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Selenotrisulfides. Formation by the Reaction of Thiols with Selenious Acid*

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ABSTRACT: Selenious acid combines with cysteine, 2-mercaptoethanol, glutathione, or coenzyme A to form moderately stable derivatives having an enhanced absorption in the 260–380-mμ region. The combining ratio for the thiols and selenious acid was found to be 4:1 by spectrophotometric analysis. The over-all stoichiometry thus conforms to the reaction proposed by Painter (Painter, E. P. (1941), *Chem. Rev. 28*, 179), $4RSH + H_2SeO_3 \rightarrow RSSeSR + RSSR + 3H_2O$. The reaction mixtures were resolved by thin-layer chromatography into two spots corresponding to the disulfide and the selenotrisulfide (RSSeSR). A column chromatographic procedure based on chelated copper as a stationary phase was developed which permitted the isolation of selenodicysteine and selenodimercaptoethanol. Selenodicysteine was identified by elemental analysis and by amino acid analysis.

These results establish the above reaction as a plausible means of incorporating inorganic selenite into a stable organic moiety. The chemistry and possible biological significance of selenotrisulfides are under further investigation.

As part of a continuing investigation on the biosynthesis of organoselenium compounds from inorganic forms of selenium, it was shown in a previous study that a rather extensive enzymic conversion of sodium selenite into dimethyl selenide occurs directly in mammalian tissues (Ganther, 1966). Liver microsomal fractions showed an absolute and specific requirement for glutathione in this process. The involvement of glutathione in the metabolism of selenite made it desirable to know more about the possible nonenzymic reactions that might occur between sulfhydryl compounds and selenite.

It has been known for over 30 years that aqueous solutions of selenium dioxide react with sulfhydryl compounds to form relatively unstable derivatives. In a 1941 review, Painter proposed that the reaction between thiols and selenium dioxide takes place as seen in reaction 1. These derivatives of the type RSSeSR will be referred to as selenotrisulfides.[1] Selenotrisulfides are relatively unstable, especially in alkaline solution, decomposing to the disulfide and elemental selenium as shown in reaction 2. Although previous studies

$$4RSH + SeO_2 \longrightarrow RSSeSR + RSSR + 2H_2O \quad (1)$$

$$RSSeSR \longrightarrow RSSR + Se \quad (2)$$

of selenium dioxide–thiol reactions, summarized in the Discussion, are consistent with reaction 1, no selenotrisulfide has ever been isolated from the reaction mixture of thiols and selenious acid in pure form for proper characterization. The potential importance of selenotrisulfides in biological systems, as well as the interesting chemistry involved, made it desirable to establish this class of compounds as genuine chemical entities. The experiments in this paper confirm reaction 1 and describe the isolation and characterization of selenodicysteine.

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[1] No general name has previously been used for these compounds, although specific compounds have been referred to as selenodicysteine and selenodiglutathione (Rosenfeld and Beath, 1964). Although selenodithiol or dithioselenide might be used as trivial names for the general type, selenotrisulfide is preferred because it follows the common practice of using the prefix seleno to indicate substitution of an atom of selenium for an atom of sulfur in the sulfur analog, which in this case is a trisulfide. The names selenodicysteine, selenodiglutathione, etc., will be retained for specific selenotrisulfides.

Materials and Methods

Materials. Selenium dioxide (99.9%) was obtained from Alfa Inorganics. Cysteine and glutathione were obtained from Calbiochem, 2-mercaptoethanol (type I) from Sigma, and coenzyme A (Chromatopure grade) from P-L Biochemicals. Solutions of thiols were prepared on the day of use and stored in ice. The method of Ellman (1959) was used to standardize solutions of coenzyme A and mercaptoethanol, as well as glutathione and cysteine in some experiments. Glass-distilled water was used to prepare all solutions.

Radioactive selenium was purchased as $[^{75}Se]H_2SeO_3$ from the Nuclear Science and Engineering Corp. To purify the radioactive selenium, an appropriate amount of carrier selenious acid was added, followed by ascorbic acid to a concentration of 1% to reduce selenite to elemental selenium. The supernatant was discarded and the precipitated selenium was washed twice with distilled water. The selenium was then oxidized to selenium dioxide by adding a small amount of nitric acid and evaporating the solution to dryness in a hood at 50–60°. After addition of water and evaporation to dryness the white residue was dissolved in water and analyzed for selenite by a modification of the diaminobenzidine method of Cheng (1956), as described below.

Selenite Analysis. Samples and standards containing 1–10 μg of selenium as selenite in a volume of 5 ml were adjusted to pH 2.9–3 (glass electrode) with formic acid and ammonium hydroxide. After the addition of 2 ml of a freshly prepared 0.3% solution of 3,3′-diaminobenzidine (J. T. Baker Chemical Co.) the samples were incubated 40 min at room temperature while protected from light. The samples were then brought to pH 7–8 by the addition of ammonium hydroxide, quantitatively transferred to 40-ml glass-stoppered centrifuge tubes, and adjusted to a volume of 15.0 ml. The selenite–diaminobenzidine complex was extracted into 3.0 ml of toluene. After brief centrifugation, the optical density of the toluene layer was measured at 420 mμ against a reagent blank and the amount of selenium calculated from a standard curve prepared using the same procedure.

