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Selenotrisulfides. II. Cross-Linking of Reduced Pancreatic Ribonuclease with Selenium*

H. E. Ganther and C. Corcoran

ABSTRACT: Native or reduced pancreatic ribonuclease A having zero and eight thiol groups per mole, respectively, was treated with selenious acid to evaluate the role of thiol groups in the nonenzymic incorporation of inorganic selenite into proteins. Over the pH range of 2–7, no selenium was incorporated into native ribonuclease A, but reduced ribonuclease A treated with selenious acid at pH 2 and 4° showed rapid loss of thiol groups, spectral changes equivalent to selenotrisulfide (RSSeSR) formation, and the uptake of 2 moles of selenium/mole of ribonuclease A, in accord with the over-all reaction $4RSH + H_2SeO_3 \rightarrow RSSeSR + RSSR + 3H_2O$. At higher pH, elemental selenium was liberated during the reaction, decreasing the incorporation of selenium to 1.41 and 0.44 moles of Se per mole of ribonuclease A at pH 4.7 and 7, respectively, but pH 7 caused little or no release of selenium from the final reaction product. Gel filtration and sedimentation velocity studies of the pH 2 derivative indicate that it is homogeneous, monomeric, and more unfolded than native ribonuclease A. Spectral perturbations in the 280–290-mμ region likewise suggest that the degree of folding is intermediate between that of reduced and native ribonuclease A.

The exchange of approximately 1 mole of selenium into ribonuclease A from the selenotrisulfide derivative of 2-mercaptoethanol occurred with reduced ribonuclease A at 4° at pH 2–7, with extensive liberation of elemental selenium, but none was exchanged into native ribonuclease A. The enzyme activity of the derivatives against yeast ribonucleic acid or 2′,-3′-cyclic cytidylic acid equaled only a few per cent of that of native ribonuclease A. It is concluded that selenium was incorporated between two sulfur atoms to form an intramolecular selenotrisulfide linkage in place of a disulfide. Such derivatives may be useful for a variety of protein structure–function studies.

The reaction of selenious acid with low molecular weight thiols to form selenotrisulfides (RSSeSR) has previously been characterized in this laboratory (Ganther, 1968). We also investigated the reaction with protein thiols, with the object of creating proteins having selenotrisulfide cross-linkages. Besides clarifying a nonenzymic process of selenite incorporation into proteins, the creation of such derivatives might be useful for other reasons: (1) the conversion of a disulfide bond into the SSeS linkage could provide a useful analog for study of protein structure and function; (2) the introduction at specific sites of a selenium atom could be useful in X-ray crystallographic or electron spin resonance studies of proteins; (3) protein selenotrisulfides might participate in catalytic processes related to the biological functions of selenium.

Ribonuclease was chosen as a model protein for these studies. Besides having great stability and useful solubility properties, the native enzyme has four disulfide bonds that can be reduced to yield four pairs of sulfhydryl groups, providing a well-defined system for the precise study of selenium incorporation in the same polypeptide chain, with or without thiol groups. There was also a reasonable chance that the selenotri-

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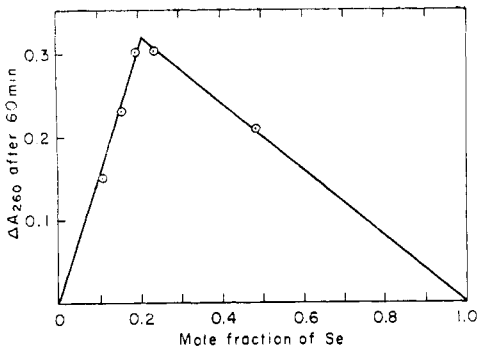


FIGURE 1: Combining ratio for selenious acid and the sulfhydryl groups of reduced RNase by the method of continuous variations. A Zeiss PMQ II spectrophotometer was used. Selenious acid and reduced RNase (sum of selenious acid and protein thiol groups = 2 μmoles) were mixed in various proportions in 3 ml of 0.01 N HCl, 25°, final pH 2.20–2.25. The reaction was mostly completed within 3 min and the A_{260} then remained constant through 60 min. The final solutions were clear and had an A_{400} of approximately 0.01 or less.

sulfide derivative might possess catalytic activity, since the reduced enzyme can be reoxidized spontaneously with re-formation of intramolecular disulfide bonds and full recovery of activity.

It is shown in this paper that selenious acid and other selenium compounds are incorporated into ribonuclease in a stable form through a reaction with the sulfhydryl groups of the protein. The products appear to be cross-linked by selenotrisulfide bridges and are largely devoid of enzyme activity.

