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Reactions of Reduced Glutathione with Some Methylated Selenium Compounds

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REACTIONS OF REDUCED GLUTATHIONE WITH SOME METHYLATED SELENIUM COMPOUNDS

BY

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REACTIONS OF REDUCED GLUTATHIONE WITH

SOME METHYLATED SELENIUM COMPOUNDS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Major Adviser Date

Head, Chemistry Department Date

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To PJT

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LIST OF ABBREVIATIONS

GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GSSeSG	Selenodiglutathione
GSSeH	Glutathione Selenopersulfide

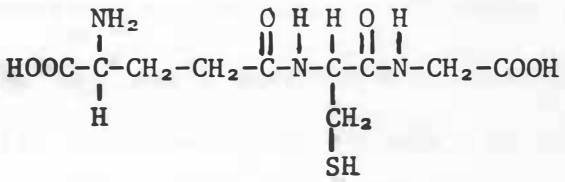
NOMENCLATURE OF SOME SELENIUM COMPOUNDS

Selenite ion	SeO_3^{2-}
Selenate ion	SeO_4^{2-}
Methylseleninic acid	$CH_3Se(=O)-OH$
Methylselenonic acid	$CH_3Se(=O)_2-OH$
Methylselenenic acid	CH_3SeOH
Dimethyl selenide	CH_3SeCH_3
Dimethyl diselenide	$CH_3SeSeCH_3$
Trimethylselenonium ion	$(CH_3)_3Se^{1+}$
Dimethyl selenoxide	$CH_3Se(=O)CH_3$
Dimethyl selenone	$CH_3Se(=O)_2-CH_3$
Methaneselenol	CH_3SeH
A selenotrisulfide	RSSeSR
Selenodiglutathione	GSSeSG
A selenopersulfide	RSSeH
Glutathione selenopersulfide	GSSeH

STRUCTURES OF GSH, GSSG, and $GSSeCH_3$

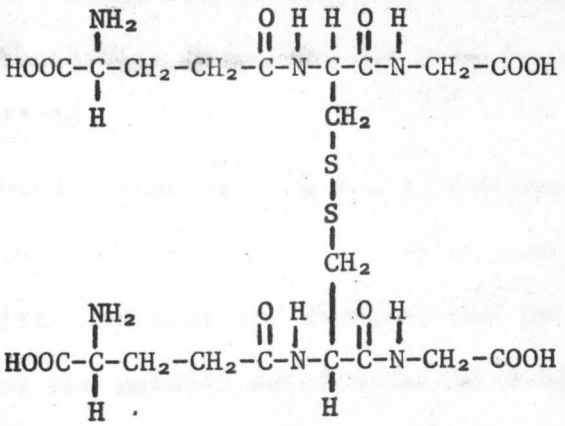
Reduced Glutathione (GSH)

or α-Glutamylcysteinylglycine

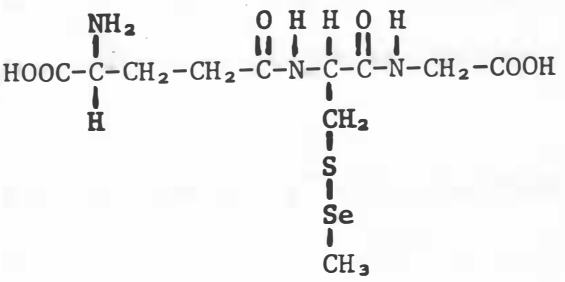


Oxidized Glutathione (GSH)

or NN' {dithiobis [1- [(carboxymethyl)carbamoyl] ethylene]} diglutamine



Methyl Derivative of Glutathione Selenopersulfide ($GSSeCH_3$)



INTRODUCTION

Selenium biochemistry has been of general interest since the discovery of selenium as the toxic agent in certain plants,[1-3] which when ingested by animals causes a definite disease syndrome[4-8] and even death.[8-11] Early scientists suggested that the logical place in the body for toxic selenium compounds to attack was at the various sites occupied by sulfur. In 1939 a study was published which indicated that reduced glutathione (GSH), a thiol, protected rats against death from a minimum fatal dose of selenium given as sodium selenite.[12] Since this discovery, the reaction of GSH with various selenium containing compounds has been the subject of much research.[13-21]

Improved technology has led to new insights about the reaction of GSH with selenium compounds. This, coupled with recent advances in metabolite and toxicity studies, has led to the proposal of a pathway for the metabolism of selenium which involves the reaction of GSH with several methylated selenium compounds.[22] The studies reported here have been done in an attempt to investigate those proposed reactions. Specifically, the reactions under investigation are:

1) The reaction of GSH with methylseleninic acid (CH_3SeO_2H),
2) The reaction of GSH with dimethyl diselenide ($CH_3SeSeCH_3$),
3) The reaction of GSH with dimethyl selenoxide [$(CH_3)_2SeO$].

LITERATURE REVIEW

Selenium

The syndrome produced by selenium has been a problem to stockmen and farmers for centuries and was the problem which first created interest in selenium biochemistry. This ancient disease, often referred to as alkali disease, has been described in widely separated areas of the world. Marco Polo[4] is perhaps the first to report a disease syndrome resulting from the ingestion of seleniferous plants. He encountered the disease in his travels in western China near the border of Turkestan and Tibet in the year 1295. He wrote:

> ....and throughout all the mountainous parts of it the most excellent kind of rhubarb is produced, in large quantities, and the merchants who procure loadings of it on the spot convey it to all parts of the world. It is a fact that when they take that road, they cannot venture amongst the mountains with any beasts of burthen [sic] excepting those accustomed to the country, on account of a poisonous plant growing there, which, if eaten by them, has the effect of causing the hoofs of the animal to drop off; but those of the country, being aware of its dangerous quality, take care to avoid it.

