molecular weight of 29,500 and does exist as a dimer. It is not possible at this time to explain the differences between these data and those of Wu and Tinker.[2]

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Reduction of the Selenotrisulfide Derivative of Glutathione to a Persulfide Analog by Glutathione Reductase*

Howard E. Ganther

ABSTRACT: The compounds formed by the reaction of selenious acid with glutathione were studied with regard to the effects of pH and glutathione:selenious acid ratio. At ratios of 4:1 or less, and a pH below 2 and above 4, the first stable product is the selenotrisulfide derivative of glutathione (GSSeSG) plus an equimolar quantity of GSSG:

$$4GSH + H_2SeO_3 \longrightarrow GSSeSG + GSSG + 3H_2O$$

GSSeSG free of GSSG was prepared by separating the two compounds on a Dowex 50 column equilibrated with 0.01 M $NiCl_2$ in 0.1 M sodium acetate (pH 4.7) followed by adsorption of the GSSeSG fraction to Dowex 50 Na^+ at pH 3 and elution with ammonium acetate at pH 5.5. In order to determine the type of selenium compound formed under conditions more nearly simulating physiological conditions of pH and reactant concentrations, ^{75}Se-labeled selenite (1×10^{-6} M) was treated with 4×10^{-3} M GSH at pH 7, 25°, followed by 50 mM iodoacetate. The major selenium compound thus formed was the *Se*-carboxymethyl derivative of glutathione selenopersulfide (GSSeH); this persulfide is believed to be formed by reduction of the initial selenotrisulfide product with excess GSH. Glutathione and elemental selenium were rapidly liberated from GSSeSG at pH 7 by 0.1 μg or less of highly purified glutathione reductase from yeast. The reduction of GSSeSG was not catalyzed by lipoyl dehydrogenase, nor was glutathione reductase active when DPNH or selenodicysteine (CySSeSCy) was substituted for TPNH or selenodiglutathione. The velocity of GSSeSG reduction was similar to that for GSSG reduction. Evidence was obtained that the initial reaction products were GSH and the selenopersulfide (GSSeH):

$$GSSeSG + TPNH + H^+ \xrightarrow{\text{glutathione reductase}} GSH + GSSeH + TPN^+$$

The selenopersulfide rapidly decomposed to GSH and elemental selenium but could be trapped in the presence of 50 mM iodoacetate as the carboxymethylated derivative, which was identified by thin-layer electrophoresis, thin-layer chromatography, and gel filtration. It is believed that this work provides the first evidence for the selenopersulfide class of compounds. These reactions may have important applications to selenium metabolism and the mechanism of action of selenium as an essential nutrient.

The fate of selenious acid, or selenite, in systems containing thiols is relevant to many problems regarding the nutritional and toxicological aspects of this substance. Compounds of the type RSSeSR (2-selena-1,3-disulfides or selenotrisulfides) have been shown in previous studies to be formed by the reaction of selenious acid with a variety of thiols (Ganther, 1968; Ganther and Corcoran, 1969) in accord with the equation first proposed by Painter (1941)

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

The most abundant thiol in biological systems is glutathione. A specific requirement for glutathione in selenite metabolism has been described (Ganther, 1966). The reaction of glutathione with selenious acid was indicated previously to be more complex than for other thiols (Ganther, 1968) and has now been studied in more detail. The results show that at least three selenium derivatives of glutathione can be formed, depending on pH and the glutathione:selenious acid ratio. One

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of these is the selenotrisulfide derivative (GSSeSG), and a method for isolating this product in pure form is described. The product formed under conditions approximating those *in vivo* has been identified as the selenopersulfide derivative, GSSeH. It is also shown that GSSeSG is readily reduced to the selenopersulfide derivative by TPNH and glutathione reductase. The relevance of these findings to selenium metabolism and the biological role of this element is discussed.

Experimental Section

Materials. Glutathione, oxidized glutathione, lipoyl dehydrogenase (pig heart), and the Dowex 50 type resin (Bio-Rad AG 50-X4, 200–400 mesh) were obtained from Calbiochem. Glutathione reductase (type III, ammonium sulfate suspension, from yeast), TPNH (type II, enzymically reduced), DPNH, DL-lipoamide, bovine serum albumin, EDTA, and iodoacetic acid were purchased from Sigma. ^{75}Se-Labeled selenious acid (selenate-free) was obtained from New England Nuclear. Selenium dioxide (Alfa Inorganics, 99.9%) was dried to constant weight and added to the radioactive selenious acid to obtain the desired specific activity. *N*-DNP-ethanolamine was synthesized by the method of James and Synge (1951). Carboxymethylglutathione was prepared by adjusting a 2×10^{-2} M solution of glutathione to pH 8 with NaOH and adding an equal volume of 0.1 M iodoacetate. After 1-hr reaction at room temperature under nitrogen the mixture was stored at 4°. Selenodicysteine was the preparation described previously (Ganther, 1968). Deionized glass-distilled water was used in all studies

Enzyme Assays. A Beckman DB spectrophotometer and Sargent SRL recorder were used for direct spectrophotometric assays of enzyme reactions. Lipoyl dehydrogenase was assayed by the oxidation of DPNH with lipoamide (Massey, 1960). The incubation medium of Massey and Williams (1965) was used for glutathione reductase except bovine serum albumin was omitted to prevent decomposition of GSSeSG by the protein thiol groups. Following equilibration of the reaction mixture at 25° in 1-cm cuvets, the reaction was started by the addition of 10–25 μl of diluted enzyme, prepared daily by diluting a stock solution of enzyme with 0.005 M sodium phosphate buffer (pH 7.6), containing 1 mM EDTA plus 0.2% bovine serum albumin. The stock solution was prepared by dialyzing the ammonium sulfate suspension of the enzyme against the buffer (albumin omitted) and diluting to a concentration of 80 μg/ml, as determined from the A_{280} using an $E_{1\%}$ of 15.4 (Mavis and Stellwagen, 1968). The specific activity of the enzyme was 160 EU/mg at 25°.

In the stop-analyze procedure for glutathione reductase, GSH was measured by the method of Ellman (1959) after stopping the reaction with metaphosphoric acid. The reaction mixtures were preincubated for 5 min at 25°, then 25 μl of glutathione reductase was added to start the reaction. Aliquots of 1.5 ml were removed 1 and 5 min later and added to 0.18 ml of 18% metaphosphoric acid on ice. After standing for 10 min an aliquot of 0.56 ml was placed in a 1-ml cuvet followed by 0.19 ml of 15% $Na_3PO_4 \cdot 12H_2O$, 0.25 ml of water, and 0.02 ml of 10^{-2} M DTNB.[1] (Elemental selenium was removed from GSSeSG reaction mixtures by Millipore filtration prior to the addition of DTNB.) The concentration of GSH was determined from the absorbance at 412 mμ (E_{mM} 13.6). Enzyme activity was calculated from the amount of GSH formed between 1 and 5 min. The amount of substrate used during the reaction did not exceed 33% of the initial value, except at the two lowest concentrations of GSSeSG where it amounted to approximately 50%.