Radiochemical Purity. The purity of the radioactive selenious acid was determined by thin-layer chromatography of an aliquot in the presence of 0.4 μmole each of nonradioactive sodium selenite and sodium selenate on precoated cellulose plates (Brinkmann Instruments, Inc.). The solvent system was composed of equal volumes of dioxane and 2 N NH_4OH (Kempe and Denn, 1966). After chromatography, a spray reagent (Paulson *et al.*, 1966) composed of 50% hypophosphorous acid and concentrated HCl (40:60, v/v) was used to detect selenite by reduction to elemental selenium. Selenate became visible after heating for 10 min at 80°. To determine the distribution of radioactivity, the cellulose was scraped into shell vials for crystal scintillation counting, using the 0.405-meV peak of ^{75}Se. Of the total radioactivity present on the plate, 94% was recovered in the selenite spot (R_F 0.31), 1% in the selenate spot (R_F 0.53), and the rest was distributed uniformly over the plate. The purity of the radioactive selenite was also determined by extracting the selenite–diaminobenzidine complex formed during the analysis of the radioactive selenite with toluene and comparing the partition of radioactivity between the toluene and the aqueous phase (selenate does not form an extractable complex). By this procedure it was found that 98.2% of the radioactivity was removed by four extractions with toluene and 98.6% after six extractions, confirming that the radioactive selenite was essentially free of selenate. This convenient extraction procedure was used for routine checking of the radioactive selenate content.

Preparation of Metal–Chelate Columns. Analytical grade chelating resin (Chelex 100, Bio-Rad Laboratories) (200–400 mesh, Na^+) was suspended in 0.01 M sodium acetate buffer (pH 4) to remove fine particles of resin. The total capacity of this resin was given as 0.33 mmole of $Cu(NH_3)_4^{2+}$/ml of resin. To prepare a given metal–chelate column, the desired volume of resin was treated with an amount of the appropriate metal salt equal to 50% of the total capacity of the resin for $Cu(NH_3)_4^{2+}$. The mixture was stirred gently and allowed to stand for 10 min. The resin was washed once and was then poured into a column partially filled with buffer, and fitted with a funnel and stirrer. Buffer was allowed to flow through the column at a slow rate while the resin packed to a firm bed. After additional washing with buffer the column was ready for use.

Spectrophotometric Procedures. The reaction of thiols with selenious acid leads to a considerably enhanced absorption in the region of 260–380 mμ which permitted the stoichiometry of the reaction between thiols and selenious acid to be determined spectrophotometrically, using two different procedures. In one method a fixed amount of selenious acid was mixed with different amounts of thiol and these reaction mixtures were monitored until no further change in absorbance was observed; the SH/Se ratio at which the absorbance change reached a maximum is the combining ratio. The second procedure used was the method of continuous variations (Chaberek and Martell, 1959) in which the final absorbance increment was determined for solutions containing different ratios of reactants but having a constant value for the sum of their molar concentrations. In the latter procedure the absorbance change is greatest when the mole fractions of the reactants correspond to the combining ratio.

A Zeiss PMQ II spectrophotometer was used for all fixed-wavelength spectrophotometric procedures. Absorption spectra were recorded with a Beckman DB-G spectrophotometer coupled to a Sargent SRL recorder.

Results

Stoichiometry. When 1-μmole quantities of selenious acid were titrated with 2-mercaptoethanol at pH 4 (Figure 1) there was a nearly linear increase in the 30-min A_{260} at SH/Se ratios up to 4, and no indication of decomposition of the selenodimercaptoethanol to elemental selenium was observed. In six other experiments the effect of pH and buffer composition was studied

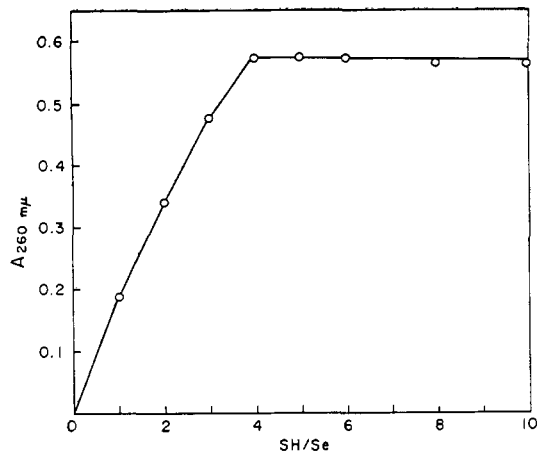

FIGURE 1: Titration of selenious acid with 2-mercaptoethanol. H_2SeO_3 (1 μmole) was added at zero time to cuvets containing, in a final volume of 3 ml, 300 μmoles of sodium acetate (pH 4.0) and 1–10 μmoles of 2-mercaptoethanol.

over a pH range of 1.2–6.4; the rate of reaction was most rapid at low pH, but the same final A_{260} was obtained in all cases. Similar results were obtained with 2-mercaptoethanol using the method of continuous variations (Figure 2); a maximum A_{260} was obtained when the mole fraction of selenious acid was 0.205, in good agreement with the expected value of 0.2 for a SH/Se combining ratio equal to 4.