Experimental Section

Materials. Ribonuclease A from bovine pancreas (type III-A, 90–95% homogeneous), yeast ribonucleic acid (type XI) purified by the method of Crestfield (1955), 2′,3′-cyclic cytidylic acid, and 2-mercaptoethanol, were obtained from the Sigma Chemical Co., St. Louis, Mo., and used without further purification. Urea (J. T. Baker) was recrystallized frequently from 95% ethanol and dissolved just prior to use. Selenium dioxide (99.9%) was obtained from Alfa Inorganics. Radioactive selenium was purchased as [^{75}Se]H_2SeO_3 from the Nuclear Science and Engineering Corp. The procedures used for purifying the radioactive selenious acid, for determining radiochemical purity, and for chemical analysis of selenious acid have been described (Ganther, 1968).

Reduction of RNase. Ribonuclease (25 mg) was treated with 25 μl of 2-mercaptoethanol in 0.7 ml of 8 M urea at pH 8.6 for 4.5 hr at 23° under nitrogen (Anfinsen and Haber, 1961). This mixture was then adjusted to pH 3.5 with acetic acid and passed rapidly through a 2 × 40 cm column of Bio-Gel P-2 equilibrated with 0.1 N acetic acid, at 4°, to separate reduced protein from the other reagents. This solution was either used immediately or stored overnight under nitrogen in the refrigerator. The concentration of reduced protein in 0.1 N acetic acid was estimated at 275.5 mμ using an extinction coefficient of 8.28×10^3 l. mole^{-1} cm^{-1}, as determined by an analysis for total nitrogen (Lang, 1958) and use of the calculated per cent nitrogen (17.5) for reduced pancreatic ribonuclease A, $C_{575}H_{909}N_{171}O_{193}S_{12}$, mol wt 13,675. An extinction coefficient of 9.8×10^3 l. mole^{-1} cm^{-1} at 277.5 mμ was used to determine the concentration of native RNase in neutral solutions (Sela and Anfinsen, 1957). The sulfhydryl content of reduced RNase determined by the method of Ellman (1959) was close to the expected value of eight sulfhydryl groups per molecule of RNase provided the reduced protein was treated with the Ellman reagent immediately after adjusting the sample to pH 8, so that a rapid spontaneous oxidation of thiol was prevented.

Assay of RNase Activity. With yeast RNA as substrate, a modification of the method of Kalnitsky *et al.* (1959) was used. Samples of enzyme to be assayed were adjusted to pH 5.0 with sodium acetate or acetic acid. Aliquots (up to 0.2 ml) of these solutions and native RNase controls were placed in polypropylene centrifuge tubes and 0.1 M sodium acetate buffer (pH 5.0) was added to bring the volume to 0.5 ml, followed by 0.25 ml of a 1% solution of yeast RNA previously dialyzed against the acetate buffer. After a 4-min incubation at 30° the reaction was stopped with 0.25 ml of 0.75% uranyl acetate in 25% perchloric acid and the samples were chilled on ice. After centrifugation in the cold, 0.1-ml aliquots of the supernatant fluid were diluted with 3.0 ml of water and read at 260 mμ, then corrected for RNase-free blanks carried through the same procedure. RNase activity was calculated from the ΔA_{260}, linearly related to the amount of RNase up to 6 μg.

RNase activity with 2′,3′-cyclic cytidylic acid as substrate was determined using the conditions specified by Crook *et al.* (1960), with 0.275 μmole of substrate, 100 μmoles of Tris-HCl buffer (pH 7.0) and enzyme in a final volume of 1.0–1.2 ml. The initial rate (up to 0.03 ΔA_{286}) was linearly related to the amount of RNase up to 10 μg.

Results

Spectrophotometric Studies. It was shown previously (Ganther, 1968) that the reaction between thiols and selenious acid can be measured by the increase in absorption in the near ultraviolet. The spectrophotometric method of continuous variations (Chaberek and Martell, 1959) was therefore used to determine the combining ratio for selenious acid and the thiol groups of reduced RNase (Figure 1). The maximum absorption increment occurred at a mole fraction of selenium equal to 0.2, corresponding to an SH–Se combining ratio of 4, thus indicating that the thiol groups of this protein react with selenious acid in a manner analogous to other thiols (Ganther, 1968) (eq 1).

$$4RSH + H_2SeO_3 \longrightarrow RSSeSR + RSSR + 3H_2O \quad (1)$$

The data shown in Figure 1 were obtained in dilute HCl, pH 2.25; similar results were obtained at pH 4.7, but as the pH of the reaction mixture was increased there was an increasing tendency of the product to decompose to elemental selenium in the presence of excess thiol, so that the turbidity of the elemental selenium precluded accurate spectrophotometric analysis.