Stein, who traveled in Turkestan and western China in 1906 to 1908 as a representative of the British Government, suspected that his ponies had eaten some of the poisonous plants about which Marco Polo had written.[5]

In the Americas, chronic selenosis resembling alkali disease in livestock, malformations in chicks and children, and loss of hair and nails of the people were first described in Columbia by Father

Pedro Simon in 1560.[6] In the neighborhood of Irapuato in Mexico, a disease was described over 200 years ago. This disease was similar to alkali disease in livestock, and among the people, loss of hair and teeth and a form of paralysis were noted.[7]

In 1857 Dr. T. C. Madison, an army surgeon stationed at Fort Randall, then located in the Nebraska Territory but now a part of South Dakota near the Nebraska border, published a sanitary report in which he described a fatal disease in horses grazing near the fort:[8]

> A very fatal disease manifested itself among the dragoon horses, which is supposed not to have been described in works on veterinary surgery. Four companies of the second dragoons arrived at this post about the 10th of August, 1856, one squadron from Fort Lookout and one from Big Sioux river, the latter accompanied by a number of new or remount horses. The four companies encamped on the east or lower side of the dry ravine, separating the dragoon and infantry camps. About the 20th of August the disease commenced simultaneously in all four companies, and many horses died, not, however, until after the lapse of weeks and months. The following symptoms were observed: first, that, among the remount horses from below, there was a sort of catarrh, or distemper, with running at the nose, and among all the horses a swelling of the skin or the throat and jaw; also, inflammation, swelling, and suppuration of the sheath, tenderness and inflammation of the feet, followed by suppuration at the point where the hoof joins the skin, the hoof, in a measure, detaching itself, and a new one forming in its place. These were accompanied by loss of the manes and tails. The appetite was uniformly good; but, from extreme tenderness of the feet, they were unable to move about in search of food, and it appears that at that time they were entirely dependent upon grazing, there being no forage at the post for issue. Sorrel horses appeared to suffer the most, but no color escaped. The private horses of officers shared the fate of the public animals. A few mules and Indian ponies were similarly affected. The acclimated suffered equally with the

> unacclimated. No treatment was effectual, or afforded permanent relief. Bleeding in the feet was tried, but its effect was merely temporary. Every case of disease originated on the lower side of the dry ravine, above alluded to. After forage was provided for the horses no new cases occured, and hence, it is fair to infer that a liberal allowance of forage in the beginning might have rendered the disease much less fatal, or have prevented it....

As the western part of this country became settled, farmers and stockmen experienced similar losses in all forms of livestock. The peculiar ailment was reported in 1893 in horses in the Shirley Basin, Carbon County, Wyoming[9] and in the livestock of Boyd County, Nebraska[10] in the 1890's. Throughout the vast grazing areas in fifteen of the western states, acute and chronic poisoning of obscure origin, frequently attributed to poisonous forage plants, have taken a heavy toll of cattle and sheep on the ranges. An example illustrating the magnitude of the losses was reported during the summers of 1907 and 1908 when more than 15,000 sheep died in a region north of Medicine Bow, Wyoming.[11]

The disease aquired the name ''alkali disease'' because of the belief that alkali (high salt) in the water and soil was responsible. In spite of the fact that the investigations of Larsen et al.[23] in 1912 and Larsen and Bailey[24] in 1913 eliminated alkali water as the cause of the disease and that later selenium was definitely shown to be the causative agent, the ailment continued to be erroneously called alkali disease.

An interbureau cooperative study of the problem was started

by the U.S. government in 1931,[1] and at one of the interbureau committee meetings H. G. Knight, Chief of the Bureau of Chemistry and Soils, was reported to have suggested that selenium should be investigated as the possible ingredient in toxic grain. In 1933, W. O. Robinson[2] obtained samples of toxic wheat and after devising a method of analysis, he found 10-12 ppm Se in one sample and 5-6 ppm in another. Nontoxic wheat, on the other hand, was free of selenium.

In 1929 Franke[3] began a series of investigations at the South Dakota Experiment Station, some of which were not published until 1934. This work established that grains and grasses grown in definite soil areas were toxic to animals, and that this toxicity was due to the presence of selenium in the grains and grasses.

Subsequent work in the area established that different species of plants differed in their selenium content[25] and researchers began analyzing different types of vegetation for selenium. As the problem was studied further, workers began studying the toxicity of various selenium containing compounds and metabolism of selenium compounds in the body.

Selenium Compounds and Their Reactions with Reduced Glutathione

Moxon,[26] in 1938, showed that arsenic, as sodium arsenite, gives protection against the toxicity of selenium. It had been shown earlier, in 1930, that albino rats could be completely protected from a lethal dose of arsenoxide by a preceeding

injection of reduced glutathione (GSH).[27] From a combination of the facts that GSH was found to prevent arsenic toxicity, and arsenic was found to protect against selenium toxicity, Dubois, Rhian, and Moxon[12] decided to carry out an experiment to study the effect of GSH on the toxicity of selenium.