Figure 2 also shows the results obtained for the reaction between coenzyme A and selenious acid. In this case the increment in A_{300} was used to measure the reaction, and was found to be approximately the same as the increment in A_{300} when other thiols were treated with selenious acid. Coenzyme A reacted less rapidly but yielded an unusually stable derivative, so that the reaction could be investigated at a neutral pH where most thiol–selenious acid mixtures show extensive decomposition to elemental selenium. It was found that coenzyme A and selenious acid react in a ratio of 4:1 at pH 2.3, 4.7, and 7.0, and 4:1 mixtures of coenzyme A and selenious acid were found in other experiments to yield the same final A_{300} values at four intermediate values of pH.

The stoichiometry of the reaction between cysteine and selenite was more difficult to evaluate. The increase in A_{260} with increasing SH/Se ratios was not as linear as for mercaptoethanol, being larger per increment of sulfhydryl at low SH/Se ratios. Although the mixtures developed a maximum A_{260} at SH/Se ratios of approximately 4, the cysteine derivative showed a marked tendency to decompose at higher SH/Se ratios, which made it more difficult to establish the equivalence point by the usual spectrophotometric methods because of the turbidity of elemental selenium. Decomposition was always observed at SH/Se ratios of 5 or more whereas little or no decomposition was observed at ratios of 4 or less. This characteristic property made it possible to estimate the equivalence point (*i.e.*, where additional cysteine would be in excess of selenite) by determining the SH/Se ratio at which elemental selenium formation first occurred. As shown in Table I,

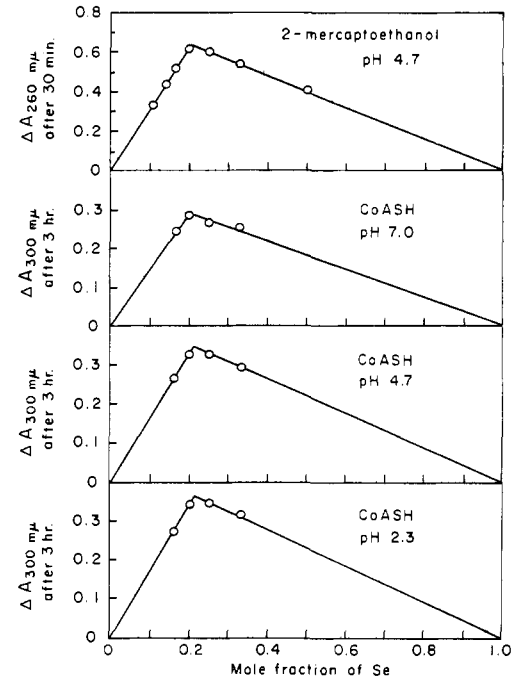

FIGURE 2: Combining ratio for selenious acid and 2-mercaptoethanol or coenzyme A by method of continuous variations. Selenious acid and the indicated thiol (sum of both reactants = 5 μmoles) were mixed in various ratios in 3 ml of solution containing 300 μmoles of buffer at the final pH indicated: sodium phosphate, pH 2.3 or 7.0; sodium acetate, pH 4.7.

decomposition occurred very rapidly at the highest SH/Se ratio and was complete at intermediate ratios after 18.5 hr, but practically no decomposition was observed after the same length of time at ratios of 1, 2, 3, or 4, where cysteine would presumably be limiting if the reaction followed 4:1 stoichiometry. The maximum amount of turbidity produced at all ratios above 4 was approximately the same, suggesting that cysteine effected the decomposition in a catalytic manner.

In a systematic study of the effect of pH on the reaction of cysteine and selenious acid (4:1), such mixtures developed the same final A_{260} over a pH range of 1.2 5.5, with rapid decomposition observed at pH 6.4.

For glutathione, the reaction with selenious acid appears to be more complex and greatly influenced by pH, and only under certain conditions was it possible to obtain 4:1 stoichiometry. A systematic study of the reaction stoichiometry for glutathione at pH 1.3, 2- and 4.7, over a 100-fold range in total reactant concen, tration, revealed that the apparent SH/Se combining ratio approached a 4:1 value as the concentration of reactants was increased and as the pH at which the reaction was carried out was decreased. As shown in Figure 3, a homogeneous reaction corresponding to a 4:1 combining ratio was obtained at pH 1.3, using a total concentration of reactants of 50 μmoles/3 ml, which is ten times the usual concentration. At pH 4.7 and a total reactants concentration of 0.5 μmole/3 ml, the apparent SH/Se combining ratio decreased to approximately 1:1. At pH 2 and 50 μmoles of reactants/3 ml, the apparent combining ratio was 4:1, but the final

TABLE I: Instability of Selenodicysteine in the Presence of Excess Cysteine.