The spectra of native RNase, reduced RNase, and of reduced RNase treated with a stoichiometric amount (2Se/RNase) of selenious acid are shown in Figure 2. Compared with native RNase (curve 1), the spectrum of reduced RNase (curve 2) is shifted to slightly shorter wavelengths and has a somewhat deeper trough at 250 mμ. A pronounced spectral change occurred when selenious acid was added to reduced

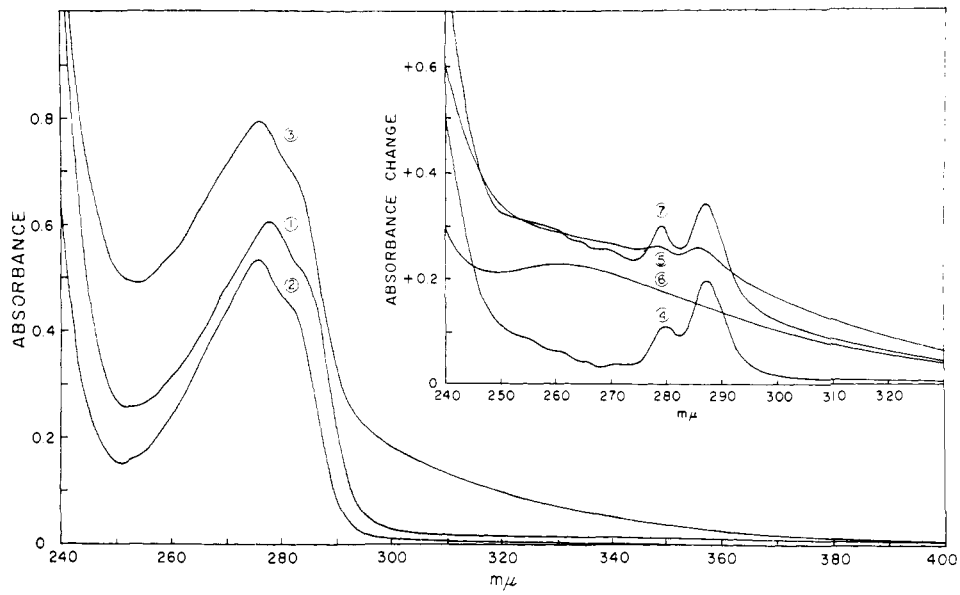


FIGURE 2: Ultraviolet absorption spectra of RNase and derivatives in 0.1 N acetic acid *vs.* solvent, 25°. The spectra were recorded with a Beckman DB-G spectrophotometer coupled to a Sargent SRL recorder. Split compartment cells (Yankeelov, 1963) having a path length of 0.88 cm were used. Each cell contained 0.125 μmole of protein in 1.8 ml of 0.1 N acetic acid. Curve 1, native RNase; curve 2, reduced RNase (8.16 SH/mole); curve 3, reduced RNase plus 0.25 μmole of H_2SeO_3 (mixed at 1°, then warmed to 25° after 1.5 hr and recorded). Insert shows difference spectra for same solutions: curve 4, native *vs.* reduced RNase (curve 1 *vs.* 2); curve 5, reduced RNase plus H_2SeO_3, mixed *vs.* unmixed (one compartment contained 0.125 μmole of RNase in 0.9 ml of solvent, the second compartment contained 0.25 μmole of H_2SeO_3 in 0.9 ml of solvent; after scanning contents *vs.* a duplicate cell to obtain base line, both cells were cooled to 1°, one cell was mixed, and after 1.5 hr both cells were warmed to 25° and spectrum of mixed was recorded *vs.* unmixed); curve 6, cysteine plus H_2SeO_3, mixed *vs.* unmixed (same procedure as described for curve 5, except 1 μmole of cysteine was substituted for 0.125 μmole of reduced RNase); curve 7 is the sum of curves 4 and 6.