The results obtained indicated quite clearly that GSH protected rats against death from a minimum fatal dose of selenium given as sodium selenite. Subsequently, there has been considerable interest in the reactions of GSH and other sulfhydryl containing compounds with selenium. Some of these reactions of interest will be reviewed.

Bersin[13] prepared the compound $HOOCCH_2S\text{-}Se\text{-}SCH_2COOH$ from thioglycolic acid and selenite in 1935, and suggested that a similar unstable compound formed in the reaction of selenite and GSH.

Painter published an excellent review in 1941[14] in which he proposed that selenite readily oxidizes sulfhydryl compounds, forming a disulfide and an unstable RS-Se-SR compound. He suggested the following three courses for reactions of selenite with sulfhydryls:

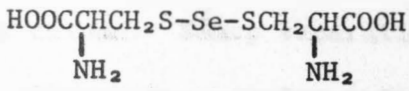
$$2RSH + SeO_2 \longrightarrow RS\text{-}\overset{\overset{O}{\downarrow}}{Se}\text{-}SR + H_2O$$

$$4RSH + SeO_2 \longrightarrow RS\text{-}Se\text{-}SR + RSSR + 2H_2O$$

$$4RSH + SeO_2 \longrightarrow 2RSSR + Se + 2H_2O$$

At the same time he also referred to some of his unpublished

work in which he had obtained an amino acid from the reaction of selenite with cysteine. He thought the acid to be



Since separation of the selenotrisulfides from the disulfide was difficult because of their instability, it took scientists nearly 30 years to prove Painter's hypothesis.

The reaction of selenite with cysteine was next studied by Stekol.[15] In 1942 he proposed the formation of selenium tetracysteine as the product of the reaction on the basis of elemental analysis. His theories were disproved by Klug and Petersen[16] in a 1949 publication.

Attempts to purify and characterize a selenium-glutathione complex led Klug and Petersen[16] to the study of selenium-cysteine compounds because analytical methods for the substances in the reaction were available. They synthesized selenium tetracysteine according to Stekol[15] and obtained a sample of selenium dicysteine from Painter. The samples were analyzed for cysteine and cystine. Cystine was found in both samples. Absorption spectra demonstrated that the two selenium-cysteine complexes were the same. That they consisted of two substances was shown by paper partition chromatography. Klug and Petersen[16] also concluded that selenium oxidizes two moles of cysteine to cystine and further, it probably bound two moles of cysteine to form selenium dicysteine. This supported the equations published by Painter[14] in 1941.

In an effort to analyze the selenium-glutathione complex Petersen[17] placed aliquots of GSH and oxidized glutathione (GSSG) standards on a starch column. Sharp separations of both the reduced and oxidized glutathione samples were obtained with positive identification of the compounds. The selenium-glutathione complex was then placed on the column and eluted. It did not contain GSH as evidenced by the absence of the reduced GSH peak. However, an GSSG peak was present along with a second peak which preceded it. Petersen concluded that his starch column chromatography had shown that the compound resulting from the reaction of GSH and selenious acid in a mole ratio of 4:1 was not homogeneous but rather it was a mixture possibly containing GSSG and selenium diglutathione (GSSeSG).

Tsen and Tappel,[18] using paper chromatography, separated the products (GSSG and GSSeSG) of the oxidation of GSH and selenite in 1958. Similarly, by paper chromatography, the final product of the oxidation was identified as GSSG by comparison with a standard.

Finally, in 1968, Ganther[19] confirmed the reaction proposed by Painter[14] in 1941:

$$4\ RSH + SeO_2 \longrightarrow RSSeSR + RSSR + 2H_2O$$

and described the isolation and characterization of selenodicysteine. Ganther found that selenious acid (H_2SeO_3) combined with cysteine, 2-mercaptoethanol, GSH, or coenzyme A to form moderately stable derivatives having enhanced absorption in the 260-380 nm region.

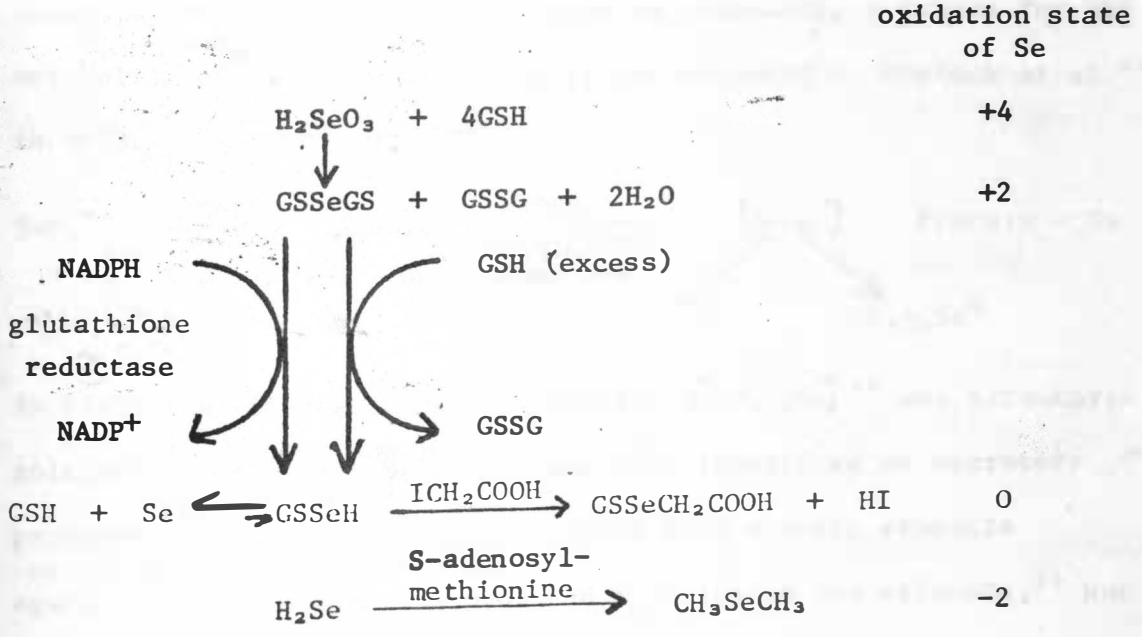
The combining ratio for the thiols and selenious acid was found to be 4:1 by spectrophotometric analysis. Unfortunately, the case for GSH is not as simple as for the other thiols. For GSH, the reaction with H_2SeO_3 was greatly influenced by pH and only under certain conditions was it possible to obtain 4:1 stoichiometry. A systematic study 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 decreased.