Successive Treatments (hr)[a]	Absorbance at 400 mμ							
SH/Se =	1	2	3	4	5	6	8	10
At 4° (1.5)	0	0	0	0	0	0.003	0.047	0.165
At room temperature (1)	0	0	0	0	0.077	0.230	0.372	0.360
At room temperature (2)	0	0	0	0	0.167	0.352	0.380	0.325
At room temperature (18.5)	0	0	0	0.020	*b*	*b*	*b*	*b*

[a] Selenious acid (1 μmole) was mixed at zero time with the appropriate amount of cysteine to give the indicated SH/Se ratio in a final volume of 3 ml containing 0.05 M sodium acetate (pH 4). After 1.5 hr at 4° samples were brought to room temperature (23°) and the absorbance at 400 mμ was determined immediately and at subsequent intervals as a measure of the decomposition of selenodicysteine to elemental selenium. [b] The $A_{400\ m\mu}$ was not determined due to coalescence of the elemental selenium.

A_{260} was approximately double that obtained at 1.3, indicating the formation of a different type of product. The reaction of glutathione with selenious acid is being investigated further.

Thin-Layer Chromatography of Reaction Products. The separation by thin-layer chromatography of products formed by the reaction of various thiols with selenious acid is shown in Figure 4. When cysteine is mixed with selenious acid, cysteine disappears; cystine, and a component with a mobility intermediate between that of cysteine and cystine, appear in approximately equal amounts. Analogous results were obtained with coenzyme A. Mixtures of glutathione and selenious acid at various pH values likewise showed in all cases a nearly complete loss of glutathione accompanied by the formation of oxidized glutathione, and a second ninhydrin spot that would correspond to the selenotrisulfide derived from other thiols. The reaction mixtures were also subjected to thin-layer chromatography in a solvent system composed of 1-butanol–acetic acid–H_2O (2:1:1), with similar results.

Chromatography on Chelated-Metal Columns. The ability of sulfides and selenides to form stable complexes with various metals (Livingstone, 1965) suggested that a suitable metal fixed to a stationary phase might bring about a differential retardation of the disulfide and the selenotrisulfide formed in the thiol–selenious acid mixture. A number of different metal–chelate columns were therefore prepared, as described in the experimental section. The first column, prepared with Ni^{II}, gave complete separation of the mercaptoethanol–selenious acid products and fairly good separation of cystine from selenodicysteine. $Copper^{II}$ was found to give superior resolution and the results of typical separations on chelated copper are shown in Figure 5. When 2-mercaptoethanol (Figure 5A) was treated with [^{75}Se]selenious acid, only a small amount of radioactivity came out at the void volume for selenious acid. The first ultraviolet-absorbing component, which was free of ^{75}Se, came out somewhat later, followed still later by a second major peak which contained nearly all of the radioactive selenium applied to the column and which showed a close correspondence between radioactive selenium content and absorption at 240 mμ. It is concluded that the radioactive substance is selenodimercaptoethanol and the less-retarded peak is 2-hydroxyethyl disulfide (2,2′-dithiodiethanol). The separation of a cysteine–selenious acid reaction mixture on the same column is shown in Figure 5B. In this case a quantitative ninhydrin procedure (Spies, 1957) was used to monitor the effluent, in addition to optical density at 240 mμ and ^{75}Se content. Three ninhydrin-positive peaks were obtained, one small peak (unidentified, but presumably unreacted cysteine) which also contained a small amount of radioactivity, a second peak (cystine) that was devoid of radioactivity, and a third peak (selenodicysteine) which contained most of the radioactivity but which had a somewhat lower total ninhydrin color relative to peak 2.

The ninhydrin constant relative to leucine was found to be 0.30 for selenodicysteine and 0.53 for cystine (both intensities expressed on the half-molar basis), thus explaining the smaller amount of ninhydrin color

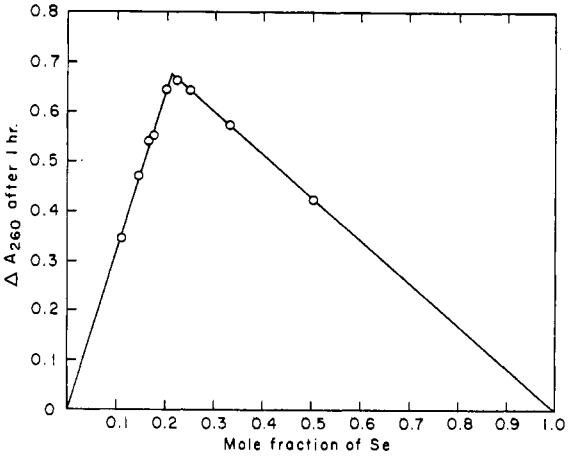

FIGURE 3: Combining ratio for selenious acid and glutathione at tenfold higher concentrations and pH 1.3. The reactants (sum of both reactants = 50 μmoles) were mixed in various ratios in 3 ml of dilute HCl (pH 1.3). The absorbance at 260 mμ was determined for aliquots diluted 1:10 with water.