RNase, which was complete within a short time. This derivative, Se–RNase, showed enhanced absorption throughout the near-ultraviolet spectrum with a band extending to approximately 400 mμ (curve 3), suggestive of selenotrisulfide formation. In order to compare the spectral changes in Se–RNase with those which occur during selenotrisulfide formation, at least two effects must be considered. One effect involves the formation of a new chromophore, SSeS. The other concerns pertubations of existing chromophores, including tyrosine residues, related to folding of the RNase when disulfide or selenotrisulfide linkages are formed. The folding pertubations are evident in curve 4, the difference spectrum for native *vs.* reduced RNase; the characteristic peaks at 279 and 287.5 mμ are seen, plus some enhancement at lower wavelengths as well. The spectral change that occurs in forming the selenotrisulfide chromophore from cysteine is shown in curve 6; a peak at 262 mμ and a trough at 248 mμ are characteristic of this class of compounds (Ganther, 1968). The spectral changes that occur in forming Se–RNase are then shown in curve 5, the difference spectrum of Se–RNase *vs.* reduced RNase. It shows some degree of pertubation of the tyrosine residues, indicating a folding effect. It shows long-wavelength absorption similar to that of selenotrisulfides, but lacks the characteristic peak at 262 mμ. The absence of the peak at 262 mμ would be expected however, if the observed difference spectrum (curve 5) is a composite of the two effects described previously, because the trough at 248 mμ in the SSeS chromophore would be obliterated by the sharp increase in absorption beginning at about 250 mμ which is associated with the difference in spectrum between folded (native) and reduced RNase (curve 4). Certainly the folding in Se–RNase is not the same as in native RNase, but if one adds curves 4 and 6 to obtain curve 7, a reasonable approximation to the observed difference spectrum (curve 5) is obtained. Furthermore, it is quite possible that the spectrum of the S–Se–S structure, like other chromophores, may be slightly different in a protein when compared with simple selenotrisulfides. For these reasons we believe that the observed spectral changes are consistent with the formation of a selenotrisulfide cross-linkage in RNase.

Uptake of ^{75}Se by RNase. The amount of radioactive [^{75}Se]-H_2SeO_3 taken up by reduced or native RNase at pH 2, 4.7, and 7 was then investigated. Even though stoichiometric proportions of protein thiol and selenious acid were used, and the reactions carried out on ice, some elemental selenium was liberated during the course of the reaction at pH 7, although little was noticeable at pH 4.7 and none at pH 2. After several days storage at 4° the reaction mixtures were passed through a 2.5 × 90 cm column of Sephadex G-75 equilibrated with 0.1 N acetic acid, at room temperature. Figure 3 shows the resulting distribution of selenium in relation to A_{240}. It is clear that selenite becomes firmly incorporated into reduced RNase in all cases, whereas selenite added to native RNase is completely separated from protein by gel filtration. At pH 2, nearly all of the selenite added to reduced RNase was present in the protein fraction and almost none emerged at the elution volume for free selenite. At higher pH, the amount of selenium bound to protein decreased and the amount of free selenium increased. The elemental selenium formed at higher pH remains on the Sephadex column.

Besides showing that selenium was firmly associated with RNase protein, the gel filtration studies revealed that the protein which contained selenium always emerged ahead of the