Preparation of Dowex 50 Ni^{2+} Column. Approximately 1400 ml of Dowex 50 Na^+ resin was treated batchwise with sufficient 0.1 M sodium acetate buffer (pH 4.7), containing 0.01 M $NiCl_2$, to saturate the resin with nickel. The suspension was poured into a 5-cm jacketed column and allowed to pack under gravity to a height of 68 cm, then equilibrated with the acetate–nickel buffer at 4° prior to application of the sample. After using the column to separate glutathione–selenious acid reaction mixtures, it was found necessary to wash the column just prior to subsequent runs with additional buffer to remove ultraviolet-absorbing material. This material was ninhydrin positive and had a spectrum similar to that of GSSG. It is believed that a trace of unreacted GSH in the glutathione–selenious reaction mixtures is initially bound tightly to the column, but gradually oxidizes to GSSG, which is washed off.

Thin-Layer Chromatography and Electrophoresis Procedures. Precoated cellulose plates obtained from Brinkmann Instruments were used. Electrophoresis was conducted at 10° for 1 hr at 400 V (20 V/cm) and approximately 10 mA in a Camag thin-layer electrophoresis cell.[2] The buffers used were formic–acetic–H_2O (150:100:750, v/v, pH 1.3) and pyridine–acetic acid–H_2O (6:20:974, v/v, pH 4.25). After spotting the samples, each end of the plate was dipped in the buffer to a point just below the origin, then blotted on filter paper and placed in the apparatus. Thin-layer chromatography was carried out in sandwich chambers or tanks containing 1-butanol–acetic acid–H_2O (2:1:1, v/v, system I) or isobutyric acid–H_2O–NH_4OH (66:33:1, v/v, system II). After drying in air to remove buffer or solvents, the plates were sprayed with ninhydrin (Sigma) or the combined nitroprusside–cyanide reagent of Smith (1960). *N*-DNP-ethanolamine was used as a marker for electrophoresis.

Other Methods. Elemental selenium was determined by passing the incubation mixtures through a Millipore VS filter, average pore size 0.025 μ, mounted in a Swinnex syringe assembly. After two washes with water the filters were transferred to counting vials for counting of the ^{75}Se in a gamma scintillation counter. Ine xperiments on stoichiometry the difference in A_{340} between the filtered and unfiltered solutions was used to correct the apparent A_{340} change so that TPNH oxidation could be calculated. Quantitative ninhydrin analysis was carried out according to the method of Spies (1957).

Results

Preparation of GSSeSG. Optimum conditions for obtaining GSSeSG in stoichiometric yield were previously shown to involve the reaction of glutathione and selenious acid in a 4:1 ratio at pH 1.3, 4°, at relatively high reactant concentrations (Ganther, 1968). A fresh solution of 0.1 M GSH was cooled to 0°, and 4 ml (400 μmoles) was added (all in one portion) to 100 μmoles of ^{75}Se-labeled selenious acid in 6 ml of 0.1 M HCl previously cooled to 0°. The final pH of this solution was approximately 0.9. After 15 min the reaction mixture was titrated to pH 4.5 by the addition of approximately 1.5 ml of 2 M sodium acetate (stronger bases caused liberation of elemental selenium).

The next step was separation of GSSeSG from the GSSG

[1] Abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoate).

[2] Purchased from funds supplied by The Nutrition Foundation.

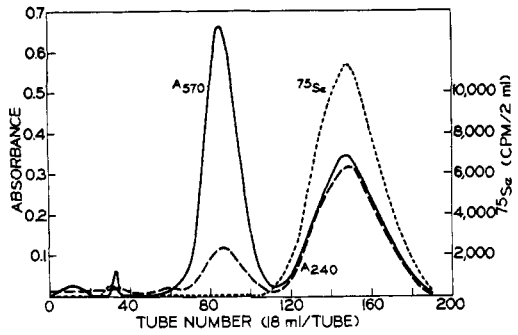


FIGURE 1: Separation of selenodiglutathione from oxidized glutathione. A 10-ml aliquot of the reaction mixture of glutathione and selenious acid (see text) was applied to a 5 × 68 cm column of Dowex 50 equilibrated with 0.1 M sodium acetate containing 0.01 M $NiCl_2$ (pH 4.7) and eluted with the same buffer at 4° at a flow rate of 6.1 ml/cm² per hr. Fractions of approximately 18 ml were collected and read at 240 mμ *vs.* buffer (– – –). Aliquots were also taken for ninhydrin analysis (—) and for ^{75}Se counting in a well-type scintillation counter (- - - -).

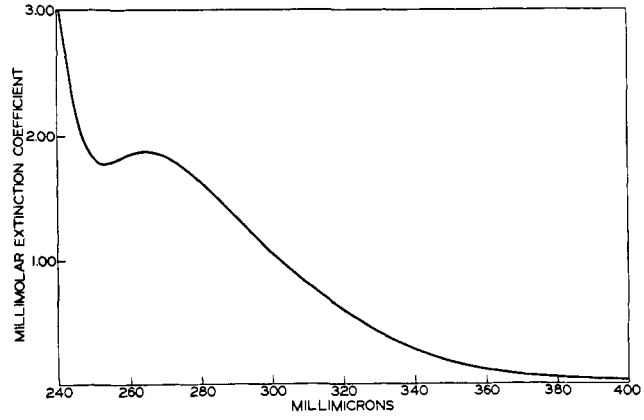


FIGURE 2: Ultraviolet absorption spectrum of selenodiglutathione. A 1:4 dilution of the nickel-free eluate from the second Dowex 50 column (see text), pH 4.2, was scanned in a 1-cm cell *vs.* water.

produced in equimolar amounts in the reaction. Chromatography on columns composed of copper bound to Chelex 100 was unsuccessful, even though these columns readily separated lower molecular weight disulfides from selenotrisulfides. It was found, however, that by operating a Dowex 50 column with sodium acetate buffer having a pH slightly higher than the pH where GSSG was retained on the resin, a differential retardation of GSSeSG and GSSG could be produced by incorporating Ni^{2+} into the buffer. Figure 1 shows the use of such a column to separate the products of the reaction between GSH and radioactive selenious acid. A minor peak (3–5% of the total radioactivity applied to the column), believed to be unreacted selenious acid, emerged from the column at about 45% of the bed volume. This was followed by two major ninhydrin-positive peaks. The first ninhydrin peak contained no ^{75}Se, and was shown to be GSSG by thin-layer chromatography in solvent system II after concentration of the fraction and removal of nickel by means of Dowex 50. The second ninhydrin peak (GSSeSG) had a greater absorption at 240 mμ and contained about 90% of the radioactivity applied to the column.

To concentrate the GSSeSG peak and remove nickel, the pooled fractions were adjusted to pH 3 with 5 N HCl, then adsorbed to a 5 × 8 cm column of Dowex 50-X4, sodium form, equilibrated with 0.1 N formic acid. After washing with several bed volumes of 0.1 N formic acid, GSSeSG was eluted with ammonium acetate buffer, pH 5.5 (0.1 M with respect to acetate). GSSeSG began to be eluted when the pH of the eluate reached 3.3, and the most concentrated fractions had a pH of 4.2. Recovery of GSSeSG in this step was quantitative. Although a volatile buffer was used with the intention of obtaining the peptide in salt-free form after lyophilization, the ammonium ion apparently displaced sodium from the resin so that GSSeSG came off in nearly 0.1 M sodium acetate. The concentration of nickel in these solutions was 0.45 μM or less, as determined by atomic absorption. The concentration of GSSeSG in the peak tubes was sufficiently high (1–1.5 mM) that further concentration was not necessary. The solution could be lyophilized, however, without decomposition of GSSeSG (provided the process was not interrupted until all traces of moisture were eliminated), yielding several grams of a white powder that dissolved in cold 0.1 N acetic acid without decomposition. Although it was found possible to obtain salt-free GSSeSG by using a Dowex 50 NH_4^+ column and eluting with ammonium acetate, the GSSeSG partially decomposed during lyophilization.