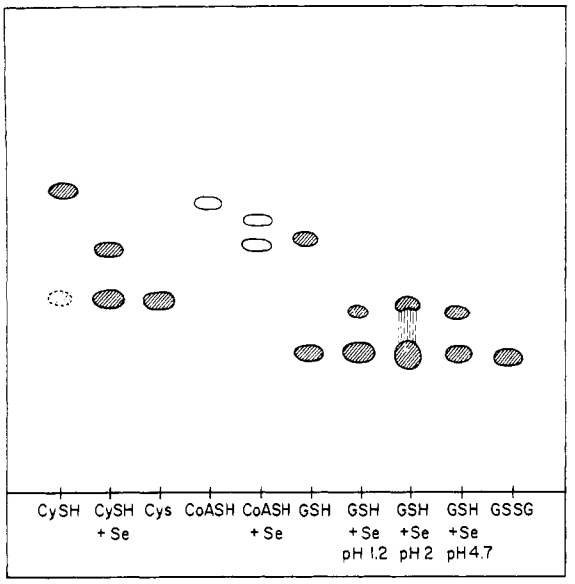

FIGURE 4: Chromatography on cellulose thin-layer plates of reaction mixtures of various thiols and selenious acid. Solvent system = isobutyric acid–H_2O–NH_4OH (66:33:1, v/v). Solutions spotted, reading left to right, were as follows: two 1-μl portions of 0.004 M cysteine (old solution); two 1-μl portions of reaction mixture of 1.5 μmoles of selenious acid with 6 μmoles of cysteine in 1 ml of 0.1 M sodium acetate (pH 4); 1 μl of 0.005 M cystine in 0.05 N HCl; two 1-μl portions of 0.004 M coenzyme A; two 1-μl portions of reaction mixture of 1.37 μmoles of selenious acid with 5.48 μmoles of coenzyme A in 1 ml of 0.1 M sodium acetate buffer (pH 4); 1 μl of 0.004 M glutathione (old solution); 1 μl of reaction mixture of 0.1 μmole of selenious acid reacted with 0.4 μmole of glutathione in 0.3 ml of dilute HCl (pH 1.2) then lyophilized to dryness and taken up in 30 μl of H_2O (partially decomposed); same as previous solution, except taken up in 60 μl of H_2O after being reacted in 0.05 M sodium phosphate buffer (pH 2); same as previous solution except reacted in 0.05 M sodium acetate buffer (pH 4.7); 1 μl of 0.002 M oxidized glutathione. Chromatogram was developed in a sandwich chamber for 20 hr so that solvent front ran off the end of the plate; this was necessary to get maximum resolution of the disulfides and selenotrisulfides. (The R_F of reduced glutathione in this system is 0.44.)

observed in the selenodicysteine peak relative to the cystine peak.

The ultraviolet absorption spectra of selenodicysteine and selenodimercaptoethanol are shown in Figure 6, along with the spectra for the appropriate disulfides. The spectra of both selenotrisulfides are very similar, confirming that the –S–Se–S– grouping is the chromophore. As expected, the selenotrisulfides show a much greater ultraviolet absorption than the disulfides. For selenodicysteine the absorption maximum is at 263 mμ with a molar extinction coefficient of 1650 l. mole^{-1} cm^{-1}; for selenodimercaptoethanol these values are 265 mμ and 1600 l. mole^{-1} cm^{-1}. Both extinction coefficients were calculated from the specific activity of the radioactive selenious acid used to prepare the selenotrisulfides.

At the relatively high concentrations of reactants used to obtain easily detectable amounts of material in the eluates, decomposition was observed unless the reactions were carried out in ice. Once the reaction was complete, the mixtures were stable and subsequent