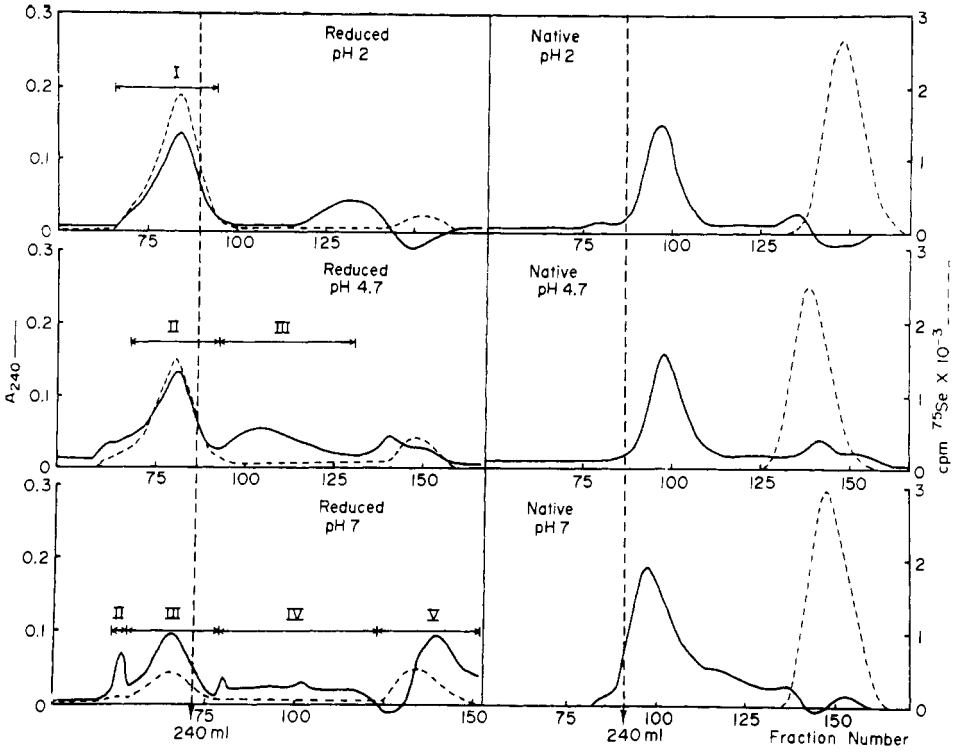


FIGURE 3: Sephadex G-75 chromatography of RNase treated with selenious acid. To a solution containing 7.5 mg of reduced RNase (8.16 SH/mole) or native RNase at 4° in a final volume of 25 ml was added sufficient [^{75}Se]H_2SeO_3 to give a molar Se/RNase ratio of 2; solvents used, and the final pH obtained, were 0.01 N HCl, pH 2.2; 0.1 M sodium acetate, pH 4.7; and 0.1 M sodium phosphate, pH 7.2. After 2.5 hr aliquots of the reaction mixtures were frozen for later assay of enzyme activity (Table III). The remainder was stored at 4° and a 15-ml aliquot subsequently chromatographed at room temperature on a 2.5 × 90 cm column of Sephadex G-75 (previously calibrated with a 15-ml aliquot of native RNase) equilibrated with 0.1 N acetic acid and eluted with the same solvent at a flow rate of 6 ml/cm^2 per hr. A_{240} (protein was monitored at 240 mμ instead of 280 mμ to increase detection sensitivity, see Figure 2) and ^{75}Se content were determined for each fraction of approximately 3 ml (to compensate for variations in the volume of the fractions, the cumulative volume of effluent was measured; the vertical dashed line at 240-ml marks the critical effluent volume, *i.e.*, after Se–RNase is mostly eluted and before native RNase is eluted). The peaks were pooled as indicated in the figure, lyophilized, and analyzed for total nitrogen and ^{75}Se content (Table I).

point where native RNase would emerge. For the derivative prepared at pH 2, all of the protein and selenium behaved in this fashion, and there was no peak corresponding to native RNase. At pH 4.7 two protein peaks were seen, one that contained selenium emerging first, followed by a peak corresponding to native RNase that was practically devoid of selenium. In the pH 7 sample the major peak of protein-bound selenium corresponded to those observed at pH 2 and 4.7, with a diffuse distribution of protein in the region of native RNase. Preceding the main pH 7 peak was a small A_{240} peak having a low content of selenium, which might be aggregated RNase (in other experiments, insoluble material sometimes precipitated from pH 4.7 mixtures during subsequent dialysis at pH 7, similar to the behavior reported for RNase aggregates; Steinberg and Sperling, 1967). The changes in A_{240} near the elution volume for selenious acid in both the native and reduced RNase chromatograms are artifacts related to the emergence of sample buffers, with the possible exception of the pH 7 reduced RNase sample.

Although the gel filtration studies led to the preliminary conclusion (Ganther and Corcoran, 1969) that selenium might be present only in dimeric RNase, further studies of such material in the ultracentrifuge showed that this was not the case. When reduced RNase (0.3 mg/ml) was treated with selenious acid in 0.01 N HCl at 4°, then brought to a concentration of 4.4 mg/ml in 0.1 M sodium acetate, pH 4.7, and sedimented at 59,780 rpm at 20°, an apparent sedimentation velocity of 1.5 S was obtained, with no sign of dimeric or higher aggregates. Native RNase run simultaneously in the opposite position of the rotor (same buffer and protein concentration) had an apparent sedimentation velocity of 1.7 S. These results indicate that the selenium derivative is a monomer of RNase, apparently more unfolded than native RNase in view of the lower sedimentation value and the smaller elution volume on Sephadex G-75. Crestfield *et al.* (1963) have previously shown that RNase derivatives in which the disulfide bonds have been ruptured are excluded more than native RNase by Sephadex G-75 and emerge in a position comparable with aggregates of RNase. Both the gel filtration and the sedimentation studies indicate that the derivative formed at pH 2 is quite homogeneous.

The peaks separated by Sephadex G-75 chromatography were lyophilized to dryness and analyzed for nitrogen and ^{75}Se (Table I) in order to calculate moles of selenium incorporated per mole of protein, which amounted to 2.07, 1.41, and 0.44 for the major protein derivative formed at pH 2, 4.7, and 7, respectively. A value of 1.80 moles of Se/mole of RNase was obtained for a second preparation at pH 2. In other experiments where a 100% excess of selenious acid was used (Se/RNase = 4), approximately 50% of the ^{75}Se could be removed by dialysis and the uptake of ^{75}Se into total RNase was not

TABLE I: Selenium Content of RNase after Sephadex G-75 Chromatography of Reduced RNase Treated with Selenious Acid.

