNase with increasing concentrations of DTE and subsequent treatment with iodoacetic acid. Reduction was carried out at RNase concentration of 1 μmole/ml in 1 M Tris buffer (pH 8.0) for 60 min at 23°. DTE concentrations were 20 μmoles/ml (C), 100 μmoles/ml (D), 200 μmoles/ml (E), 600 μmoles/ml (F), and 1000 μmoles/ml (G). For comparison, the results for 8CM-RNase (A) and RNase (B) are included.

acid. The fractions corresponding to the protein peak were pooled and lyophilized.

AMINOETHYLATION. Ethyleneimine (20-fold excess over each sulfhydryl group) was added to the stirred reaction mixture, under nitrogen, and aminoethylation was carried out for 30 min. The reaction mixture was purified by gel filtration as above.

Identification of the Modified Disulfide Bond. A protein sample (5 mg/ml) was digested by pepsin (2.5% w/w) in 5.0% formic acid (20 hr at 37°). The peptic digest was applied to Whatman No. 3MM paper (approximately 1–1.5 mg/cm) and subjected to electrophoresis at 60 V/cm for 45 min at pH 6.5 (Katz *et al.*, 1959). In all cases, the peptic digests were applied to the paper immediately after digestion. After electrophoresis, the paper was dried by a stream of cold air and narrow strips (3 cm) were cut from both sides of the length of the paper, developed with ninhydrin–cadmium reagent (Dreyer and Bynum, 1967), and nitroprusside–cyanide reagent (Toennies and Kolb, 1951), respectively. The strips were then checked for their radioactive patterns.

Analysis of the radioactive regions was carried out as follows. The radioactive strips were cut out of the remaining paper and stitched to another piece of Whatman No. 3MM paper. The paper was subjected to descending chromatography in 1-butanol–acetic acid–water (25:6:25, v/v), upper phase, for 48 hr, and dried in a stream of cold air. After the run, narrow strips (1 cm wide) were cut from both sides of the length of the paper and stained with ninhydrin–cadmium. These strips were then cut into small pieces about 1–2 cm in length, and the counting rate was determined for each piece of paper immersed in 10 ml of Bray's (1960) solution. The spots which were both ninhydrin positive and radioactive were marked and the corresponding bands on the remainder of the paper were eluted with 7% (w/v) ammonium bicarbonate, lyophilized, hydrolyzed, and subjected to amino acid analysis.

In some experiments, after chromatography of small amounts of the peptide and elution of the radioactive peptide from the chromatogram by 7% ammonium bicarbonate, carboxymethylcysteine was found to be oxidized to carboxymethylcysteine sulfone (Harris, 1967), which was the only radioactive amino acid found by analysis.

Crystallization of [RNase·1Hg] and [RNase·2Hg]. The crystallization was performed in 55% 2-methyl-2,4-pentanediol at pH 7.0 according to method D of King *et al.* (1956).

Results

Partial Reduction of Ribonuclease. The extent of the reduction of disulfide bonds in ribonuclease (RNase) by (DTE)[1] in aqueous solution was investigated as a function of the reaction time and the molar ratio of DTE to RNase. The effect of urea and phosphate ions on the reaction course was also studied.

RNase was dissolved in 5–10 ml of 1 M Tris-chloride buffer (pH 8.0; previously deaerated by a stream of nitrogen), to a final concentration of 1.0 μmole/ml, and placed in a stirred Radiometer titration vessel under a slow stream of nitrogen gas. Measured amounts of DTE solutions were then added and the reduction was allowed to proceed for 60 min. The reduced protein was then carboxymethylated and isolated as described under Methods, and subjected to total hydrolysis and amino acid analysis. Figure 1 illustrates the extent of reduction and carboxymethylation of the disulfide bonds as a function of DTE concentration. The enzymic activity of the carboxymethylated protein preparations toward RNA is also presented in this figure. It can be seen that at a DTE concentration of 20 μmoles/ml, corresponding to a molar ratio of DTE to S–S bonds of 5, an average of approximately one carboxymethylcysteine was found per protein molecule after 60-min reaction. This preparation will be designated in the following as CM-RNase. No perceptible loss of enzymic activity was detected at this stage. With increasing amounts of reducing agent, the extent of reduction is somewhat increased, and is accompanied by appreciable loss of enzymic activity.

The various preparations of partially reduced and carboxymethylated RNase were subjected to acrylamide gel electro-

[1] Abbreviations used are: DTE, dithioerythritol; DTT, dithiothreitol; 8CM-RNase, fully reduced and carboxymethylated RNase; 2CM-RNase, ribonuclease reduced at cystine IV–V and carboxymethylated.

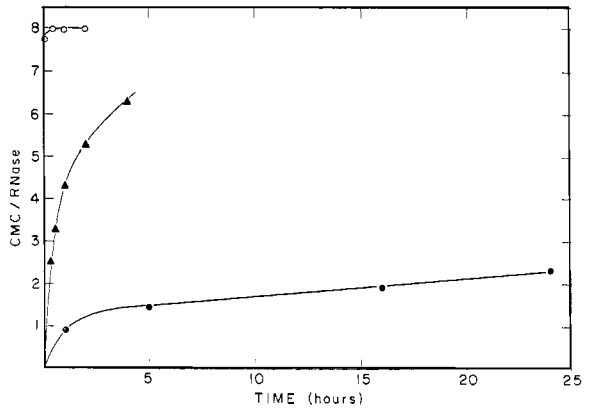

FIGURE 3: Kinetics of formation of CMC residues in RNase (1 μmole/ml) upon reduction with DTE (20 μmoles/ml) and subsequent treatment with iodoacetic acid. Reduction was performed at 23° in: 1 M Tris-chloride buffer (pH 8.0) (●); 1 M Tris-chloride buffer (pH 8.0)–8 M urea (○); 0.2 M phosphate buffer (pH 7.3)–8 M urea (▲).