Ganther resolved his reaction mixtures by thin-layer chromatography into two spots corresponding to the disulfide and the selenotrisulfide. A column chromatographic procedure based on chelated copper as a stationary phase was developed and it permitted the isolation of selenodicysteine and selenodimercaptoethanol. Selenodicysteine was identified by amino acid analysis.

Ganther[20] followed this work with another publication in 1971 which provided evidence for a new class of selenium compounds - the selenopersulfides. He studied the compounds formed as affected by pH and glutathione:selenious acid ratio. At ratios of 4:1 or less, and a pH below 2 or above 4, the first stable product is the selenotrisulfide derivative of glutathione (GSSeSG) plus an equimolar quantity of GSSG. GSSG was separated from GSSeSG on a Dowex 50 column. In order to determine the type of selenium compound formed under conditions more nearly simulating

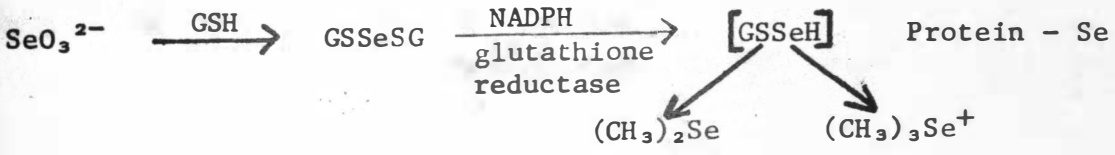
physiological conditions of pH and reactant conditions, ^{75}Se-labeled Selenite (1×10^{-16} M) was treated with 4×10^{-3} M GSH at pH 7.25, followed by 50 mM iodoacetate. The major selenium compound thus formed was the Se-carboxymethyl derivative of glutathione selenopersulfide (GSSeH). This persulfide is believed to be formed by reduction of the initial selenotrisulfide product with excess GSH. GSH 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 selenopersulfide rapidly decomposed to GSH and elemental selenium but it 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.

Ganther summarized this work with the following scheme:[20]



Sandholm and Sipponen[21] studied reactions between GSH and selenite ion, SeO_3^{2-}, using absorption spectrophotometry and redox titrations in water at pH 7.3. At various times the reactants and products were separated and identified by thin layer chromatography. Autoradiography was performed to detect selenium on plates where $^{75}SeO_3^{2-}$ was used as a reactant. When the ratio of GSH/selenium was low (under 4) the main reaction products were GSSG, selenium, GSSeGS, and possibly a compound of four glutathiones bound to one atom of selenium. When the ratio was raised, the main products were selenium and GSSG. It was established that the GSH and selenite reacted in different molar proportions possibly up to 4 moles of GSH to one of selenite. The selenotrisulfide seemed to be the most stable intermediate.

In an effort to relate this new information about selenium chemistry to the metabolism of selenium compounds, a scheme for the metabolism of selenium in rat liver was proposed by Diplock et al.[28] in 1973.



In biological studies dimethyl selenide $[(CH_3)_2Se]$[29] and trimethylselenonium ion $[(CH_3)_3Se^+]$[30,31] had been identified as excretory products. Also it had been discovered that arsenic protects against the toxic symptoms produced by selenite and selenate,[26] but

greatly increases the normally low toxicities of $(CH_3)_3Se^+$ and $CH_3SeSeCH_3$.[32] With this and other data Palmer and Halverson[33] suggested that the pathway postulated by Challenger[34] for the metabolism of selenium compounds may be extended to include the methylated excretory products of selenium.

$$SeO_3^= \text{ or } SeO_4^= \longrightarrow CH_3SeO_2H \longrightarrow CH_3SeCH_3 \longrightarrow CH_3SeCH_3 \begin{cases} \nearrow CH_3SeCH_3 \\ \searrow (CH_3)_3Se^+ \end{cases}$$

Then, to accommodate the pathway suggested by Diplock, et al., Palmer and Olson[22] suggested an alternate pathway consistent with the data of both groups. (See Figure I)

The objective of this work has been to examine the proposed reactions: The reaction of methylseleninic acid (CH_3SeO_2H) with GSH, the reaction of dimethyl diselenide ($CH_3SeSeCH_3$) with GSH, and the reaction of dimethyl selenoxide (CH_3SeCH_3) with GSH.