Fraction[a]	Total N (μg)	Total ^{75}Se (cpm)	Moles of Se/Mole of RNase
pH 2, I	471	27,220	2.07
pH 4.7, II	354	13,910	1.41
III	38	1,180	*b*
pH 7, II	56	400	*b*
III	340	4,130	0.44
IV	204	1,860	0.25
V	78	6,420	*b*

[a] See chromatograms in Figure 3. [b] The amounts of N or ^{75}Se are too small to permit a reliable ratio to be calculated.

TABLE II: Exchange of Selenium from a Selenotrisulfide into RNase.

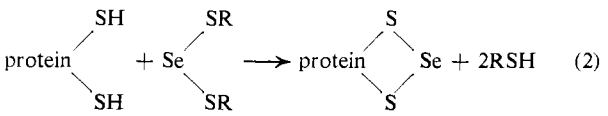
Treatment[a]		Moles of Se/Mole of RNase
Enzyme Form	pH	
Reduced	2.2	1.11
Reduced	3.2	1.09
Reduced	4.1	0.85
Reduced	7.0	1.26
Native	2.2	0.002
Native	7.0	0.002

[a] Reduced RNase (7.68 SH/mole) or native RNase at a concentration of 0.3 mg/ml was treated at 4° with sufficient [^{75}Se]selenodimercaptoethanol (Ganther, 1968) to give a molar ratio of selenotrisulfide/RNase equal to 4; pH 2.2, 0.01 N HCl; pH 3.2, 0.1 N acetic acid; pH 4, 0.1 M sodium acetate; and pH 7, 0.1 M sodium phosphate. Red turbidity (Se°) appeared immediately in the reduced enzyme treated at pH 7, and within 0.5 hr at pH 4.1; increasing turbidity developed after 1 and 24 hr at pH 3.2 and 2.2, respectively, but none was formed with the native RNase. All samples were dialyzed against dilute acetic acid until free of dialyzable ^{75}Se, then passed through short columns of Bio-Gel P-2 equilibrated with 0.1 N acetic acid until free of turbidity. The fractions were concentrated by lyophilization and analyzed for nitrogen and ^{75}Se to determine moles of Se per mole of RNase.

appreciably increased. Since there are eight SH groups per molecule of reduced RNase, the uptake at pH 2 is in good agreement with the stoichiometry of the reaction determined spectrophotometrically. At pH 7 the amount of selenium incorporated was only about 20% of that at pH 2, but even this is a very substantial amount, corresponding to 2540 ppm.

The oxidation of protein SH groups by selenious acid was determined in another experiment. Reduced RNase treated with a stoichiometric amount of selenious acid at pH 2, 4°, under nitrogen, had 0.61 and 0.18 SH groups per molecule of RNase after 30 min and 1 hr, respectively. The corresponding values for a control sample not treated with selenious acid were 7.26 and 7.27. Since the nearly complete oxidation of SH groups in the presence of selenious acid occurred under anaerobic conditions, selenium was not catalyzing an oxidation of SH groups by oxygen.

In the over-all reaction of various thiols or reduced RNase with selenious acid, the reduction of Se^{IV} to the selenotrisulfide requires the oxidation of a second pair of sulfhydryl groups to a disulfide. It was therefore of interest to treat reduced RNase with a reduced form of selenium, so that protein sulfhydryls would not have to serve as a source of reducing equivalents. With this in mind, the selenotrisulfide derivative of 2-mercaptoethanol was prepared as described previously (Ganther, 1968) to see if selenium could be incorporated into reduced RNase by a sulfhydryl–selenotrisulfide-exchange reaction. If the over-all exchange occurred as in eq 2, one



(2)

might expect to incorporate one selenium between every two sulfurs, or 4 Se/RNase. Reduced or native RNase was therefore treated with [^{75}Se]selenodimercaptoethanol at 4° over a pH range from 2.2 to 7.0 (Table II). With reduced enzyme a reaction occurred in all cases, as shown by the appearance of turbid elemental selenium, although the reaction proceeded quite slowly at pH 2.2 and 3.2. There was no sign of reaction with native RNase. After dialysis, and repeated passage of the turbid samples through Bio-Gel P-2 to remove elemental selenium, the samples were analyzed for nitrogen and ^{75}Se. The selenium content of the purified derivatives of reduced RNase ranged from 0.85 to 1.26 moles of Se per mole of protein, whereas native RNase took up almost no selenium. These results indicate that a substantial exchange of selenium into RNase did occur, but the yield was lower than expected because decomposition of some reaction intermediate occurred (stability of both the reactant and the product forms of selenium under the reaction conditions was demonstrated). Other experiments with elemental selenium (not shown) revealed that very little of this form of selenium was incorporated into either reduced or native RNase.