Properties of GSSeSG. The ultraviolet absorption spectrum of GSSeSG is shown in Figure 2. The peak at 263 mμ with absorption extending to 400 mμ is very similar to the spectrum of other selenotrisulfides (Ganther, 1968), although the millimolar extinction coefficient of 1.87 at λ_{max} is somewhat higher. Thin-layer chromatography in system II of a 1.47 mM solution of freshly prepared GSSeSG revealed one ninhydrin-positive spot having the mobility of GSSeSG (Ganther, 1968) and no detectable GSSG. When ^{75}Se-labeled GSSeSG was subjected to thin-layer electrophoresis in pyridine–acetate buffer (pH 4), there was a single ninhydrin-positive spot with an anodic mobility equal to 90–95% of that for GSSG. This is in accord with a peptide having the same charge as GSSG but a slightly higher molecular weight. ^{75}Se was present in the same zone as the ninhydrin-positive material and none was present at the origin or in the zone corresponding to selenite. After 2-months storage of GSSeSG solutions at 4°, pH 4.5, slight decomposition of GSSeSG to GSSG and elemental selenium was noted.

Reaction of Selenite with Excess GSH. Solutions of selenious acid and most thiols liberate elemental selenium when the thiol:Se ratio exceeds 4:1, especially at neutral pH (Ganther, 1968). With a sufficient excess of GSH, however, elemental selenium might be converted into the selenopersulfide in accord with the following equilibrium

$$GSH + Se \rightleftharpoons GSSeH \qquad (2)$$

In order to establish what type of compound might exist under conditions of reactant concentrations and pH approximating conditions *in vivo* ^{75}Se-labeled selenious acid (10^{-6} M) was treated with 4 mM GSH at pH 7, 25°. After 1–2 hr iodoacetate was added to convert GSH and any selenopersulfide compounds that might be present to their carboxymethyl derivatives. This solution will be referred to as "sample solution." As a control for possible adsorption of radioactive selenite to the products, a second reaction mixture was set up in which iodoacetate was added to the GSH 1–2 hr before the addition of selenite ("control solution"). A portion of each solution was then applied directly to thin-layer cellulose plates for electrophoresis, and the remainder used for column chromatography.

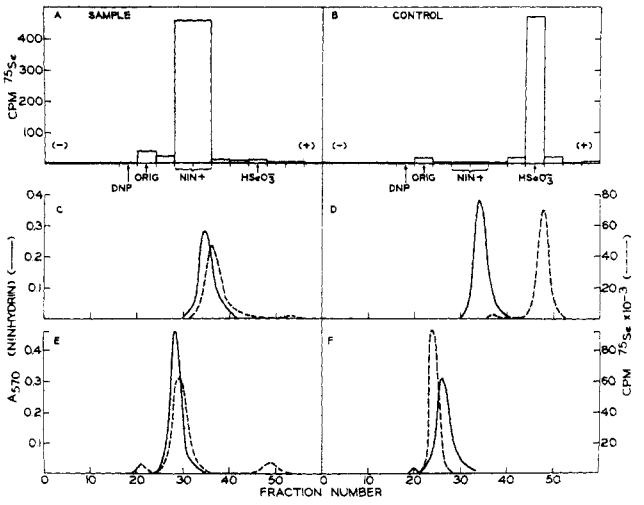


FIGURE 3: Electrophoretic and chromatographic analysis of product formed from selenite (10^{-6} M) and GSH (4×10^{-3} M) at pH 7. Sample (3A,C,E): ^{75}Se-labeled H_2SeO_3 (0.005 μmole) was added to 20 μmoles of GSH in 5 ml of 0.25 M potassium phosphate buffer, pH 7, 25°. After 2 hr an equal volume of 0.1 M iodoacetate was added and the mixture left at room temperature for 30 min, then stored at 4°. Control (3B,D,F): same as sample, except iodoacetate was added at zero time, followed by selenite after 2 hr. A and B: the reaction mixtures (20 μl) were applied directly to cellulose plates for electrophoresis in pH 4 buffer (see Methods) for 1 hr at 10°, 20 V/cm (10 mA). DNP indicates location of *N*-DNP-ethanolamine marker for electroosmosis. Orig = origin. C and D: reaction mixtures were passed through short beds of Sephadex G-10,[3] then applied to a 2.5 × 63 cm column of G-10 equilibrated with 0.05 M sodium acetate (pH 5.5) and eluted with the same buffer at 5 ml/cm² per hr at 4°. Fractions of 4 ml were collected and counted for ^{75}Se (- - - -) or assayed by quantitative ninhydrin analysis (——). E and F: same as C and D except sample not given preliminary G-10 filtration. Aliquots were chromatographed on a 2.5 × 41 cm column of Dowex 50 equilibrated and eluted with 0.1 M sodium acetate (pH 4.7) containing 0.01 M $NiCl_2$, at 4°. Flow rate was 5 ml/cm² per hr and 4-ml fractions were collected. Recovery of ^{75}Se applied to the column was 87 and 93% for sample and control, respectively.

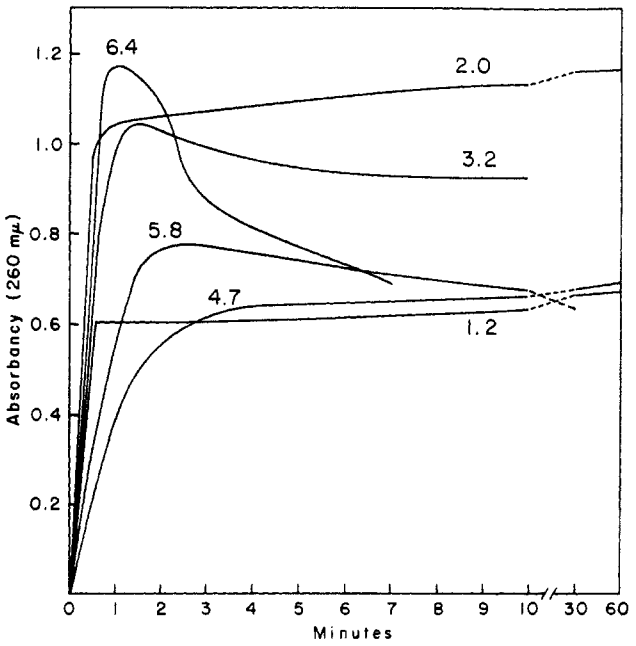


FIGURE 4: Effect of pH on the reaction of glutathione with selenious acid. H_2SeO_3 (1 μmole) was added at zero time to cuvets of 1-cm path length containing, in a final volume of 3 ml, 4 μmoles of GSH and 300 μmoles of buffer at the final pH indicated: 1.2 = phosphoric acid:HCl; 2.0 and 6.4 = sodium phosphate; 3.2 = sodium citrate; 4.7 and 5.8 = sodium acetate; temperature = 23°.