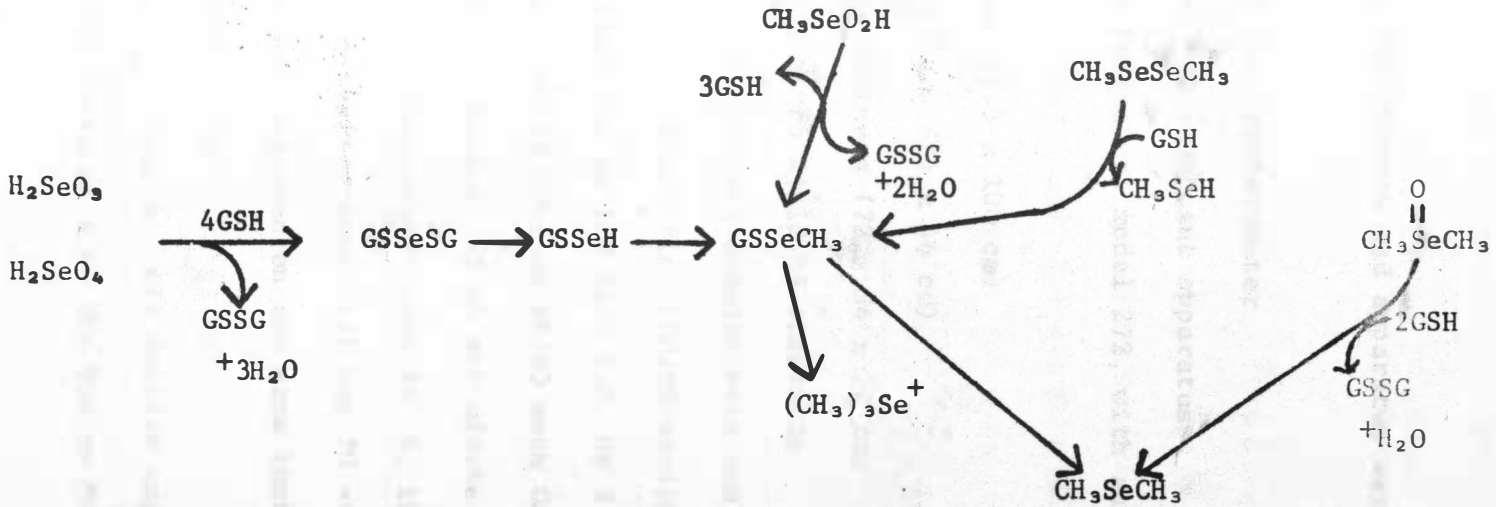


Figure I. Metabolic Scheme of Palmer and Olson[22]

EXPERIMENTAL

Apparatus

The following instruments and apparatus were used during this research:

Beckman DK-2A Spectrophotometer

Fisher - Johns melting point apparatus

ISCO fraction collector, model 272, with an ISCO volumeter, model 400

Sephadex column (1.5 x 100 cm)

Chromatography jars (24 x 46 cm)

Chromatography cabinets (72 x 54 x 77 cm)

Lab Con Company Micro Kjeldahl apparatus

Mass Spectrometer: Liquid samples were analyzed with a Finnigan Model 3000 Peak Identifier GLC/MS equipped with a Gohlke separator. The column was an 1/8 inch i.d. by 5 foot stainless steel column packed with 3% OV-1 on 60/80 mesh Gas Chrom Q with a helium carrier gas flow rate of 40 ml per minute. Temperatures of the column, injector and separator were 90° C, 100° C and 150° C respectively. The ionization potential was 70 ev.

Solid samples were analyzed on the same instrument, but were injected with a solid probe.

Nuclear Magnetic Resonance: All nuclear magnetic resonance (nmr) spectra were obtained on a 60 MHz Varian Model A60-A

spectrometer at room temperature. All samples were analyzed as solutions, and all chemical shifts were reported as δ values relative to tetramethylsilane (TMS) which was used as an external standard.

Materials

Reduced glutathione was purchased from Nutritional Biochemicals Corporation and from Calbiochem, and was used as received.

Oxidized glutathione was purchased from Nutritional Biochemicals Corporation and from Calbiochem, and was used as received.

Methylseleninic acid (CH_3SeO_2H) was obtained from the Department of Station Biochemistry, South Dakota State University. The compound had been prepared according to the method of Bird and Challenger.[35] The neutralization equivalent was found to be 127 (theoretical, 127.0) and the melting point was found to be 122-123° C.

Dimethyl diselenide ($CH_3SeSeCH_3$) was obtained from the Department of Station Biochemistry, South Dakota State University, and had been prepared according to the method of Bird and Challenger.[35] The nmr spectrum in CF_3COOH showed a singlet at 2.1 δ. The mass spectrum showed a molecular ion pattern at m/e = 185. The line intensities of the pattern were consistent with the presence of two selenium atoms. The presence of strong M-15 and M-30 fragments was consistent with the loss of one and two methyl groups, respectively. The fragment intensity patterns were also consistent with the presence of two selenium atoms.