Enzyme Activity of Derivatives. The enzyme activity of reduced or native RNase treated with selenious acid was determined with both yeast RNA and cytidine 2′,3′-cyclic cytidylic acid substrates (Table III). The activity of native enzyme was not appreciably altered by the treatment but the reduced RNase showed almost no recovery of activity when treated with selenious acid at low pH, where selenium was incorporated stoichiometrically. Although significant activity was present in the sample reacted at pH 7, this might have resulted from the re-formation of a small amount of selenium-free RNase by spontaneous oxidation, since we observed complete recovery of activity in other experiments when reduced RNase was allowed to reoxidize under the same conditions. The condition of near neutral pH required for high spontaneous recovery of activity (Haber and Anfinsen, 1962) is least favorable for high yields of the selenotrisulfide, thus we cannot say

TABLE III: Enzymic Activity of Reduced or Native RNase Treated with Selenious Acid at Varied pH.

Treatment[a]		Amt Assayed	Activity[b]	
Enzyme Form	pH	(μg)	RNA	Cytidine 2′,3′-Cyclic Phosphate
Reduced	2.2	60	0.5	0
Reduced	4.7	60	1.8	0.8
Reduced	7	60	8.6	2.3
Native	2.2	6	93	100
Native	4.7	6	92	98
Native	7	6	120	96

[a] The reaction mixtures which were assayed are those described in Figure 3. [b] Activity per μg of treated RNase/activity per μg of native RNase × 100.

whether or not the formation of such derivatives necessarily leads to loss of enzyme activity.

Discussion

This work was undertaken with two objectives in mind. One was to evaluate the role of SH groups in the nonenzymic incorporation of selenium into proteins, in order to clarify the fate of inorganic selenite in biological systems. The second objective was to specifically insert a selenium atom between the sulfur atoms of a protein disulfide bridge, thus creating a selenotrisulfide derivative useful for a variety of protein structure–function studies.

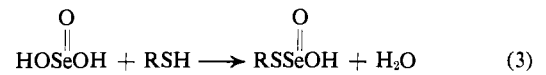
It is clear that the nonenzymic incorporation of selenious acid into ribonuclease involves a reaction with SH groups. Selenium is not bound to native RNase, which lacks SH groups, but reduced RNase treated with selenious acid shows rapid spectral changes consistent with selenotrisulfide formation, complete loss of SH groups, and uptake of selenium in accord with the over-all reaction

$$4RSH + H_2SeO_3 \longrightarrow RSSeSR + RSSR + 3H_2O$$

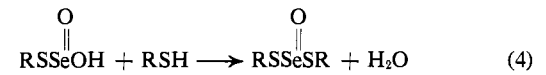
At low pH this reaction stoichiometry holds precisely, whereas at pH 7 the reaction is accompanied by extensive liberation of elemental selenium, considerably decreasing the final selenium incorporation. The fact that the final reaction product does not liberate selenium at pH 7 and 4° indicates that elemental selenium formed under such conditions arises from decomposition of a labile intermediate. Similar effects were noted previously in the reaction of selenious acid with the majority of low molecular weight thiols (Ganther, 1968). It is very unlikely that the failure of native RNase to react with selenious acid results from folding and masking of some reactive group other than sulfhydryl. The conclusion that selenium is incorporated as a selenotrisulfide is based on the analogies between the reaction of selenious acid with reduced RNase and the previously characterized reactions of selenious acid with low molecular weight thiols (Ganther, 1968); reaction stoichiometry (determined spectrophotometrically and also by ^{75}Se uptake), spectral changes, and chemical stability of the compounds are very similar in both cases.