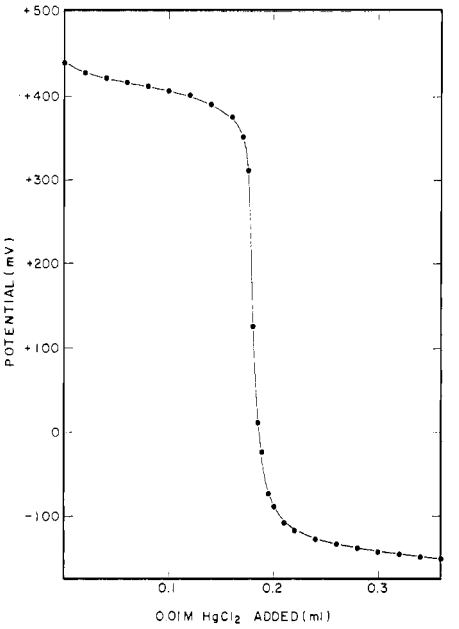

FIGURE 4. Potentiometric titration of a reduction mixture containing 0.09 μmole of RNase and 1.8 μmoles of DTE with 0.01 M mercuric chloride. All titrations were carried out with a gold amalgam-saturated calomel electrode pair at 25° in 1 ml of 0.1 M Tris-chloride buffer (pH 8.0).

phoresis (Reisfeld *et al.*, 1962). Some of the results are presented in Figure 2. The carboxymethyl RNase obtained at a DTE concentration of 20 μmoles/ml migrated as a single band with a mobility similar to that of the native protein. However, on preparative acrylamide gel electrophoresis CM-RNase was partially resolved. The leading edge contained no CMC while the trailing edge contained up to 1.6 CMC/protein molecule. At concentrations of DTE higher than 20 μmoles/ml an additional band characteristic of fully reduced-carboxymethylated RNase starts to appear. No intermediate bands could be detected.

Figure 3 describes the course of the reduction of RNase by DTE in aqueous solution (pH 8.0) and in 8 M urea. The concentration of DTE was 20 μmoles/ml corresponding to a molar ratio of DTE to S–S bonds of 5. The ordinate represents the amount of CMC per RNase molecule obtained, as described above, after termination of the reaction. In the aqueous solution, about one CMC was found per RNase molecule after 1-hr reduction. Subsequently, the reaction slowed down considerably. Acrylamide gel electrophoresis of the carboxymethylated products obtained after various times of reduction were performed as those presented in Figure 2. After 1-hr reduction only a single band could be detected with a mobility similar to that of the native protein. A band corresponding to 8CM-RNase appeared in the acrylamide electrophorograms after 2 hr of reduction and its intensity increased with duration of reduction. Also in these experiments no intermediate bands could be detected.

In the presence of 8 M urea the reduction was dramatically faster than in the aqueous solution, resulting in total reduction of the disulfide bonds in 15-min reaction. This was accompanied by a complete loss of enzymic activity and complete disappearance of native RNase in the acrylamide gel electrophorograms. It is of interest that 0.2 M phosphate provides some protection of RNase against reduction in the 8 M urea solutions and slows down the rate of reduction (Figure 3). In the presence of phosphate ions, 50% of the disulfide bonds were reduced in about 1 hr in 8 M urea. Reduction of RNase with DTT led to results similar to those obtained with DTE. DTE is, however, somewhat more convenient to handle than DTT, since it is less hygroscopic. Most experiments were therefore carried out with DTE.

Preparation of a 1:1 Ribonuclease–Mercury Complex. A mixture of RNase (1 μmole/ml) and DTE (20 μmoles/ml) was incubated at room temperature in 1 M Tris-chloride buffer (pH 8.0) as described above, for 60 min. A portion of the reduction mixture was then diluted tenfold with deaerated water, flushed with nitrogen gas, and titrated with 0.01 M $HgCl_2$, a gold amalgam-saturated calomel electrode pair serving as indicator (Cecil, 1955). Figure 4 illustrates such a potentiometric titration. As can be seen, there is a very sharp end point in the titration curve, the jump in the electrode potential being about 500 mV. The end point corresponds to the addition of one mercury ion per two sulfhydryl groups in the titrated solution (Cecil, 1955; Steinberg and Sperling, 1967). This titration curve served to identify the electrode potential at the equivalence point. [^{203}Hg]$HgCl_2$ solution was then added to the remainder of the reduction mixture in slight excess relative to the sulfhydryl groups (up to the point where the electrode potential difference dropped to a value of two-thirds of the total change), as monitored by a gold amalgam-saturated calomel system. The DTE–mercury complex precipitated and was removed by centrifugation. The supernatant solution was subjected to gel filtration on a Sephadex G-25 column. The mercury content in the protein varied somewhat across the protein peak. However, after lyophilization of the protein solution obtained, and a second gel filtration on Sephadex G-25, the ratio of mercury to RNase was homogeneous across the protein peak and was found to be 2 (±0.15). This ribonuclease derivative will be designated [RNase·2Hg] in the following. It is of interest that when the above preparative procedure was repeated with the use of *p*-mercuribenzoate instead of mercuric ion, none of the mercurial was found to be attached

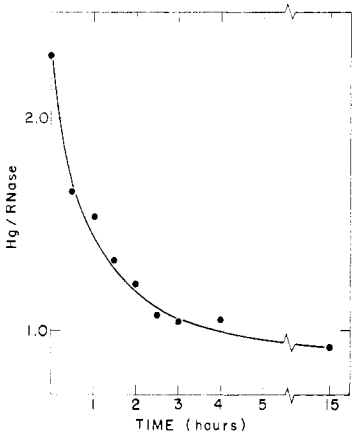

FIGURE 5: Rate of escape of ^{203}Hg from the solution of [RNase·2Hg]. Samples of [RNase·2Hg] (0.4 mg in 2 ml of 0.2 M acetic acid) were dialyzed in preheated cellulose tubing against 6 l. of distilled water at pH 7.0 (adjusted by NaOH). Samples were taken at listed intervals and measured for their protein and ^{203}Hg content.

to the protein. Steric factors possibly exclude the relatively large mercurial from the vicinity of the sulfhydryl groups of the partially reduced protein.