Dimethyl selenide (CH_3SeCH_3) was obtained from the Department of Station Biochemistry, South Dakota State University, and had been prepared according to the method of Bird and Challenger.[35] The nmr spectrum in CF_3COOH showed a singlet at 1.6 δ, and in CCl_4 a singlet at 2.0 δ. The mass spectrum showed a molecular ion pattern at m/e = 106. The line intensities of the pattern were consistent with the presence of one selenium atom. Strong M-15 and M-30 fragments were consistent with the loss of one and two methyl groups, respectively. The fragment intensity patterns are also consistent with the presence of one selenium atom.

Dimethyl selenoxide was obtained from the Department of Station Biochemistry, South Dakota State University, and had been prepared according to the method of Bird and Challenger.[35] The mass spectrum showed a molecular ion pattern at m/e = 122. The line intensities of the pattern were consistent with the presence of one selenium atom. Strong M-15, M-30 and M-46 fragments were consistent with the loss of one methyl group, two methyl groups, and two methyl groups and one oxygen, respectively. The fragment intensity patterns were also consistent with the presence of one selenium atom. Attempts to dry the compound were unsuccessful and it was used in crude form.

Anhydrous sodium selenite was purchased from K and K Laboratories, Incorporated, and was used as purchased.

Dueterium oxide was purchased from Stohler Isotope Chemicals.

All other reagents were analytical grade and were used as purchased.

Sephadex G-10 was obtained from Pharmacia Fine Chemicals.

Whatman No. 1 and Whatman No. 3 MM chromatograph paper were used for all paper chromatography.

Selenium analysis: All selenium analyses were performed by a technician at the Department of Station Biochemistry, South Dakota State University, according to the Fluorometric procedure of Olson.[36]

Nitrogen analysis: All nitrogen analyses were done according to the official method of the Association of Official Analytical Chemists[37] with the following exceptions: 1.0 gram of a 10:1 mixture of K_2SO_4:$CuSO_4$ was used as catalyst, instead of HgO, and 1.0 ml of H_2SO_4 was used for digestion instead of 2.0 ml.

Reaction of Reduced Glutathione with Methylseleninic acid

Spectrophotometric Study: The reaction of selenite and reduced glutathione at 25° C was followed using a Beckman DK-2A Scanning spectrophotometer.[21] Solutions of 0.125 mM Na_2SeO_3 and 0.125 mM GSH were prepared in a pH 7 pHydrion buffer and combined in a 1:1 molar ratio. The spectral scan, from 360 nm to 190 nm, was made with buffer in the reference cuvette and it was started immediately after the reactants were mixed. The whole scan required approximately two minutes.

Using this study as a guide, the reaction of CH_3SeO_2H and GSH

was followed in the same manner. Solutions of 0.125 mM GSH and 0.125 mM CH_3SeO_2H were prepared in a pH 7 pHydrion buffer and combined in a 3:1 molar ratio (GSH:CH_3SeO_2H). Again, the scanning from 360 nm to 190 nm was made with buffer in the reference cuvette and was started immediately after the reactants were mixed.

Paper chromatography: Paper chromatography was first used to follow the reaction of CH_3SeO_2H and GSH; secondly, to identify the ingredients of individual fractions from the Sephadex G-10 column; and thirdly, to attempt to isolate one of the products of the reaction.

Mixtures of different molar ratios of GSH:CH_3SeO_2H were studied chromatographically to determine the stoichiometry of the reaction. Solutions of 0.01 M GSH, 0.01 M GSSG, and 0.01 M CH_3SeO_2H were prepared in distilled water. Whatman No. 1 chromatography papers were spotted one inch from the bottom of the sheet with 10 microliter samples of each of the following:

Spot Number	GSH:CH_3SeO_2H molar ratio	Sample
1		GSSG control
2		GSH control
3		CH_3SeO_2H control
4	1:1	1 ml of GSH + 1 ml of CH_3SeO_2H
5	2:1	2 ml of GSH + 1 ml of CH_3SeO_2H

Spot Number	$GSH:CH_3SeO_2H$ molar ratio	Sample
6	3:1	3 ml of GSH + 1 ml of CH_3SeO_2H
7	4:1	4 ml of GSH + 1 ml of CH_3SeO_2H
8	5:1	5 ml of GSH + 1 ml of CH_3SeO_2H

One dimensional paper chromatograms were developed by the ascending technique. The papers were developed for 25 hours at room temperature with a solution containing 50 ml of 1-butanol, 10 ml of pyridine, 20 ml of glacial acetic acid, 20 ml of ethyl acetate, and 20 ml of distilled water.[21]

Some chromatograms were treated with fluorescein mercuric acetate (FMA) 10^{-4} M which was dissolved in pH 6.8 phosphate buffer[21] to detect GSH. The chromatograms were sprayed and examined under ultraviolet light. The FMA gives bright yellow fluorescent background whereas the GSH quenches ultraviolet light causing dark spots on the chromatogram.

Other chromatograms were sprayed with FMA (10^{-4}) dissolved in 1.5 N NaOH.[21] After spraying, the chromatograms were examined under ultraviolet light to detect both oxidized and reduced glutathione. Again, the GSH and GSSG appeared as dark spots in the yellow fluorescent background.

To detect CH_3SeO_2H and/or SeO_3^{2-}, chromatograms were sprayed with 3% H_2O_2 and then heated in the oven for 5 to 10 minutes at 100° C. They were then sprayed with 0.1 M $SnCl_2$ with 5 ml of concentrated HCl added per 100 ml of solution. The chromatograms

were then heated again at 100° C for 5 to 10 minutes. Two sprayings with each of the above reagents were necessary to detect CH_3SeO_2H.