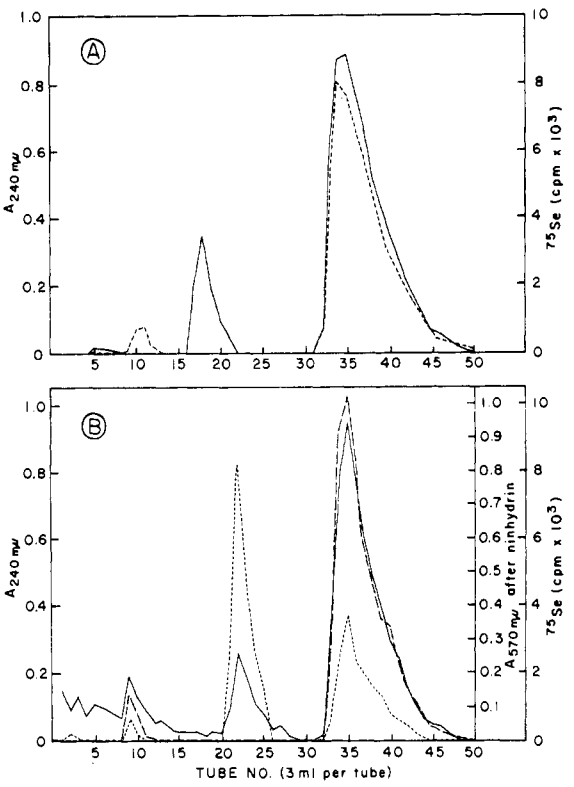

FIGURE 5: Separation on copper–Chelex column (1.2 × 30 cm) of reaction products of [^{75}Se]H_2SeO_3 and 2-mercaptoethanol (A) or cysteine (B). The indicated thiol (30 μmoles) and selenious acid (7.5 μmoles) were reacted in 0.5 ml of 0.01 M sodium acetate (pH 4), at 0° for 15 min (2-mercaptoethanol) or 5 min (cysteine), then transferred to the column and eluted with the same acetate buffer at room temperature at a flow rate of 16 ml/cm^2 per hr. (——) A_{240} mμ; (– – –) ^{75}Se; (· · · ·) A_{570} mμ after ninhydrin.

chromatography was carried out at room temperature. Cysteine and selenious acid were allowed to react for only 5 min; the reaction was nearly complete by this time, as shown by measurement of the A_{260} on a diluted aliquot, and the products had a great tendency to precipitate after a longer interval.

Columns prepared from a number of other metals were all less satisfactory than copper, either affording no separation, or, as in the case of Hg^{II}, causing decomposition of the compounds. While glutathione–selenious acid reaction mixtures emerged from the copper–Chelex columns as a single peak, the isolation of selenodiglutathione has since been accomplished using Dowex 50 columns equilibrated with Ni^{II}, and will be reported in a later paper.

Preparation of Selenodicysteine. To obtain sufficient selenodicysteine for more complete characterization a larger copper–Chelex column (5 × 10.8 cm) was prepared in the same way described for the analytical column. Cysteine (480 μmoles) and selenious acid (120 μmoles) in 8 ml of 0.01 M sodium acetate buffer (pH 4) were allowed to react at 0° for 5 min. A 7-ml aliquot was then transferred to the column equilibrated with 0.01 M sodium acetate buffer (pH 4) and eluted at room temperature with the same buffer at a flow rate of 10 ml/hr per cm^2 column surface. Complete separation

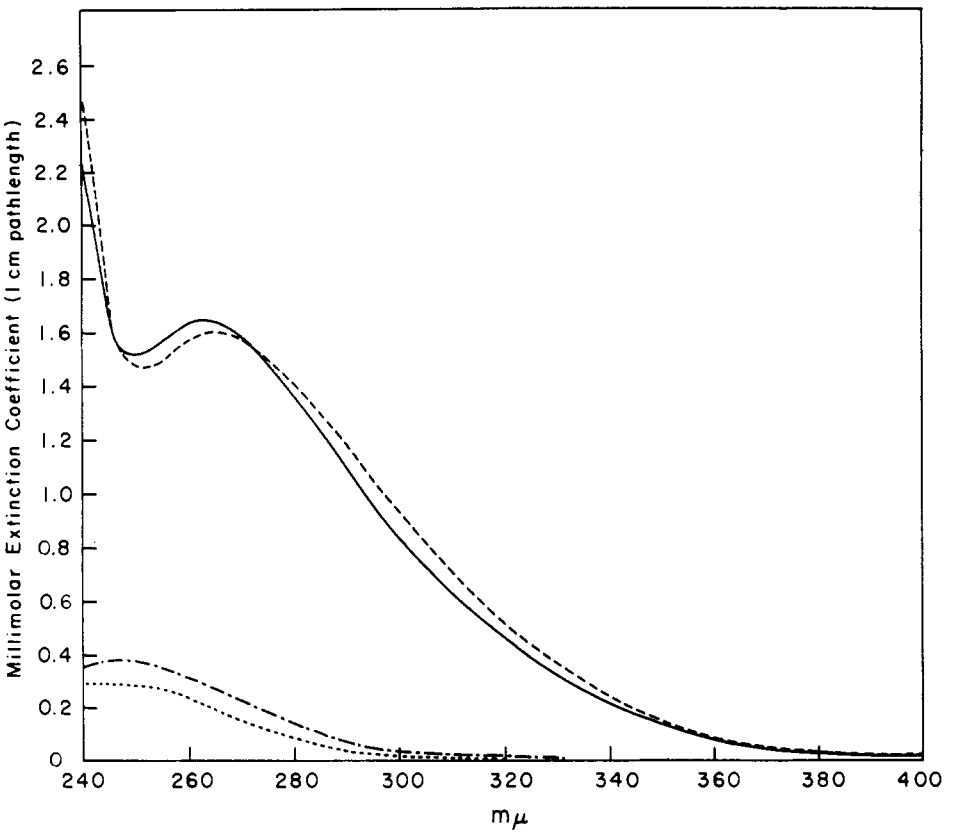

FIGURE 6: Ultraviolet absorption spectra of selenodimercaptoethanol, selenodicysteine, and 2-hydroxyethyl disulfide in 0.01 M sodium acetate buffer (pH 4) *vs.* buffer. The spectrum of cystine is for a solution in 0.05 N HCl *vs.* 0.05 N HCl. (——) Selenodicysteine, (– – –) selenodimercaptoethanol, (· · · ·) cystine, and (–·–·–·) 2-hydroxyethyl disulfide.