[RNase·2Hg] can be made to give up part or all of its mercury by dialysis under the appropriate conditions. When dialyzed for several hours against water adjusted to pH 7.0 by NaOH, [RNase·2Hg] lost one mercury atom, while the other mercury atom remained firmly bound. Figure 5 shows that even prolonged dialysis against water does not remove this second mercury atom from the protein. The resultant RNase derivative will be referred to in the following as [RNase·1Hg]. It should be noted that dialysis of [RNase·2Hg] against 0.2 M acetic acid for several hours hardly removed any mercury from the complex. On the other hand, dialysis of [RNase·2Hg] against 0.1 M Tris buffer (pH 8.0) or against 10^{-3} M EDTA for 12 hr removed all of the mercury from the complex.

When native ribonuclease (0.1 μmoles/ml) was mixed with [^{203}Hg]$HgCl_2$ or with DTE–mercury complex (2 μmoles/ml), in the absence of free DTE, and then subjected to gel filtration on Sephadex G-25 and dialysis for several hours against water (pH 7.0), only traces of mercury remained with the protein (less than 0.1 mercury atom/RNase molecule).

Reduction experiments were performed with tritiated DTT; no traces of the reducing agent were found to be associated with the mercury complexes of RNase obtained. We may thus conclude that [RNase·1Hg], which requires some prior reduction of disulfide bonds in RNase in order to be obtained, has the mercury atom attached to some reduced cystine residues with the formation of a bond of the –S–Hg–S type.

[RNase·2Hg] can be regenerated from [RNase·1Hg] by mixing the latter derivative with [^{203}Hg]$HgCl_2$; 5 ml of [RNase·1Hg] (0.02 μmole/ml) was incubated with [^{203}Hg]$HgCl_2$ (0.02 μmole/ml) for 20 min at room temperature. The mixture was passed through a G-25 Sephadex column, and the protein obtained was found to contain 1.8 mercury atoms/protein molecule. This experiment established the reversibility of the binding of one of the mercury atoms in [RNase·2Hg].

Site of Binding of the Mercury in [RNase·1Hg] and [RNase·2Hg]. Neumann *et al.* (1967) have reported that pepsin digestion of native ribonuclease yields three peptides which contain disulfide bonds. Upon electrophoresis on paper at pH 6.5 these three peptides appear in two regions on the paper. These peptides are both ninhydrin and cyanide–nitroprusside positive. In one region, which we shall call region 1, two cystine-containing peptides are located: one containing the S–S bridge IV–V, and the other the S–S bridge III–VIII (the notation used here is that of Spackman *et al.*, 1960). In the second region, region 2, one large peptide containing the remaining two cystine residues is located, *i.e.*, the S–S bridges I–VI and II–VII.

TABLE I: Amino Acid Composition of the Radioactive Peptide from the Peptic Digest of Partially Reduced Carboxymethylated Ribonuclease.

Amino Acid	μmoles × 10^2	Residues Found[a]	Residues Theor[b]
Lysine	3.26	1.93	2
Histidine			
Arginine	tr		
Carboxymethylcysteine	3.10	1.84	2
Aspartic acid	5.03	2.98	3
Threonine	2.13	1.26	1
Serine	2.37	1.40	1
Glutamic acid	5.06	3.00	3
Proline			
Glycine	2.51	1.49	1
Alanine	2.37	1.40	1
Valine	1.72	1.02	1
Methionine			
Isoleucine			
Leucine			
Tyrosine	1.03	0.61	1
Phenylalanine			

[a] Based on three residues for glutamic acid. [b] According to Smyth *et al.* (1963); Neumann *et al.* (1967).

[RNase·1Hg] was similarly subjected to peptic digestion. The digest was then analyzed by high-voltage electrophoresis as described under Methods. The pattern obtained after development with ninhydrin–cadmium reagent was similar to that obtained by Neumann *et al.* (1967) for the peptic digest of native RNase. Furthermore, when the paper was developed with the cyanide–nitroprusside reagent, which reveals S–S, SH (Toennies and Kolb, 1951), and S–Hg–S (Y. Burstein and R. Sperling, in preparation) groups, region 1 and region 2, as defined above, appeared also in the case of [RNase·1Hg] (see Figure 6). On the other hand, radioactivity measurements of the electrophorograms revealed only one major peak for the location of ^{203}Hg, which coincided with region 1.

These experiments indicate that the mercury atom in [RNase·1Hg] is associated either with the cysteine residues IV and V or with the cysteine residues III and VIII. Analysis of the peptic digest of [RNase·2Hg] by the procedure outlined above, yielded results similar to those obtained with [RNase·1Hg].