Figure 3A,B shows the distribution of ^{75}Se after electrophoresis of sample and control reaction mixtures, respectively, at pH 4. The major ^{75}Se fraction in the sample moved with the ninhydrin-positive material (*S*-carboxymethylglutathione) toward the anode, and almost no ^{75}Se was present in the zone corresponding to selenite. In contrast, all of the radioactivity in the control moved with selenite and no activity was found in the ninhydrin-positive zone. These results show that selenium from selenite was firmly incorporated into a compound having the same electrophoretic mobility as *S*-carboxymethylglutathione, and that the process involved the SH group of glutathione. Similarly, gel filtration on Sephadex G-10 (Figures 3C,D) showed that ^{75}Se in the sample solution was eluted in the region corresponding to *S*-carboxymethylglutathione whereas that in the control was practically all eluted in the lower molecular weight region corresponding to selenite. None of the radioactivity was present in the region where GSSG was eluted during column calibration, showing that selenium compounds of 600 molecular weight or larger were probably not present in the sample solution. *S*-Carboxymethylglutathione, assayed by the ninhydrin method, served as a convenient internal marker; the elution of the ^{75}Se peak just slightly behind the ninhydrin peak during chromatography on Sephadex G-10 (Figure 3C) was probably caused by an interaction of the selenium atom with the gel that was sufficiently strong to offset the molecular weight contribution of selenium (Christ *et al.*, 1970). A similar retardation of other selenotrisulfide compounds compared to the analogous disulfide compounds has been observed during Sephadex G-10 chromatography (H. E. Ganther, unpublished data).

For additional characterization the sample and control solutions were chromatographed on a Dowex 50 nickel column. On such columns selenite was found in preliminary studies to be eluted before *S*-carboxymethylglutathione. Figure 3E shows that the major portion of the ^{75}Se in the sample solution was eluted just behind the ninhydrin peak of *S*-carboxymethylglutathione, as expected for $GSSeCH_2COOH$, with a small portion in an earlier peak at the position expected for selenite, plus an unidentified third peak. In contrast, most of the ^{75}Se in the control solution (Figure 3F) was eluted ahead of the ninhydrin peak, although somewhat behind the position expected for selenite. The reason for this discrepancy is not known.

Additional studies (not shown) using thin-layer chromatography in solvent systems I and II showed that the ^{75}Se-labeled carboxymethylated derivative formed from selenite in the presence of excess GSH could not be distinguished from the carboxymethylated product formed by the action of glutathione reductase on GSSeSG (see a later section). It is concluded that the compound produced by either method is *Se*-carboxymethylglutathione selenopersulfide, $GSSeCH_2COOH$.

Derivative Formed at pH 2.25. The reaction of selenious acid

[3] Initial experiments showed that a portion of the ^{75}Se in the sample solution was retained on Sephadex G-10 and could not be eluted. This is believed to be elemental selenium, which is known to be retained on Sephadex (Ganther and Corcoran, 1969). For this reason both sample and control solutions were given a preliminary filtration through 2.5 × 3 cm columns of G-10 equilibrated with 0.05 N acetic acid. From 84 to 91% of the ^{75}Se in the sample solution and 100% of that in the control solution could be eluted from the short G-10 columns.

with glutathione is greatly influenced by pH (Ganther, 1968). Figure 4 shows the absorbance changes at 260 mμ when glutathione and selenious acid are mixed in a 4:1 ratio over the pH range 1.2–5.8. At pH 1.2 the absorbance change is very rapid and reaches a stable value in less than 1 min. At pH 4.7 and above the transitory appearance of an elevated A_{260} suggests that one or more intermediates having a higher extinction coefficient accumulates before decaying to a final value similar to that obtained at pH 1.2. The relative rates of formation and decay of such an intermediate presumably depend on pH. At intermediate pH values between 1.2 and 4.7, however, the final A_{260} stabilizes at a considerably higher value. Such effects were not observed with other thiols, and were not caused by a reversible effect of pH on the spectrum of the chromaphore (adjusting the product formed at pH 1.2–4 with sodium acetate had only a minor effect on A_{260}, as did acidification of mixtures reacted at pH 4.7). Length of time for reaction did not seem to be a major factor, since the A_{260} of mixtures reacted at pH 2.25 was still nearly twice that of pH 4.7 mixtures after 7 days at 4°. The high extinction product was obtained at pH 2 in the absence of phosphate buffer and therefore was not related to the presence of phosphate.

Further study revealed that the effect of pH on the extinction coefficient of selenium in the product was greatest at pH 2.25 and dependent on total reactant concentration, being maximal (E_{mM} 4.05) at a concentration of 0.4 mM GSH and 0.1 mM selenious acid and much decreased (E_{mM} 2.30) when the total concentration of reactants was 100-fold higher (50 mM). Chromatography of the ^{75}Se-labeled pH 2.25 derivative on Sephadex G-10 showed that it was eluted just slightly after GSSG, in the same position as GSSeSG, and thus had an apparent molecular weight in the neighborhood of 600. Upon thin-layer electrophoresis, the derivative moved toward the cathode with GSSeSG at pH 1.3, but had no anionic mobility at pH 4, in contrast to GSSeSG. These studies indicate that the high extinction selenium derivative of glutathione formed at pH 2.25 is closely related to GSSeSG, but its structure remains to be determined.

GSSeSG Reduction by Glutathione Reductase. The TPNH-linked reduction of GSSeSG by 0.1 μg of glutathione reductase at pH 7.1 is demonstrated in Figure 5. TPNH oxidation and elemental selenium formation (turbidity) were monitored in separate experiments by following absorption changes at 340 and 400 mμ, respectively. Oxidation of TPNH ensued promptly upon the addition of enzyme. After a brief lag period absorption at 400 mμ also began to increase, and fine red particles of selenium could be seen in the cuvet. As the turbidity increased the loss of absorption at 340 mμ became slower and finally stabilized, as did the absorption at 400 mμ. In the absence of enzyme there was no spontaneous liberation of elemental selenium from GSSeSG, demonstrating the inherent stability of this compound at pH 7 and 25°. Similarly, the addition of the solution used to dilute the enzyme failed to cause any absorbance changes at 340 or 400 mμ, thus ruling out the possibility that decomposition of GSSeSG resulted from the thiol groups of bovine serum albumin present in the diluent.

The lag in the appearance of elemental selenium suggested that it was a secondary product formed by the decomposition of a labile product formed initially in the enzymic reaction. By analogy to the reaction for GSSG (reaction 3), the initial products of GSSeSG reduction should be GSSeH and GSH (reaction 4).

$$\text{GSSG} + \text{TPNH} + \text{H}^+ \longrightarrow \text{GSH} + \text{GSH} + \text{TPN}^+ \quad (3)$$

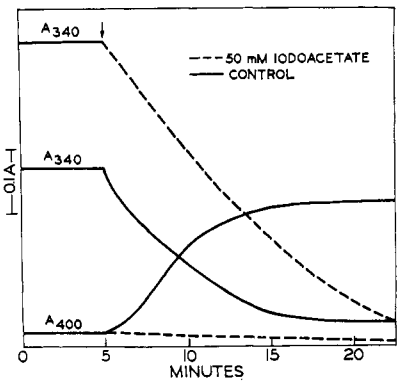


FIGURE 5: Reduction of GSSeSG by glutathione reductase. The control reaction mixtures contained 0.1 μmole of GSSeSG, 0.1 μmole of TPNH, 50 μmoles of potassium phosphate, and 3 μmoles of EDTA, in a total volume of 0.995 (pH 7.1). After a 5-min preincubation at 25°, 0.1 μg of enzyme in 5 μl of diluent was added at the arrow. There were no absorbance changes when diluent alone was added. Control (——); control + 50 mM iodoacetate (- - -).

$$\text{GSSeSG} + \text{TPNH} + \text{H}^+ \longrightarrow \text{GSSeH} + \text{GSH} + \text{TPN}^+ \quad (4)$$

$$\text{GSSeH} \longrightarrow \text{GSH} + \text{Se}^0 \quad (5)$$

Decomposition of the unstable GSSeH would liberate elemental selenium and GSH (reaction 5).