of cystine and selenodicysteine was obtained. The selenodicysteine peak was concentrated by lyophilization, dissolved in dilute HCl, and filtered through a 0.45-μ Millipore filter. The filtrate was adjusted to pH 4 with 2 M sodium acetate to precipitate the selenodicysteine. The fine yellow precipitate was collected by filtration, washed with cold water and cold ethanol, and dried *in vacuo* over phosphorus pentoxide for 24 hr at 56°. The yield was approximately 66%. *Anal.* Calcd for $C_6H_{12}N_2O_4S_2Se$: C, 22.57; H, 3.79; N, 8.77. Found: C, 22.59; H, 3.64; N, 8.64; ash, 0. When heated on a Fisher-Johns melting point apparatus the selenodicysteine decomposed, becoming red-brown at 195–200°.

Analysis for Half-cystine Content of Selenodicysteine. To remove selenium from the selenodicysteine prior to amino acid analysis, a portion of the ^{75}Se-labeled fraction isolated on the copper–Chelex column (Figure 5) containing 0.82 μmole of selenodicysteine was mixed with 1.25 μmoles of cysteine in a volume of 4.3 ml. This solution was heated at 70° for 15 min to ensure complete decomposition of the selenotrisulfide to elemental selenium and cystine. To remove the precipitated selenium the solution was quantitatively transferred to a 5-cc syringe, equipped with a Swinnex unit containing a 0.01-μ VF filter (Millipore Filter Corp.), and filtered. After washing the filter with 0.5 N HCl the filtrate was counted and found to be free of radioactive selenium. The filtrate was brought to dryness by rotatory evaporation and stored at 0°.

Performic acid oxidation of the above sample was carried out essentially by the procedure of Moore (1963). A 0.01-ml aliquot of a solution containing 20 mg of alanine and 30 mg of methionine was added, followed by 2 ml of performic acid. This mixture was allowed to stand at 4° for 4 hr, then 0.3 ml of 48% HBr was added and the reagents were removed by rotatory evaporation. The residue was dissolved in buffer (sodium citrate, 0.067 M, pH 2.2) and an aliquot was analyzed in the Technicon amino acid analyzer. An aliquot of cysteine identical with that added to decompose the selenodicysteine was treated as above, omitting the filtration step. The ninhydrin color in the cysteic acid peak derived from oxidation of 0.82 μmole of selenodicysteine plus 1.25 μmoles of cysteine was equivalent to 2.27 times the amount derived from 1.25 μmoles of cysteine alone, thus the amount of cysteic acid derived from 0.82 μmole of selenodicysteine is equivalent to (1.25 μmoles × 2.27) − 1.25 μmoles, or 1.59 μmoles. The half-cystine/Se ratio for selenodicysteine is thus 1.59/0.82 = 1.94, in good agreement with the expected value of 2.

Discussion

The purpose of this work was to investigate the reac-

tions between thiols and selenious acid that lead to the formation of selenotrisulfides, and to establish the existence of this class of compounds by isolating and characterizing selenodicysteine. In this regard the supporting chemical evidence can now be summarized.

The earlier view that the stoichiometry for the reaction of thiols with selenious acid is 4:1 has been confirmed and extended. There appear to be two published interpretations of this stoichiometry for the reaction between cysteine and selenious acid. One view, proposed by Painter in 1941, is that two atoms of sulfur become bound to one atom of selenium (reaction 3). The second, proposed by Stekol in 1942, is that four atoms of sulfur become bound to one atom of selenium (reaction 4). Painter (1941, p 186) indicated that he ob-

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

$$4RSH + H_2SeO_3 \longrightarrow (RS)_4Se + 3H_2O \quad (4)$$

tained from cysteine and selenious acid a substance having a S/Se ratio slightly greater than 2. The compound isolated by Stekol, named selenotetracysteine, was based on a once-recrystallized reaction product that had an elemental analysis consistent with $(RS)_4Se$. Stekol's view has been criticized on the basis that elemental analysis, while excluding many other possible derivatives of selenium, would not differentiate between the compound $(RS)_4Se$ and the mixture $[(RS)_2Se + (RS)_2]$. In order to choose between these two possibilities it is therefore necessary to determine whether one product or two is formed in the reaction.