Further Studies on the Location of the Disulfide Bond Reduced by DTE. In principle, one can resolve the question as to

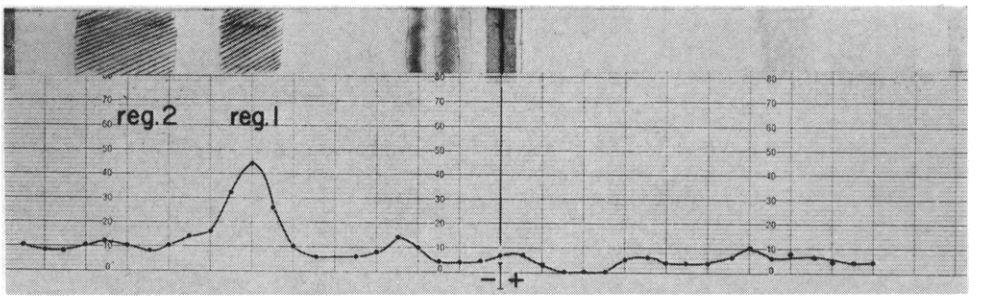

FIGURE 6: Electrophoretic pattern of the peptic digest of [RNase·1Hg]. The digest was fractionated at pH 6.5, 60 V/cm. Strips were cut out, developed with ninhydrin (upper pattern, grey areas) and cyanide–nitroprusside (upper pattern, hatched areas), and assayed for their ^{203}Hg content (lower pattern). Regions 1 and 2 were the only ninhydrin- and cyanide–nitroprusside-positive spots. The radioactivity was concentrated in region 1.

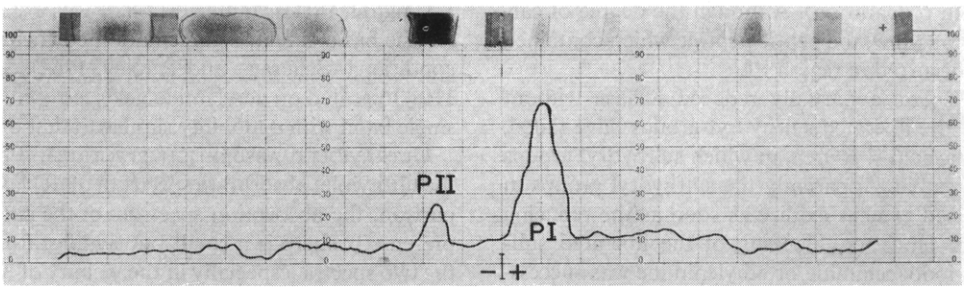

FIGURE 7: Electrophoretic pattern of the peptic digest of CM-RNase (prepared with [2-^{14}C]ICH_2COOH). The peptic digest was fractionated at pH 6.5, 60 V/cm. Strips were cut out, developed with ninhydrin–Cd (upper pattern), and scanned for their ^{14}C radioactivity content (lower pattern).

which of the peptides in region 1 the mercury is complexed, by separation and analysis of the various peptides in this region. In practice, however, serious difficulties have been encountered in the application of this approach. This was due to the fact that the S–Hg–S bonds, though strong, have a finite dissociation constant. Thus, the yield of radioactive mercury which migrates with the cysteine-containing peptides was found to be poor (20%) (Y. Burstein and R. Sperling, in preparation). Furthermore, because of the dissociability of the S–Hg–S bonds, exchange reactions involving these bonds may occur during the analytical procedures. To avoid these difficulties, the conclusive identification of the disulfide bridge, or bridges, which had been reduced by DTE was carried out on alkylated, partially reduced RNase, as described below.

Ribonuclease (1 μmole/ml) was treated with DTE (20 μmoles/ml) in Tris buffer (pH 8.0) for 60 min. All the sulfhydryl groups in the reaction mixture were then alkylated with [2-^{14}C]ICH_2COOH, and the protein fraction was isolated by gel filtration, as described under Methods; 0.8–1.2 residues of *S*-carboxymethylcysteine were found per RNase molecule by amino acid analysis. The partially reduced and alkylated RNase was then digested by pepsin and subjected to high-voltage electrophoresis (see Methods).

Figure 7 illustrates the electrophoresis pattern of the peptic digest. It can be seen that two spots contain the labeled carboxymethyl group: PI, which contributes approximately 80% of the counts, and PII, which contributes approximately 20% of the counts. PII showed an intense ninhydrin reaction, consisted of many peptides, and, on hydrolysis and amino acid analysis, was found to contain relatively small amounts of *S*-carboxymethylcysteine (about 10% of the total CMC found in CM-RNase). Even prolonged electrophoresis and subsequent paper chromatography of PII failed to yield a defined radioactive peptide. PII was found to diminish somewhat in magnitude after prolonged digestion with pepsin. It was therefore concluded that some of the carboxymethyl groups in PII were not attached to cysteine and that some of the peptides in PII were precursors of PI. Because of its complexity, no further analysis was performed on PII, although it possibly contained minor amounts of *S*-carboxymethylcysteine peptides different from the one which appears in PI.

The spot PI was subjected to paper chromatography with 1-butanol–acetic acid–water as solvent (Figure 8). It can be seen that PI separated into a number of ninhydrin-positive spots; however, only one of them was radioactively labeled, PIa. This radioactive peptide was eluted and subjected to acid hydrolysis and to amino acid analysis. The results are presented in Table I. This table also includes the amino acid analysis expected for the peptide containing cystine IV–V, "the small loop," obtained by peptic digestion of RNase (Smyth *et al.*, 1963; Neumann *et al.*, 1967). The agreement between the two sets of data is good. It may thus be concluded from Table I that on mild reduction of RNase by DTE under the conditions described, the disulfide bond closing the small loop is preferentially ruptured.