It has been shown that analogous compounds of the type RSSH, referred to as alkyl hydrogen disulfides or persulfides, are formed as intermediates in certain enzyme-catalyzed reactions of sulfur compounds (Hylin and Wood, 1959; Flavin, 1962). Such compounds are unstable and immediately decompose to elemental sulfur and the corresponding thiol. Flavin (1962) showed, however, that the persulfide intermediate in the reaction catalyzed by the cystathionine-cleavage enzyme could be trapped by means of iodoacetate. After establishing that relatively high concentrations of iodoacetate could be tolerated by glutathione reductase, the reduction of GSSeSG was carried out in the presence of 50 mM iodoacetate to see if the postulated GSSeH intermediate could be trapped, thus preventing its decomposition to elemental selenium. As shown in Figure 5, iodoacetate completely suppressed the appearance of elemental selenium but not the oxidation of TPNH; the decline in A_{340} proceeded smoothly, in contrast to the biphasic curve seen when iodoacetate was not present.

Since thiols are known to catalyze the decomposition of selenotrisulfides (Ganther, 1968), an experiment was next carried out to determine whether the decomposition of GSSeSG observed in the presence of glutathione reductase was caused by direct action of the enzyme on the selenotrisulfide, or by glutathione formed from traces of GSSG that might be present in the GSSeSG. By lowering the pH to 6 to suppress the GSH-catalyzed process it could easily be demonstrated (Figure 6) that the decomposition of GSSeSG catalyzed by glutathione reductase was much more rapid than could be accounted for by the presence of GSH. The addition of 0.04 or 0.1 μmole of GSH (an amount equivalent to that which would be formed if GSSG contamination amounted to 20 or 50% of the GSSeSG substrate) caused practically no decomposition over a 5-min period, whereas GSSeSG decomposition and TPNH oxidation in the presence of 2 μg of enzyme were largely complete in the same length of time.

Stoichiometry of GSSeSG Reduction. The stoichiometric relationship between the amount of TPNH oxidized and the

TABLE I: Stoichiometry of GSSeSG Reduction by TPNH and Glutathione Reductase.[a]

Expt	Enzyme (μg)	GSSeSG (μmole)	TPNH (μmole)	TPNH Oxidized ΔA_{340}[b]	TPNH Oxidized μmole	TPNH Oxidized μmoles/μmole of GSSeSG	GSH Formed (μmole)	Elemental Se Formed ΔA_{400}	Elemental Se Formed ^{75}Se on Filter cpm	Elemental Se Formed ^{75}Se on Filter μmole
1	0.2	0.04	0.20	−0.166	0.053	1.32	0.088	0.021	2227	0.032
2	0.2	0.08	0.20	−0.346	0.111	1.39	0.172	0.058	4347	0.062
3	0.2	0.12	0.20	−0.511	0.164	1.37	0.217	0.100	7087	0.100
4	0.2	0.16	0.20	−0.579	0.186	1.16	0.287	0.154	8710	0.124
5		0.16	0.20	−0.002	0.001	0.00	0.00	0.000	8	0.000

[a] Cuvets were set up which contained, in a final volume of 1.99 ml, 100 μmoles of potassium phosphate (pH 7.4), 0.2 μmole of TPNH, 6 μmoles of EDTA, and 0.04–0.16 μmole of GSSeSG. After recording the initial A_{340} and A_{400}, 10 μl of a glutathione reductase solution containing 0.2 μg of enzyme and 0.02 mg of bovine serum albumin was added to start the reaction, and the absorbance at 340 and 400 mμ was recorded until no further changes took place (20–125 min, depending on GSSeSG concentration). The samples were then filtered to remove elemental selenium, and returned to the spectrophotometer to record the absorbance of the filtrates at 340 and 400 mμ. The filtrates were then stored on ice for subsequent determination of SH content. The Millipore filters were washed twice with water and placed in vials for ^{75}Se counting. [b] Calculated from the change in absorbance during the initial phase (see text) and corrected for turbidity of elemental selenium: $\Delta A_{340} = \Delta A_{340}$(initial) − [$A_{340}$(final, unfiltered) − A_{340}(final, filtered)].

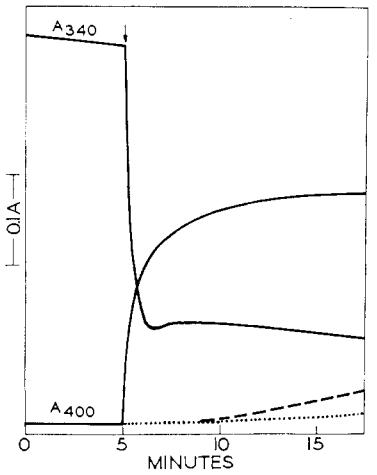


FIGURE 6: Reduction of GSSeSG at pH 6 by glutathione reductase and TPNH or by glutathione. Same conditions as Figure 1 except final pH was 6.05. At the arrow, 2 μg of glutathione reductase (——), 0.04 μmole of GSH (· · · ·), or 0.1 μmole of GSH (- - -) was added. The slow rate of decline in A_{340} during preincubation is caused by the instability of TPNH in acidic medium.

amount of GSH and elemental selenium produced was determined for various amounts of GSSeSG in the presence of excess TPNH (Table I). Upon the addition of enzyme there was a rapid phase of TPNH oxidation, followed by a slower phase; elemental selenium formation, monitored by the increase in turbidity at 400 mμ, ceased after the rapid phase of TPNH oxidation was completed. The slow phase of TPNH oxidation was apparently caused by spontaneous oxidation of GSH to GSSG by dissolved oxygen, which allowed oxidation of TPNH to proceed until the TPNH was exhausted.[4]

[4] In the course of these experiments it was observed that the slow phase of TPNH oxidation was five to seven times faster with GSSeSG than with the same amounts of GSSG, indicating that the presence of selenium somehow accelerated the spontaneous rate of GSH oxidation.

TABLE II: Stoichiometric Relationships in the Presence of Iodoacetate.[a]

GSSeSG (μmole)	μmoles of TPNH Oxidized/μmole of GSSeSG: Control[a]	+ Iodoacetate[b]
0.02	1.20	0.75
0.04	1.30	0.85
0.06	1.30	0.73
0.08	1.11	0.79
	With GSSG 0.79–0.85	

[a] Same conditions as Table I. [b] 50 mM.

The calculation of TPNH oxidized was therefore based only on the ΔA_{340} for the rapid initial phase, corrected for the increase in A_{340} caused by the turbidity of elemental selenium. The turbidity correction at 340 mμ was based on the loss in A_{340} in the final reaction mixture after elemental selenium was removed by filtration (see Experimental Section).

As shown in Table I, for each mole of GSSeSG present initially, approximately 2 moles of GSH and 1 mole of elemental selenium were liberated in the presence of glutathione reductase. The amount of TPNH oxidized relative to GSSeSG, however, was definitely in excess of the expected 1:1 stoichiometry; about 1.3–1.4 moles of TPNH was oxidized (initial phase) for each mole of GSSeSG, up to the point where TPNH was no longer in sufficient excess (expt 5), in which case the ratio was 1.15. In control experiments with comparable amounts of GSSG carried through the same procedure, each mole of GSSG oxidized 0.92–0.96 mole of TPNH, thus confirming the validity of the procedure.