In all investigations where the reaction mixture of thiols and selenious acid has been subjected to chromatography, evidence of product heterogeneity has been obtained (Klug and Petersen, 1949; Petersen, 1951; Tsen and Tappel, 1958). These studies have generally involved paper chromatography, a mild procedure that would not be expected to induce such heterogeneity. The present study likewise shows that the reaction mixtures for three different thiols, when subjected to thin-layer chromatography in two solvent systems, invariably yield two substances, one corresponding to the disulfide, with complete disappearance of the thiol.

The above results suggest strongly that selenotrisulfides are formed in the manner suggested by Painter, but the isolation of pure selenotrisulfides in amounts sufficient for further characterization had never been accomplished. A column chromatographic procedure was sought which could achieve separations by interactions directly related to the S–S and the S–Se–S structures, and thus be applicable to the isolation of a wide variety of these derivatives. A column composed of cupric ion chelated to a stationary phase proved to be suitable and readily separated the products of the cysteine–selenious acid reaction into two ninhydrin-positive peaks, one free of selenium, and the other containing nearly all of the selenium. The fact that the products of both ionic (cysteine) and nonionic (2-mercaptoethanol) thiols separated in a similar way on the same column suggests that the separations do indeed occur primarily on the basis of metal–disulfide and metal–selenotrisulfide interactions.

The selenium-containing derivative of cysteine obtained by column chromatography was subjected to elemental analysis after a simple work-up and found to have a content of C, H, and N in excellent agreement with that calculated for $C_6H_{12}N_2O_4S_2Se$. This elemental analysis is consistent with $(RS)_2Se$ and excludes $(RS)_4Se$.

The next analytical procedure employed was designed to establish directly the ratio of half-cystine to selenium in the compound. The starting material was prepared from ^{75}Se-labeled selenious acid in order to establish the selenium content. The compound was then treated with performic acid to obtain the cysteic acid derivative (selenium was removed prior to this step to prevent a possible interference of the selenium in the oxidation procedure). Complete amino acid analysis of the derived product showed an increment only in the cysteic acid fraction, in an amount equivalent to 1.94 moles of cysteic acid/g-atom of selenium. The possibility that the starting material was already in the oxidized state is ruled out by the elemental analysis for selenodicysteine.

On the basis of the above evidence (stoichiometry, thin-layer and column chromatography, C, H, N, and Se analysis, and amino acid analysis), the identification of selenodicysteine is established with a reasonable certainty. There is also additional evidence available for the existence of selenotrisulfides from the spectral studies described next.

The final reaction products of a variety of thiols with selenious acid all have a very similar final ultraviolet absorption spectrum, suggesting that they possess a common chromophore. The chromophore is not a loose complex of the reactants because the experimental points obtained with the method of continuous variations show almost no deviation from the intercept of the two extrapolated lines at the combining ratio. The absorption spectrum of the chromophore may be compared to that of compounds having a structure most like that of selenotrisulfides, namely, trisulfides. The trisulfide analog of selenodicysteine, thiocystine (Szczepkowski and Wood, 1967), has a spectrum very similar to that of selenodicysteine, with a comparable extinction coefficient at λ_{max}, but the spectrum of the selenotrisulfide is displaced to longer wavelengths than the trisulfide, as would be expected.

It should be emphasized that the ultraviolet absorption spectra of the unseparated reaction mixtures of selenious acid with cysteine or 2-mercaptoethanol can be fully accounted for, both qualitatively and quantitatively, by the sum of the spectra of the chromatographically separated selenotrisulfide and disulfide. This is perhaps the strongest evidence that the final derivative of cysteine, identified as selenodicysteine, is not simply generated from an initial tetracysteine product during the process of thin-layer or column chromatography.

The possible formation of selenotrisulfides is relevant to many studies involving selenium and must always be taken into account whenever selenious acid and thiols

are both present in a system. Although the studies in this paper have been limited to symmetrical selenotrisulfides, it is to be expected that unsymmetrical selenotrisulfides of the type RSSeSR′ would be formed as well if selenious acid is added to two or more species of thiol compounds (Cummins and Martin, 1967). While selenotrisulfides are often rather unstable this is not always the case, as shown for selenocoenzyme A in this paper, and for selenotrisulfide derivatives of proteins (H. E. Ganther, in preparation). Even selenodicysteine, which readily decomposes in the presence of excess cysteine, proved to be very stable as the isolated solid and showed almost no decomposition when examined by thin-layer chromatography after several months storage at room temperature.

The significance of selenotrisulfides in biological systems is presently under investigation. It has been found (H. E. Ganther, unpublished data) that selenodiglutathione is apparently an excellent substrate for glutathione reductase, as shown by the liberation of elemental selenium from this compound in the presence of TPNH[2] as soon as a small amount (0.5 μg) of highly purified yeast glutathione reductase was added. Besides providing an interesting exception to the rather stringent substrate specificity of this enzyme, such a reaction may be related to the activity of cell-free extracts that reduce selenite to elemental selenium (Nickerson and Falcone, 1963) or to dimethyl selenide (Ganther, 1966).

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[2] Abbreviation is listed in *Biochemistry 5*, 1445 (1966).

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