The conclusion that the mercury in [RNase·2Hg] and [RNase·1Hg] is bound to cysteines IV and V was substantiated by simultaneous elimination of the mercury and carboxymethylation of the released sulfhydryl groups in a solution containing EDTA and [2-^{14}C]ICH_2COOH (Y. Burstein

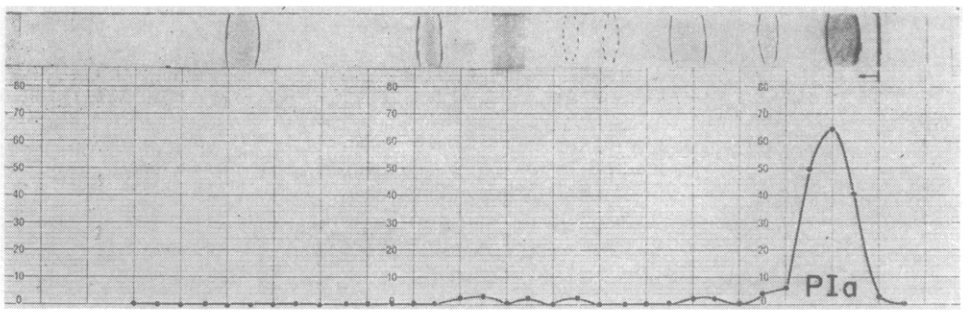

FIGURE 8: Chromatographic pattern of PI. PI was eluted from the electrophorogram (see Figure 7) and subjected to paper chromatography in butanol–acetic acid–water (25:6:25, v/v, upper phase). Strips were cut out, developed with ninhydrin–Cd (upper pattern), and assayed for radioactivity (lower pattern).

and R. Sperling, in preparation). Although the degree of carboxymethylation was low, the peptic peptide which contained CMC invariably occurred in region PI.

All efforts to isolate a partially reduced RNase without prior blocking of the liberated sulfhydryl groups have failed, even in 0.2 M acetic acid–4 M urea, in which sulfhydryl groups are usually quite stable. Apparently the sulfhydryl groups in the partially reduced protein oxidize as soon as the reducing agent is removed. However, blocking of the liberated sulfhydryl groups by iodoacetamide or ethylenimine was successful. The number of alkylcysteine groups per RNase molecule, found on amino acid analysis, was 0.7–1.2 for iodoacetamide treatment and 0.9–1.4 for ethylenimine treatment. As mentioned above, *p*-mercuribenzoate failed to block these sulfhydryl groups.

Properties of the RNase Derivatives. [RNase·1Hg]. SEDIMENTATION AND ELECTROPHORETIC MIGRATION. [RNase·1Hg] sedimented as a single symmetrical peak in the ultracentrifuge. The sedimentation coefficient at a protein concentration of 1.3 mg/ml, in Tris buffer (pH 8.0) was $s_{20,w} = 1.73$ S. Native RNase has a similar sedimentation coefficient under the same conditions (Scheraga and Rupley, 1962). Similarly, [RNase·1Hg] migrated on acrylamide gel electrophoresis (pH 4.3) as a single band with a mobility similar to that of native RNase.

ULTRAVIOLET ABSORPTION SPECTRUM. Figure 9 illustrates the ultraviolet absorption spectrum of [RNase·1Hg]. For comparison, the absorption spectrum of the native protein is also presented. There is a small but significant difference between the two spectra, especially in the vicinity of 250 mμ. The difference is probably due to the difference in absorption between the S–Hg–S group in the modified protein compared with the S–S group it replaces. This assumption is substantiated in Figure 9, where the difference between the absorption spectrum of GS–Hg–SG and GS–SG (R. Sperling and I. Z. Steinberg, in preparation) was added to that of RNase. The result coincides with the spectrum of [RNase·1Hg].

THE FLUORESCENCE QUANTUM YIELD of [RNase·1Hg] was compared with that of the native protein in an excitation wavelength of 280 mμ and an emission wavelength of 310 mμ. No difference could be detected between the quantum yields of the native and modified ribonuclease.

SPECTROPHOTOMETRIC TITRATION. Figure 10 illustrates the change in the molar extinction coefficient at 295 mμ (ϵ_{295}) of [RNase·1Hg] as a function of pH. A similar titration of native

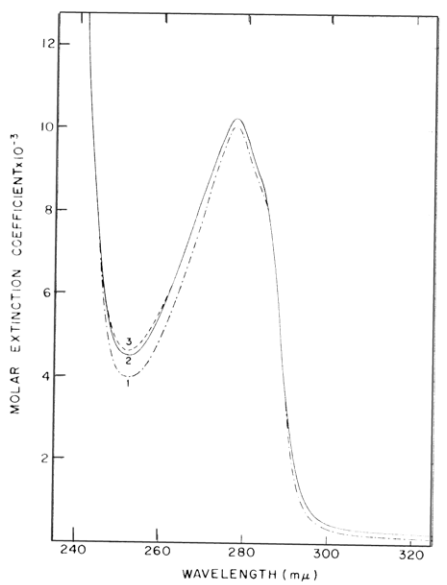

FIGURE 9: Ultraviolet absorption spectrum of RNase (curve 1) and [RNase·1Hg] (curve 2). Measurements were performed in water at pH 7.0. Curve 3 represents the calculated absorption spectrum of [RNase·1Hg], in which the contribution of the S–Hg–S bond to the absorption spectrum has been accounted for from data on model compounds (see text).

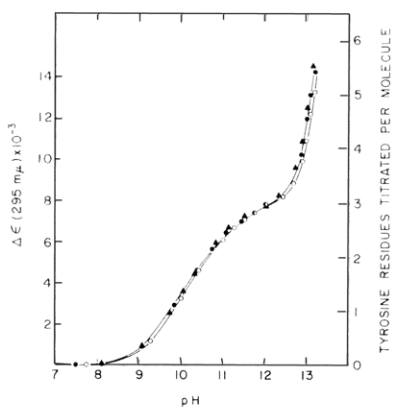

FIGURE 10: The change in molar extinction coefficient, $\Delta\epsilon_{295\ m\mu}$ of [RNase·1Hg] (●), [RNase·2Hg] (▲), and native ribonuclease (○), upon increase of pH above neutrality. Titrations were carried out at 23° in 0.15 M NaCl with NaOH solution. $\Delta\epsilon_{295\ m\mu}$ per tyrosine residue is 2630 l. mole^{-1} cm^{-1} (Tanford *et al.*, 1955).