R_f values were determined for GSH, GSSG, CH_3SeO_2H and other reaction products.

Chromatograms similar to those previously described were developed for the reaction of selenite ion with GSH. The papers were developed in the same solvent system and treated with the same sprays for detection of the compounds. They were then used for comparison with the chromatograms of the reaction products of CH_3SeO_2H with GSH.

The paper chromatography system was also used to follow the separation of reaction products by Sephadex G-10 column chromatography. After the collection of 2 ml fractions was complete, one drop from each fraction was spotted one inch from the bottom on Whatman No. 1 chromatography paper. These chromatograms were developed by the ascending technique according to the procedure previously described. FMA in NaOH was used for detection of reaction products.

Paper chromatography was also used as an isolation and purification procedure for the reaction products. A mixture of GSH and CH_3SeO_2H was prepared in a 3:1 molar ratio and dissolved in a minimal amount of water. The reaction mixture was then applied to Whatman No. 3 MM paper along an origin line three inches from the bottom of the paper. Enough of the reactant mixture was

applied to each paper to theoretically yield 0.1 grams of the product. Chromatograms were allowed to develop approximately 16 hours by the descending technique in the solvent system previously used.

The chromatograms were dried and strips 1 1/2 inches wide were cut from each side of each chromatogram. These strips were then sprayed with FMA in NaOH to detect the product. The location of the product was then marked on each chromatogram by comparison with the sprayed strips cut from the sides of the chromatogram. The sprayed strips were discarded. Lines were drawn across the chromatograms to indicate upper and lower edges of the product streak. These strips were then cut from the papers and eluted with 0.06 N acetic acid. The solvent was collected as it dripped off the strips. The volume of the combined solvent was reduced to approximately 15 ml with the aid of a flash evaporator with the rotating flask positioned over a steam bath. Absolute ethanol was then added until signs of crystallization could be noticed, and the mixture was allowed to set in the freezer to complete crystallization.

Sephadex G-10 Chromatography: After unsuccessful attempts at separation of the GSH + CH_3SeO_2H reaction products by cation exchange chromatography, Sephadex G-10 chromatography was used to achieve the separation. The gel was prepared by allowing it to stand in excess water overnight. The swollen gel was then packed in a column 1.5 cm in diameter to a height of 100 cm.

Reactants were weighed (0.003 mole or 0.921 gram of GSH and 0.001 mole or 0.127 gram of CH_3SeO_2H) and dissolved in a minimal amount of water (3 to 5 ml). The mixture was applied to the column with blue dextran to mark the void volume. Two milliliter fractions were collected with the aid of a fraction collector. One drop from each fraction was applied to a paper for chromatographic identification of its components. To those fractions containing relatively uncontaminated product, approximately three times the volume of absolute ethanol was added to aid crystallization. It should be noted that crystallization of the product could be observed before the addition of ethanol in the fraction tubes in which it was most concentrated. The ethanol solutions were then placed in the cold room at 4° C to complete crystallization. The crystalline product was filtered with the aid of a Buchner funnel and washed with water, ethanol and diethyl ether.

Reaction of Reduced Glutathione with Dimethyl Diselenide

Dimethyl diselenide ($CH_3SeSeCH_3$) and GSH were combined in a 1:1 molar ratio as follows: 1 ml of a 1 M $CH_3SeSeCH_3$ solution in dimethyl formamide (DMF) was combined with 0.3 gram of GSH dissolved in distilled water. The mixture was kept on an ice bath and a mass spectrum was obtained on the cold mixture in an effort to detect any CH_3SeH that may have been formed in the reaction.

The reaction mixture was then heated on a steam bath with a

stream of nitrogen gas passing over it and into an aqueous $AgNO_3$ solution. The solid was recovered by filtration and analyzed for selenium.

A silver nitrate solution was then added directly to a portion of the reaction mixture. Again a yellow precipitate formed which was filtered off for selenium analysis.

Another solution of $AgNO_3$ was added directly to a solution of $CH_3SeSeCH_3$ in DMF. Again, a yellow solid product was obtained and filtered off for selenium analysis.

The $CH_3SeSeCH_3$ - GSH reaction was then attempted as described above except that the pH was adjusted to 10 by the addition of TRIS buffer.[39] Again, 1 ml of 1 M $CH_3SeSeCH_3$ in DMF was added to 0.3 gram of GSH dissolved in distilled water. A stream of nitrogen gas was passed over the reaction mixture and into an aqueous $AgNO_3$ solution; a yellow solid formed which was filtered off for selenium analysis. After allowing the reaction mixture to set at room temperature overnight to insure completion of any reaction, paper chromatography was used to attempt to identify reaction products. The paper was spotted with the reaction mixture, and authentic GSH and GSSG standards for comparison. The paper was developed one dimensionally by the ascending technique as described previously. FMA in NaOH was used for detection of reaction products.

Reaction of Reduced Glutathione with Dimethyl Selenoxide

Crude dimethyl selenoxide $[(CH_3)_2SeO]$ was added to an aqueous GSH solution in a separatory funnel. A weight of the $(CH_3)_2SeO$

could not be obtained because of inability to dry the product without decomposition. CCl_4 was added to the reaction mixture and it was shaken. A mass spectrum was obtained on the CCl_4 layer of the mixture. The water layer of the mixture was chromatographed to attempt to identify the components. A paper was spotted with the reaction mixture, and authentic GSH and GSSG standards for comparison. The paper was developed one dimensionally by the ascending technique according to the procedure previously described. The developed paper was treated with FMA in NaOH for detection of reaction products.