By including iodoacetate in the reaction mixture, the stoichiometry between GSSeSG reduction and TPNH oxidation could be studied directly by means of the A_{340} changes. Table II shows that under these conditions slightly less than 1 mole

TABLE III: Specificity of GSSeSG Reduction.[a]

Substrates	ΔA_{340}/min
Expt 1: Glutathione Reductase	
GSSG, TPNH	−0.026
GSSeSG, TPNH	−0.020
GSSeSG, DPNH	0.000
CySSeSCy, TPNH	+0.006 before enzyme
	+0.009 after enzyme[b]
GSSeSG, TPNH, + 10^{-3} M $NaAsO_2$	−0.024
Expt 2: Lipoyl Dehydrogenase	
Lipoamide, DPNH	−0.085
GSSeSG, DPNH	−0.0002
—, DPNH	−0.0002
Lipoamide, GSSeSG, DPNH	−0.085

[a] Absorbance changes at 340 mμ were recorded as described in the Experimental Section after the addition of glutathione reductase (0.1 μg) or lipoyl dehydrogenase (0.5 μg), in the presence of 10^{-4} M concentrations of the indicated substrates. [b] A_{340} increase was due to spontaneous decomposition of selenodicysteine to elemental selenium without concomitant oxidation of TPNH. Decomposition was somewhat faster after enzyme addition, probably because of the thiol groups of bovine serum albumin in the enzyme diluent.

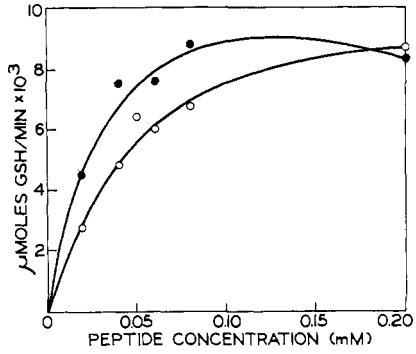


FIGURE 7: Effect of substrate concentration on enzyme activity. The rate of GSH liberation from GSSeSG or GSSG was determined by the stop-analyze procedure (see Methods). The reaction mixtures contained 0.1 mM TPNH, 50 mM potassium phosphate, 3 mM EDTA, 30 mM sodium acetate, 0.06 μg of glutathione reductase, and the indicated concentration of GSSeSG (closed circles) or GSSG (open circles) in a final volume of 4 ml, pH 6.9 ± 0.1, 25°.

of TPNH was oxidized for each mole of GSSeSG, the same value obtained in the control experiment with GSSG. Thus the reaction obeys the stoichiometry expected from reaction 4 when GSSeH is not allowed to accumulate. Just how GSSeH brings about the oxidation of considerably more than one TPNH per GSSeSG in the usual system is not known; this effect occurs in the initial phase of the reaction, before elemental selenium liberation is complete, and is thus distinguished from the effect of selenium on the rate of TPNH oxidation in the slow phase that follows. Possibly the following series of reactions, terminating in the spontaneous oxidation of hydrogen selenide to elemental selenium, could occur:

$$GSSeH + GSH \rightleftharpoons GSSG + H_2Se \quad (6)$$

$$GSSG + TPNH + H^+ \xrightarrow{\text{glutathione reductase}} 2GSH + TPN^+ \quad (3)$$

$$H_2Se + 0.5O_2 \longrightarrow Se^0 + H_2O \quad (7)$$

$$\text{Sum: } GSSeH + TPNH + H^+ + 0.5O_2 \xrightarrow{\text{glutathione reductase}} Se^0 + GSH + TPN^+ + H_2O \quad (8)$$

The thermodynamically unfavorable reduction to hydrogen selenide in reaction 6 might be offset by the favorable equilibrium for the removal of products through reactions 3 and 7. Taken together, reactions 4 and 8 provide for the oxidation of 1–2 moles of TPNH/mole of GSSeSG, depending on the extent to which reaction 8 might occur.

Enzyme Specificity for GSSeSG Reduction. DPNH was not oxidized in the presence of GSSeSG and glutathione reductase (Table III), showing that specificity for the pyridine nucleotide in the reduction of GSSeSG was comparable to that for reduction of GSSG. A high specificity for the disulfide substrate is also characteristic of glutathione reductase. Selenodicysteine (CySSeSCy), the selenotrisulfide formed from cysteine, was therefore tested and found to be a very poor substrate, as is known to be the case for cystine, the analogous disulfide. It is also shown in Table III that the reduction of GSSeSG was not inhibited by 10^{-3} M arsenite, in accord with the observation that glutathione reductase is not readily inhibited by arsenite (Tietze, 1970).

Effect of Substrate Concentration. The effects of GSSeSG and GSSG concentration on glutathione reductase activity, as determined by the stop-analyze procedure, are compared in Figure 7. Under the conditions of this experiment, it is apparent that at concentrations of substrate near the K_m value, GSSeSG is reduced somewhat more rapidly than GSSG. With increasing substrate concentration, the velocity reaches a maximum and then begins to decline in the case of GSSeSG, whereas GSSG shows no evidence of substrate inhibition. GSSeSG therefore appears to be a slightly better substrate for glutathione reductase, having a slightly lower K_m and a comparable V_{max}. In other studies (not shown) where iodoacetate was used to prevent elemental selenium formation, so that reaction velocities could be determined directly by following TPNH oxidation, the velocity at a given concentration of substrate was also higher with GSSeSG than with GSSG.

Characterization of the Iodoacetate Derivative. The identification of the ^{75}Se-labeled compound formed by the enzymic reduction of GSSeSG in the presence of iodoacetate was undertaken using gel filtration chromatography, thin-layer electrophoresis, and thin-layer chromatography. Chromatography of a lyophilized reaction mixture on a Sephadex G-10 column previously calibrated with GSSG and GSH showed the presence of two radioactive peaks (Figure 8). The first peak came off in a volume comparable to that for GSSG and was presumably residual GSSeSG. The elution volume of the second peak was similar to that for GSH, as expected for the carboxymethylated derivative of the proposed intermediate.

A second preparation of the intermediate was carried out in the same way, except additional TPNH and glutathione reductase were added to the reaction mixture after 1.5 hr. An aliquot of this reaction mixture was transferred directly to the G-10 column and eluted with 0.01 M acetic acid. All of the radioactivity was eluted in a single peak (data not shown)

TABLE IV: Characterization of Carboxymethylated Intermediate.[a]

	Mobility of Substance							
	Carboxymethylated Glutathione		Carboxymethylated Product of GSSeSG Reduction					
Procedure	Zone	cm	Zone	cm	cpm	% of Total ^{75}Se	GSSG (cm)	DNP[b] (cm)
Tle at pH 1.3[c]	8	1.5	8	(1.5)	653	77	2.2	0.2
Tle at pH 4.25[d]	12	2.1	12	(2.05)	889	71	1.4	−0.7
Tlc in system I[e]	6	4.85	6		15	1	2.55	
	7		7	(5.90)	774	79		
Tlc in system II[e]	5	4.0	5		75	8	3.0	
	6		6	(5.0)	631	72		