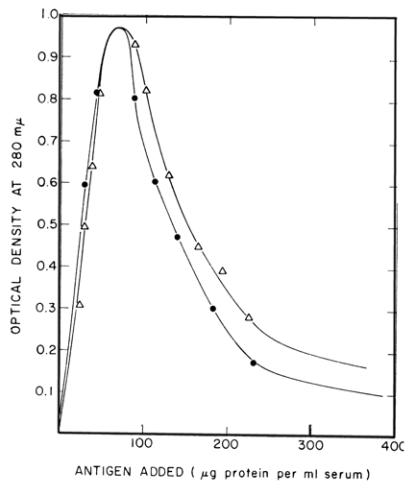

FIGURE 11: Quantitative precipitin curves of 0.3 ml of a solution of γ-globulin fraction of rabbit antiserum to bovine pancreatic ribonuclease with [RNase·1Hg] (●) and with native RNase (△). The volume of the incubation mixture was 2.0 ml. The ordinate represents the optical density at 280 mμ (light path 1.0 cm) of the immune precipitate dissolved in 1.1 ml of 0.1 N NaOH.

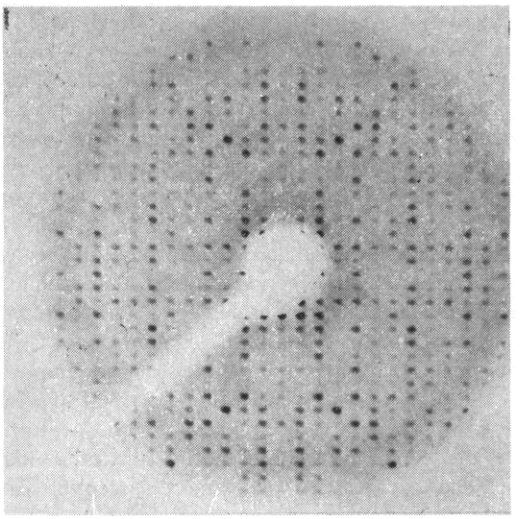

FIGURE 12: 15° precession camera photograph of the hko zone of [RNase·1Hg] monoclinic crystals.

ribonuclease is presented for comparison. Only three tyrosine residues of [RNase·1Hg] titrate normally in the pH range of 9.5–11.5, while the other three tyrosine residues titrate abnormally above pH 11.5. The titration curves for the tyrosine residues of [RNase·1Hg] and the native protein are identical.

OPTICAL ROTATORY DISPERSION was measured in the spectral range 230–300 mμ in neutral aqueous solution (2-mg/ml protein concentration). RNase and [RNase·1Hg] had identical dispersion curves in this region.

THE ENZYMIC ACTIVITY of [RNase·1Hg] toward yeast RNA and cytidine 2′,3′-cyclic phosphate was measured and compared with that of native RNase. There was no difference between the activity of the native and modified enzyme.

DIGESTION BY TRYPSIN. The susceptibility of [RNase·1Hg] to tryptic digestion was measured with a pH-Stat at pH 8.0. It was found that [RNase·1Hg] is resistant to digestion by trypsin as is the native protein.

IMMUNOLOGICAL PROPERTIES OF [RNase·1Hg]. The precipitin curve of the reaction of [RNase·1Hg] with the purified γ-globulin fraction of rabbit antiserum to RNase is presented in Figure 11. For comparison, the precipitin curve of native RNase is included. Though the curves are not superimposable, they are quite similar. It should be pointed out, however, that on precipitation [RNase·1Hg] loses most of its mercury, and only 20% of the counts could be found in these precipitate. It is pertinent to note that the anti-RNase solution used was found to contain 0.25 μmole of free sulfhydryl groups/ml.

CELL DIMENSIONS OF MONOCLINIC CRYSTALLINE [RNase·1Hg]. A single crystal of [RNase·1Hg] was photographed, using a 15° precession camera and Cu Kα radiation (1.5405 Å) (Figure 12). By use of radioactive mercury it was found that the mercury derivative retains its full mercury content upon crystallization. [RNase·1Hg] crystals were found to be monoclinic with space group $P2_1$. The unit cell dimensions were: a = 30.50 Å, b = 38.11 Å, c = 53.88 Å, and β = 107.5°. This resembled RNase modification II, crystallized by King *et al.* (1956) under similar conditions, with unit cell dimension of: a = 30.31 Å, b = 38.31 Å, c = 53.25 Å, and β = 105.78°. Comparison with results on native RNase showed differences in intensities in the precession photographs of [RNase·1Hg] and native RNase.

CM-RNase. As was shown above, CM-RNase is not a homogeneous preparation. However, the following properties are significant. It is as active as native RNase toward RNA, and it is completely resistant to digestion by trypsin at 30°.

[*RNase·2Hg*]. [RNase·2Hg] migrated as a single band on acrylamide gel disc electrophoresis. The spectrophotometric titration of [RNase·2Hg] was identical with that of the native protein and showed that three of the tyrosine hydroxyl groups titrate abnormally above pH 11.5 (see Figure 10). The optical rotatory dispersion of [RNase·2Hg] was measured in the spectral range of 240–300 mμ and was found to be identical with that of native RNase. [RNase·2Hg] was found to be as active enzymically as the native enzyme toward both RNA and cytidine 2′,3′-cyclic phosphate. It was completely resistant to digestion by trypsin at 30°, pH 8.0. [RNase·2Hg] crystallized readily by method D of King *et al.* (1956), yielding crystals of more than 5 mm in length.