Ribonuclease was a suitable protein for pursuing the second objective, having four disulfide linkages that can be reduced to four pairs of SH groups, each SH group derived from a given disulfide bond being adjacent to the other SH group derived from the same disulfide. Under appropriate conditions (pH 7–8, low protein concentrations), reduced RNase thus can reoxidize spontaneously to a form which is very similar to native RNase and which has comparable enzyme activity (Anfinsen and Haber, 1961). A molecule of selenious acid might have reacted with only one or two SH groups, forming derivatives

of the type RSSeOH or RSSeSR.



$$HOSeOH + RSH \longrightarrow RSSeOH + H_2O \quad (3)$$



$$RSSeOH + RSH \longrightarrow RSSeSR + H_2O \quad (4)$$

Reactions 3 and 4 would lead to the uptake of 8 and 4 moles of selenium, respectively, per mole of reduced RNase. These possibilities are clearly ruled out by the reaction stoichiometry, which shows that only 2 moles of selenium are incorporated per mole of RNase. It appears that such derivatives, if formed, have a strong tendency to undergo further reduction to the selenotrisulfide. The necessary reducing equivalents presumably arise by oxidation of a second pair of SH groups to a disulfide. Although intermolecular cross-linking was expected to occur, and was observed in some cases, the derivative formed at pH 2 appears to be a monomer on the basis of sedimentation behavior, even though it is excluded to a greater extent than native RNase on Sephadex G-75. It is possible to arrange a structural model of RNase so that two sets of four juxtaposed SH groups are present, by pairing certain pairs of sulfur atoms already known to be associated in the specifically folded enzyme. This could explain the fact that an average of two atoms of selenium are present in each molecule of RNase derivative, but is not a necessary condition, because one RNase molecule might donate reducing equivalents to another molecule without intermolecular bond formation. Attempts to increase the number of selenotrisulfide bridges to four per molecule by using reduced forms of selenium were unsuccessful, but did show that a sulfhydryl–selenotrisulfide exchange of some type can take place, even at pH 2.2, where sulfhydryl–disulfide interchange is minimal. The fact that the presence of selenium between two sulfur atoms confers a much greater sensitivity (compared with disulfides) to attack by thiols may be of great importance in connection with disulfide interchange phenomena and the biological role of selenium.

It remains to be determined whether or not the insertion of a selenium atom between the sulfur atoms of disulfide bridges necessarily leads to loss of enzyme activity. In simple compounds containing the C–S–S–C structure, the dihedral angle between the two C–S bonds is very nearly 90°, apparently to avoid overlap between the unshared p electrons on the two sulfur atoms (Calvin, 1954). In CSSeSC, similar considerations would cause the C–S bonds to be in nearly parallel planes, slightly offset. By changing the length and especially the ge-

ometry of the original cross-linkage, the selenotrisulfide modification might be expected to alter the protein conformation so much that enzyme activity would be lost. We found that the activity of RNase treated with selenious acid at low pH, where incorporation of selenium was stoichiometric, was indeed low. Unfortunately, however, the recovery of enzyme activity in the reoxidation of reduced RNase is markedly dependent upon pH (Haber and Anfinsen, 1962), and the condition of low pH that is most favorable for producing the selenotrisulfide derivative in high yield is least favorable for the recovery of enzyme activity. Steinberg and Sperling (1967) reported on the cross-linking of reduced RNase with mercuric chloride, forming linear S–Hg–S bonds. Although the slightly lengthened bonds thus obtained might have distorted the conformation only minimally, no catalytic activity could be detected. Their derivative was prepared at pH 4.6, however, and these authors failed to consider that the absence of activity may have been due in large part to the effect of pH on conformation, causing incorrect pairing of the sulfur atoms.

Although our results provide what is probably the most conclusive evidence for selenotrisulfide formation in proteins, Holker and Speakman (1958) were the first to investigate such a reaction. They demonstrated that cysteine residues, generated by the reduction of disulfide bridges in wool, reacted with selenious acid with loss of SH groups and incorporation of large amounts of selenium into the protein, but the stoichiometric relationships were uncertain. Jenkins (1968), working with proteins labeled *in vitro* or *in vivo* with only trace amounts of ^{75}Se, also obtained evidence of selenotrisulfide formation. Cummins and Martin (1967), however, assumed that selenious acid was simply adsorbed to proteins because protein-bound ^{75}Se could be removed by dialysis at pH 11, but this treatment would have decomposed any selenotrisulfide originally present in the protein and caused the selenium to be oxidized back to selenite by atmospheric oxygen.

While it is clear that selenium can be incorporated into proteins as a selenotrisulfide bridge, other forms may also occur. When radioactive selenite of high specific activity is used, it is obvious that radioactivity equivalent to only traces of selenium may be adsorbed to proteins, but this would have little significance chemically. There is little doubt that species capable of converting inorganic sulfur to sulfur amino acids also utilize inorganic selenite for the biosynthesis of selenoamino acids that are incorporated into proteins. Even in species incapable of the reductive utilization of sulfur, the greater chemical reactivity of selenium may lead to the formation of reduced selenides which find their way into the amino acid residues of proteins, perhaps in part by nucleophilic displacement (Theodoropoulos *et al.*, 1967) of groups such as the hydroxyl residues of serine.

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