[a] See text for description of experiment; thin-layer electrophoresis (tle) and thin-layer chromatography (tlc) methods are described in the Experimental Section. [b] *N*-DNP-ethanolamine marker for electroosmosis. [c] Distance moved toward cathode (uncorrected for electroosmosis); parentheses indicate weak ninhydrin test. [d] Distance moved toward anode (uncorrected for electroosmosis). [e] Distance moved from origin (solvent front moved 14.2 cm with solvent system I, 12.5 cm with system II).

corresponding to the second peak of Figure 8, indicating that the GSSeSG substrate had been completely converted into the lower molecular weight derivative. An aliquot of the same reaction mixture was applied as a band to a cellulose thin-layer plate and electrophoresed at pH 1.3; there was one major ninhydrin-positive band with a mobility 95% of that for carboxymethylated glutathione. A corresponding nitroprusside-positive band could be detected in a duplicate band applied elsewhere on the plate, after spraying with the combined cyanide–nitroprusside reagent. This ninhydrin-positive and nitroprusside-positive band contained 95% of the total radioactivity on the plate. A minor ninhydrin-positive component present in both carboxymethylated derivatives had a mobility identical with that of GSSG. In order to obtain sufficient material for further thin-layer studies, an additional 100 μl of reaction mixture was subjected to a preliminary electrophoresis at pH 1.3, as described, to remove salts and other reagents. The radioactive band was scraped from the plate, eluted with dilute acetic acid (pH 4.5), filtered through a Millipore filter, and lyophilized. This material was taken up in 45 μl of water and applied to four cellulose plates. Two plates were electrophoresed at pH 1.3 and 4.25 and the other two chromatographed in solvent systems I and II (Table IV). After being sprayed with ninhydrin the plates were divided into 17–20 zones, which were scraped into vials for ^{75}Se counting.

In all four cases there was a single radioactive band which had the expected mobility relative to a marker of carboxymethylated glutathione. The radioactive derivative moved toward the cathode at pH 1.3 and toward the anode at pH 4.25; the mobility in both cases was essentially the same as that for the carboxymethylated derivative of glutathione, providing strong evidence that the carboxymethylated product of GSSeSG reduction by glutathione reductase had the expected structure, G–S–Se–CH_2COOH. In the case of thin-layer chromatography, the radioactive derivative moved slightly ahead of the carboxymethylated glutathione marker in both solvent systems. This effect on mobility was presumably caused by the atom of selenium present in G–S–Se–CH_2COOH but not in carboxymethylated glutathione, G–S–CH_2COOH; it has been shown that GSSeSG, which differs from GSSG only by the presence of one selenium atom, moves ahead of GSSG in system II (Ganther, 1968).

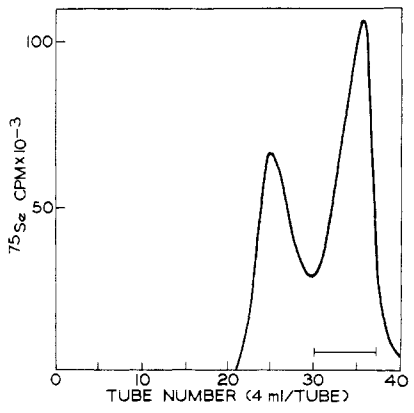


FIGURE 8: Chromatography of iodoacetate derivative on Sephadex G-10. The reaction mixture contained, in micromoles, [^{75}Se]-GSSeSG 4, TPNH 4.4, potassium phosphate 1600, iodoacetate 964, plus 1.2 μg of glutathione reductase, in a total volume of 19.3 ml, pH 6.85, 25°. Aliquots were removed at intervals to determine TPNH oxidation, which ceased after 2.5 hr. The mixture was then lyophilized and taken up in 1 ml of water and a 0.9-ml aliquot applied to a 2.5 × 60 cm Sephadex G-10 column equilibrated with water. Fractions of approximately 4 ml were collected at a flow rate of 5 ml/cm^2 per hr and counted for ^{75}Se.

Discussion

Reaction of Selenious Acid with Glutathione. The papers in this series have clarified some of the chemical reactions which selenious acid undergoes with biologically important thiols. One of the most important factors influencing the type of product formed is the thiol:selenious acid ratio. If this ratio is 4:1 or less, the first relatively stable product is a selenotrisulfide, RSSeSR, plus an equimolar quantity of the disulfide (reaction 1). The stability of selenotrisulfides is less than that of trisulfides or disulfides, and varies considerably with the nature of the alkyl moiety. The intermediate compounds formed in reaction 1 have not been identified, but they appear to be less stable than the selenotrisulfide product since elemental selenium is liberated during the reaction under conditions in which the product is stable. Decomposition of intermediates

is minimized by carrying out the reaction at low temperature and pH, and by adding thiol to selenious acid (rather than the reverse order) so that an excess of thiol is avoided.

In this paper a method is described for preparing the selenotrisulfide derivative of glutathione in high yield, and free of oxidixed glutathione. The evidence that GSSeSG is the compound obtained under these conditions, in accord with reaction 1, includes previous spectrophotometric analysis of the combining ratio, and thin-layer chromatography (Ganther, 1968), similarity of the ultraviolet absorption spectrum to other selenotrisulfides characterized previously, separation of GSSeSG from GSSG on Dowex 50 nickel columns, behavior on electrophoresis, and the finding that GSSeSG is reduced by glutathione reductase and TPNH to a selenopersulfide (GSSeH). The method for separating GSSeSG from GSSG is based on the greater retardation caused by the extra selenium atom when a mixture of the two compounds is passed through a Dowex 50 column equilibrated with nickel. The exact nature of the interactions with metal which produce the differential retardation is not known.

When the thiol:selenious acid ratio exceeds 4:1, which is the case in biological systems, there is the possibility for further reduction of the selenotrisulfide to the selenopersulfide:

$$\text{RSSeSR} + \text{RSH} \longrightarrow \text{RSSeH} + \text{RSSR} \qquad (9)$$

Analogous reactions occur readily with trisulfides (Massey *et al.*, 1971) and would be expected to occur even more easily with selenotrisulfides because of the greater ease with which a S–Se bond is cleaved compared to a S–S bond. Evidence is presented in this paper that the major product formed from selenite and glutathione under conditions approximating those *in vivo* is the selenopersulfide, GSSeH. This was shown by treating the reaction mixture with iodoacetate to form the *S*- or *Se*-carboxymethyl derivatives. Since the amount of selenium involved was too small to detect by the usual chemical or spectrophotometric methods, identification was by means of the ^{75}Se label. The behavior of the ^{75}Se-labeled product formed from selenite in the presence of excess glutathione closely mimicked that of *S*-carboxymethylglutathione during chromatography on Sephadex G-10 or Dowex 50 nickel columns, and during thin-layer chromatography and electrophoresis. ^{75}Se from selenite added after the glutathione was treated with iodoacetate did not accompany the *S*-carboxymethylglutathione in these procedures, showing that selenite was not simply adsorbed to the peptide.

Besides the selenotrisulfide and selenopersulfide derivatives of glutathione, a third compound yet to be identified was shown to be produced under certain conditions of pH and total reactant concentration. It is possible that still other derivatives of glutathione may be formed under other conditions. In particular, it has been suggested from time to time that selenotetraglutathione, $(GS)_4Se$, can be formed in the reaction of glutathione with selenite, but no evidence for a compound of this size was found in the gel filtration studies.