Discussion

In the present study it has been demonstrated that the disulfide bond IV–V in ribonuclease, which closes the "small loop," is appreciably more susceptible to reduction by a neutral sulfhydryl reagent, dithioerythritol, than the other disulfide bonds in the molecule. Furthermore, disruption or elongation of this bridge by reduction and blocking, or by introduction of a mercury atom between the sulfur atoms of this bridge, has no perceptible effect on the physicochemical or biological properties of the enzyme.

The course of the reduction of the disulfide bonds in RNase by DTE was followed by carboxymethylation of the liberated sulfhydryl groups. There is an initial fast reduction followed by a much slower reduction (Figure 3). This observation and the dependence of CMC formation upon concentration of reducing agent in the reaction mixture (Figure 1), indicates that the various disulfide bonds in the protein vary widely in

their reactivity toward the reducing agent. If there were no side reactions competing with the carboxymethylation reaction, one might have expected a change in rate of carboxymethylation after incorporation of an even number of carboxymethyl groups per protein molecule. Since partially reduced RNase tends to reoxidize rapidly, only a fraction of the partially reduced enzyme molecules undergo carboxymethylation, yielding a product which contains an *average of about one* carboxymethylcysteine group per protein molecule at the end of the fast reduction period. Indeed, this product could be partially resolved by acrylamide gel electrophoresis, yielding a protein fraction with a CMC content of 1.6 CMC/protein molecule. Analysis of the peptides obtained upon peptic digestion of CM-RNase showed that nearly all the carboxymethyl groups were attached *in pairs* to the peptide which contains the "small loop" in the native protein. It may thus be concluded that cystine IV–V is more susceptible to reduction by DTE than the other cystine bridges in RNase.

The fast reduction stage, which is completed within 1 hr of reaction, at a concentration of DTE of 20 μmoles/ml (Figures 1 and 3) yields a carboxymethylated derivative, CM-RNase, which has a mobility similar to that of the native protein (Figure 2). On submission of CM-RNase to preparative acrylamide gel electrophoresis, however, the carboxymethylated protein could be partially resolved from native protein present in the preparation. More extensive reduction yielded a mixture of carboxymethylated derivatives which were resolved into two bands on acrylamide gel electrophoresis, one corresponding to 8CM-RNase and the other to native or 2CM-RNase. No intermediate bands could be detected. If may therefore be concluded that after cystine IV–V has been reduced, the remaining intact disulfide bonds are sluggish in reacting with the reducing agent. However, when a second S–S bond in the molecule is cleaved, the remaining disulfide bonds rupture readily to produce the fully reduced protein. Conditions for preparation of partially reduced RNase and its derivatives could therefore be chosen so that no fully reduced RNase is formed.

A comparison of these results with those of cleavage of the disulfide bonds in RNase by phosphorothioate (Neumann *et al.* 1967), reveals differences in the reactivity of RNase toward DTE and phosphorothioate. With the latter reagent two disulfide bonds were split, *i.e.*, IV–V and III–VIII, and no further reaction was detected even after 24 hr, while with DTE, bond IV–V splits rapidly and the remaining disulfide bonds may subsequently react, although at a much slower rate. The differences between the two reagents may be attributed to various factors. DTE is electrically neutral while phosphorothioate is negatively charged; DTE is larger than phosphorothioate and may therefore encounter more pronounced steric hindrances; and, finally, phosphorothioate is covalently bound to the cysteine residues which are formed upon the rupture of a disulfide bond, while DTE is not.

Although CM-RNase is a mixture of native RNase and 2CM-RNase, the properties of this preparation which have been described are significant since about 50% of the mixture is 2CM-RNase. CM-RNase is enzymically as active as the native enzyme and is resistant to digestion by trypsin. It may thus be concluded that reduction and carboxymethylation of RNase at the cystine bridge IV–V does not alter the activity or gross conformation of the enzyme.

Partially reduced RNase reacts readily with mercuric ions. A complex containing two mercury atoms per protein molecule, [RNase·2Hg], is obtained at the end of the fast reduction stage on reaction with mercuric chloride. Since native RNase binds negligible amounts of mercuric ions, it is evident that the mercury in [RNase·2Hg] is bound to sulfhydryl groups formed on reduction. Further proof of this conclusion is provided by the fact that [RNase·2Hg] yields carboxymethylcysteine on reaction with EDTA and iodoacetic acid. The study of the peptic digest of the carboxymethylated, partially reduced RNase discussed above has shown that only one disulfide bond, *i.e.*, IV–V, has been reduced by DTE. [RNase·2Hg] must, therefore, have the mercury atoms bound in the form $-SHg^+$ to the two sulfur atoms of this reduced bond. This conclusion is in accord with the fact that [RNase·2Hg] readily loses one mercury atom on dialysis against water at natural pH presumably as a result of a reaction of the type $2(-SHg^+) \rightleftarrows -SHgS- + Hg^{2+}$, accompanied by the production of [RNase·1Hg]. The suggestion that the two mercury atoms in [RNase·2Hg] are both bound to the sulfur atoms of cysteines IV and V gains further support by the reversibility of the above reaction which has been demonstrated by the production of [RNase·2Hg] upon the mixing of [RNase·1Hg] with equivalent amounts of mercuric chloride. This type of reaction has been well established and characterized in previous studies on the reaction of both low and high molecular weight sulfhydryl compounds with mercuric ions (Edelhoch *et al.*, 1953; Cecil, 1963; Steinberg and Sperling, 1967).

While mercuric ions bind readily to the sulfhydryl groups of RNase which has been reduced at cysteine IV–V, *p*-mercuribenzoate completely fails to bind to the partially reduced protein. Steric restrictions probably exclude the relatively bulky organic mercurial from the vicinity of the liberated sulfhydryl groups. The marked facility with which the sulfhydryl groups of the partially reduced RNase oxidize may be due to the proximity of these groups to each other, imposed by the intact conformation of the rest of the molecule.

[RNase·2Hg] is identical with RNase in enzymatic and conformational properties. The properties of [RNase·2Hg] and CM-RNase discussed above, should be contrasted with those previously reported for 4PS-RNase, in which both cystines IV–V and III–VIII had been cleaved and phosphorothiolated (Neumann *et al.*, 1967). 4PS-RNase is digested by trypsin and and is enzymically more active than RNase toward cytidine 2′,3′-cyclic phosphate. It thus follows that while cleavage of both cystine bridges IV–V and III–VIII involves some conformational changes in RNase, cleavage of the cystine bridge IV–V is not accompanied by any significant changes in the macromolecular conformation.

Prolonged dialysis of [RNase·1Hg] against water in the absence of complexing agents, or gel filtration on Sephadex, failed to remove any of the mercury from this RNase derivative. Furthermore, no loss of mercury was detected upon crystallization of [RNase·1Hg]. These findings are to be expected in view of the extremely high binding constant of mercuric ions to sulfhydryl groups, which is of the order of 10^{40}–10^{43}. Most of the properties of [RNase·1Hg] were investigated at conditions (concentration, solvent composition, and temperature) under which the mercury was thus found to be firmly bound to the protein, thus representing genuine properties of [RNase·1Hg]. These considerations apply to sedimentation velocity in the ultracentrifuge, absorption spectra, fluorescence measurements, titration of the tyrosine hydroxyl groups, and

resistance to digestion by trypsin. Furthermore, quantitative analysis of the ultraviolet absorption spectrum of [RNase·1Hg] (see Figure 9) confirms the presence of an intact S–Hg–S bond in [RNase·1Hg]. The enzymic activity of [RNase·1Hg] was measured at lower protein concentrations, and a question might therefore be raised whether the S–Hg–S bonds in the protein derivative remained intact in these experiments. It should be noted, however, that full enzymic activity was found for the protein derivative when excess of $HgCl_2$ (10^{-6} M) was added to the assay mixture, in order to minimize dissociation of the sulfur–mercury bonds. The identity of the rates of migration of [RNase·1Hg] and RNase on acrylamide gel should be accepted with caution, since it is possible that [RNase·1Hg] lost its mercury atom to the gel and oxidized to the native protein. Loss of mercury was found to occur on reacting [RNase·1Hg] with anti-RNase. In this case the γ-globulin preparation used was found to contain free sulfhydryl groups. It is probably these free sulfhydryl groups present in the reaction mixture that extracted part of the mercury from [RNase·1Hg].

The sequence of atoms S–Hg–S is known to be linear, and the distance between the sulfur atoms is about 3Å longer than in the S–S bond (Pauling, 1960; Grdenic, 1965; Yakel and Hughes, 1954; Bradley and Kunchur, 1965). It is therefore of interest that [RNase·1Hg], which contains an S–Hg–S bond system, is very similar to native RNase in all the physicochemical and biological properties mentioned above. Preliminary studies of the optical rotatory dispersion curve and circular dichroism spectrum of [RNase·1Hg] also show them to be similar to those of the native protein. It may therefore be concluded that the exact geometry of the disulfide bridge IV–V, which closes the "small loop" is of little importance to the biological activity and gross conformation of RNase. Thus, this bridge may be extended by nearly 3 Å without involving noticeable changes in the properties of the protein. These results should be compared with the properties of [RNase·4Hg], in which there are four S–Hg–S intramolecular bonds. In the latter case, marked changes in the enzymic and immunological properties of the protein have been found upon introduction of mercury atoms (Steinberg and Sperling, 1967).

Inspection of the small loop in a model of RNase-S, constructed from X-ray diffraction data by F. M. Richards and H. W. Wyckoff,[2] suggests that cystine IV–V is the most easily reduced because it is the most exposed to the solvent (see also Kartha *et al.*, 1967). Introduction of a mercury atom between the sulfur atoms of cystine IV–V requires only minor adjustments of the backbone rotational angles of the amino acid residues 65–72 of the "small loop." No change in the rest of the enzyme is required. It should be remembered that the active site of RNase does not involve the region of the "small loop." It seems to be even easier to bind two mercury ions to the sulfur atoms of the reduced cystine IV–V, with concomitant formation of $-S-Hg^+$ bonds. No steric hindrance is encountered and no adjustment of backbone angles is required. This observation explains the close similarity in properties between [RNase·2Hg] and the native enzyme. It is therefore quite conceivable that if half-cystines IV and V were replaced by other amino acids such as glycine, alanine, or serine, the resulting protein will be indistinguishable from RNase in its properties. On the other hand, introduction of mercury atoms between the sulfur atoms of the other cystine residues in the model was found to require appreciable changes in the conformation of native RNase.

The present study has demonstrated the feasibility of preparing heavy metal derivatives of proteins for X-ray diffraction studies, by the introduction of a mercury atom into a disulfide bridge. Preliminary X-ray diffraction measurements on the monoclinic crystals of [RNase·1Hg] showed the cell dimensions to be nearly identical with those of the corresponding crystals of the native enzyme. The intensities of the reflections were significantly different in the two cases, as expected. This technique may serve as a method for the preparation of heavy metal derivatives of proteins containing cystine.

[2] The conformation of RNase is known to be quite similar to that of RNase S, except where there is a chemical difference (Wyckoff *et al.*, 1967).

Acknowledgments

We thank Professor F. M. Richards and Professor H. W. Wyckoff for very helpful discussions in connection with their three-dimensional model of RNase S. Measurements with the Cary-60 were made possible by the kind hospitality of Professor H. A. Scheraga. The X-ray photographs were obtained with the help of Mr. B. Kaufman.

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