Enzymic Reduction of GSSeSG. The catalysis of GSSeSG reduction by glutathione reductase was first observed (Ganther, 1968, 1971) with the unfractionated reaction mixture of GSH and selenious acid, which contained equimolar amounts of GSSeSG and GSSG. The development of a chromatographic technique for separating these two substances has made it possible to study the reduction of GSSeSG by glutathione reductase without the complications caused by the presence of GSSG. The present study confirms that GSSeSG is an excellent substrate for yeast glutathione reductase. At pH 7, in the presence of less than 0.1 μg of enzyme, GSH and elemental selenium were rapidly liberated in stoichiometric amounts (reaction 10) from GSSeSG at concentrations as low as 2×10^{-5} M, the practical working limit for such procedures.

$$\text{GSSeSG} + \text{TPNH} + \text{H}^+ \xrightarrow{\text{glutathione reductase}} 2\text{GSH} + \text{Se}^0 + \text{TPN}^+ \qquad (10)$$

The reduction of GSSeSG by glutathione reductase is similar in all respects to the reduction of GSSG. In terms of the kinetic parameters K_m and V_{max}, GSSeSG compares favorably to GSSG as a substrate. Furthermore, a high degree of specificty could be shown for the enzymic reduction of GSSeSG. The reduction by yeast glutathione reductase was observed only with TPNH, not with DPNH. Glutathione reductase did not catalyze reduction of the selenotrisulfide derivative of cysteine. Lipoyl dehydrogenase, a dithiol flavoprotein very similar to glutathione reductase, did not catalyze the reduction of GSSeSG. Although glutathione reductase has been regarded as a relatively specific enzyme, the suitability of GSSeSG as a substrate provides an interesting exception to this specificity, along with homoglutathione (Carnegie, 1963), the mixed disulfide of glutathione and hemoglobin (Srivastava and Beutler, 1970), and possibly others.[5]

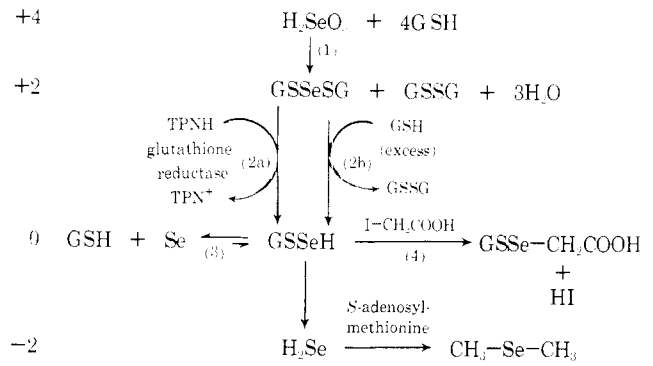
The reduction of GSSeSG is a direct action of the enzyme, rather than an indirect effect involving GSH derived from traces of GSSG. The initial product was shown to be a labile selenopersulfide, GSSeH. This labile product rapidly decomposes to GSH and elemental selenium but can be trapped as the carboxymethyl derivative with excess iodoacetate. The analogous decomposition of a persulfide intermediate of the type RSSH in the cystathionine-cleavage reaction has been demonstrated by Flavin (1962). Identification of the carboxymethyl derivative of GSSeH was based on gel filtration, thin-layer chromatography in two solvent systems, and thin-layer electrophoresis at two different values of pH.

It is believed that these studies provide the first demonstration of a selenopersulfide compound. It has been shown that GSSeH can be formed from selenite under physiological conditions in two ways. One involves the direct reduction by glutathione reductase of GSSeSG formed by the reaction of glutathione with selenious acid. The other involves the nonenzymic reduction of selenite to GSSeH in the presence of excess GSH. In either case, a TPNH-linked process requiring glutathione reductase is ultimately involved in the reduction of selenium *in vivo* to GSSeH. This labile, highly reactive selenopersulfide derivative of glutathione probably has a role in selenium metabolism and possibly in certain biological functions of selenium.

Relationships to Selenium Metabolism. In the case of yeast, inorganic selenite added to the medium is rapidly reduced and deposited in the cells as elemental selenium (Falcone and Nickerson, 1963). Nickerson and Falcone (1963) studied this process in cell-free extracts from a strain of yeast adapted to growth on high concentrations of selenite. They presented evidence that the reduction of selenite was catalyzed by an enzyme and required glutathione (oxidized), glucose 6-phosphate, and TPN, as well as a quinone. These authors proposed that glutathione was needed to keep the dithiol moiety of an enzyme in

[5] It has recently been reported (Massey *et al.*, 1971) that the trisulfide derivative of glutathione (GSSSG) may be a contaminant in GSSG. In view of the suitability of GSSeSG as a substrate for glutathione reductase, one would expect that GSSSG would also be reduced by this enzyme, forming the persulfide GSSH.

DIAGRAM I: Summary of the Reductive Metabolism of Selenium.[a]



[a] Reactions 1–4 are established with reasonable certainty. The further metabolism of selenium to the −2 oxidation state with ultimate methylation is known to occur but the pathway involved is not established.

the reduced state. In view of the studies with yeast glutathione reductase presented in this paper, however, it is clear that the TPNH-linked reduction by glutathione reductase of selenodiglutathione formed from selenious acid and glutathione could explain many of the observations of Nickerson and Falcone, with the exception of the apparent involvement of a quinone. Possibly the quinone requirement they observed was associated with the use of a strain of yeast adapted to high concentrations of selenite. In any case, the involvement of glutathione reductase in the reactions described provides a likely mechanism for the reduction of selenious acid to the zero oxidation state (Diagram I).

It is very likely that the glutathione reductase-catalyzed reactions demonstrated in the present study with the enzyme from yeast apply to the enzyme in animal tissues as well, in view of the fundamental similarity of the glutathione reductases isolated to date from a variety of sources (Icén, 1967). A specific requirement for glutathione was demonstrated in a liver enzyme system which catalyzed the conversion of selenite to dimethyl selenide (Ganther, 1966). GSSeSG is converted into dimethyl selenide by this system at the same rate and to the same extent as for the conversion of selenite (Ganther, 1971). On the basis of these and unpublished observations, a pathway is proposed (Diagram I) for the synthesis of dimethyl selenide from selenite in which GSSeH has a central role. Selenodiglutathione is the first intermediate and GSSeH the second, with glutathione reductase having a direct or indirect role in reduction to the zero oxidation state.

Relevance to the Role of Selenium. There is an increasing belief that the biochemical role of selenium may be closely tied to processes involving sulfur. This is based in part on nutritional interrelationships between selenium and sulfur amino acids, and in part on the greater chemical reactivity of selenium compounds. Compared to thiols, selenols (RSeH) are more completely dissociated at physiological pH and participate more readily in nucleophilic reactions. As a consequence, the RSe^- anion is a very effective catalyst of sulfhydryl–disulfide exchange reactions involved in the activation of certain enzymes (Dickson and Tappel, 1969). Moreover, in selenotrisulfides or diselenides, the S–Se or Se–Se bond is cleaved by thiols under conditions in which cleavage of disulfide bonds does not occur (Ganther and Corcoran, 1969; Walter *et al.*, 1969). The formation of GSSeH by the action of glutathione reductase on selenodiglutathione provides an efficient means of generating a highly reactive nucleophile through TPNH-linked processes. Such effects may be involved in the glucose-dependent protective effect of selenium against erythrocyte hemolysis recently reported by Rotruck *et al.* (1971), or possibly other functions of selenium.

Acknowledgments

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