RESULTS AND DISCUSSION

Reaction of Reduced Glutathione with Methylseleninic Acid

Paper chromatography as described in the experimental section of this thesis showed that a reaction did occur between GSH and CH_3SeO_2H and the stoichiometry of the reaction was apparently one mole of CH_3SeO_2H to three moles of GSH. An excess of GSH appeared on the chromatogram when it was added in a ratio of more than three moles to one mole of CH_3SeO_2H. (See Figures II and III). Chromatography also gave evidence for the formation of GSSG and an unidentified compound. This unidentified reaction product could be detected by spraying the chromatogram with FMA in pH 6.8 phosphate buffer or by spraying with FMA in NaOH. R_f values were calculated as follows:

Compound	R_f Value
GSSG	0.00
GSH	0.15
unidentified product	0.30
CH_3SeO_2H	0.56

The question arose as to whether the methylseleninic acid was converted to selenite in the reaction, thus yielding the same products as the reaction of GSH with selenite ion. The spectrophotometric study indicated that glutathione selenotrisulfide (GSSeSG), the product of the reaction of GSH with Na_2SeO_3, was not formed in the reaction of GSH with methylseleninic acid. The

Figure II

Paper chromatography of different mole ratios of reduced glutathione and methylseleninic acid. Detection with FMA (10^{-4}M) dissolved in 1.5 N NaOH.

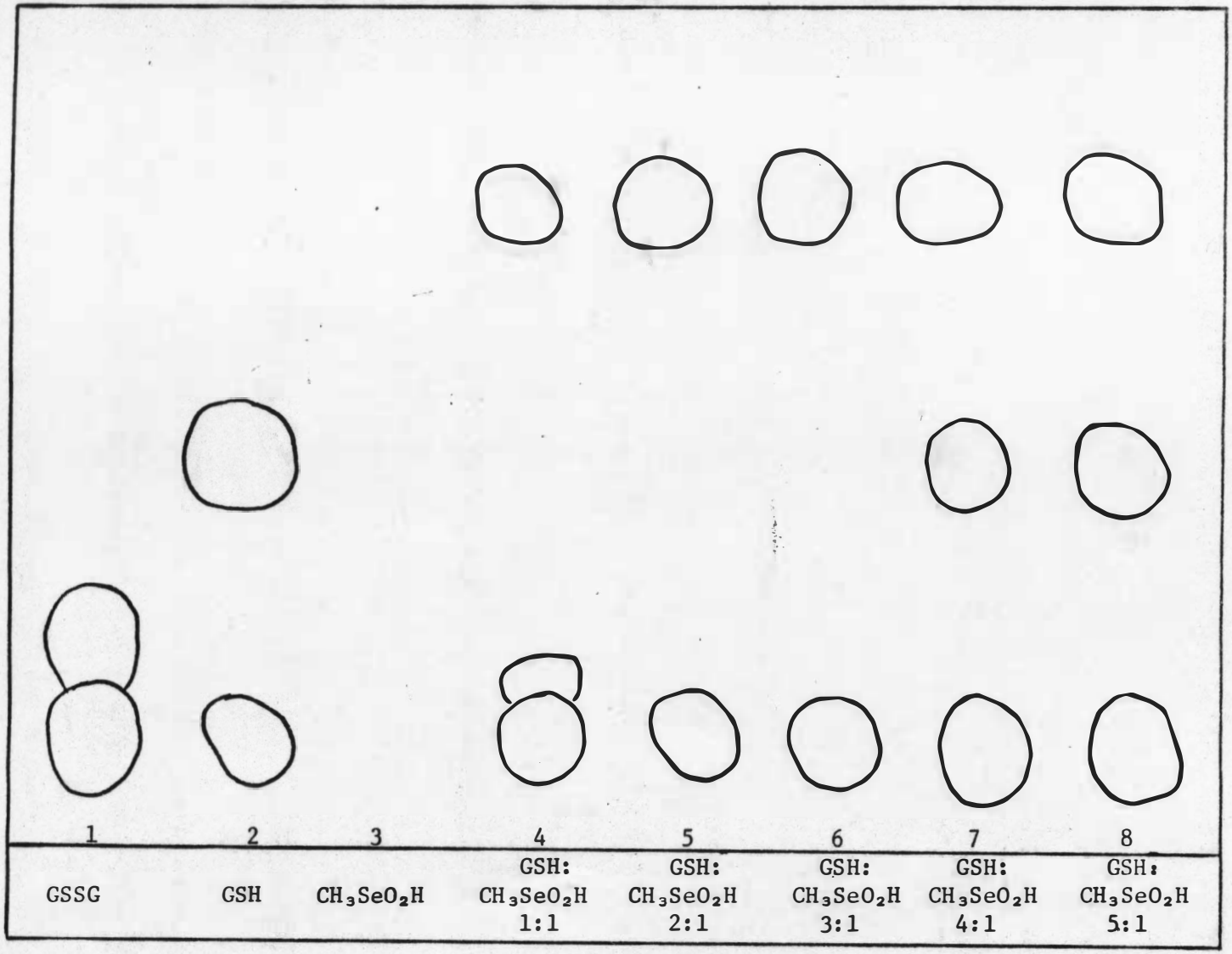
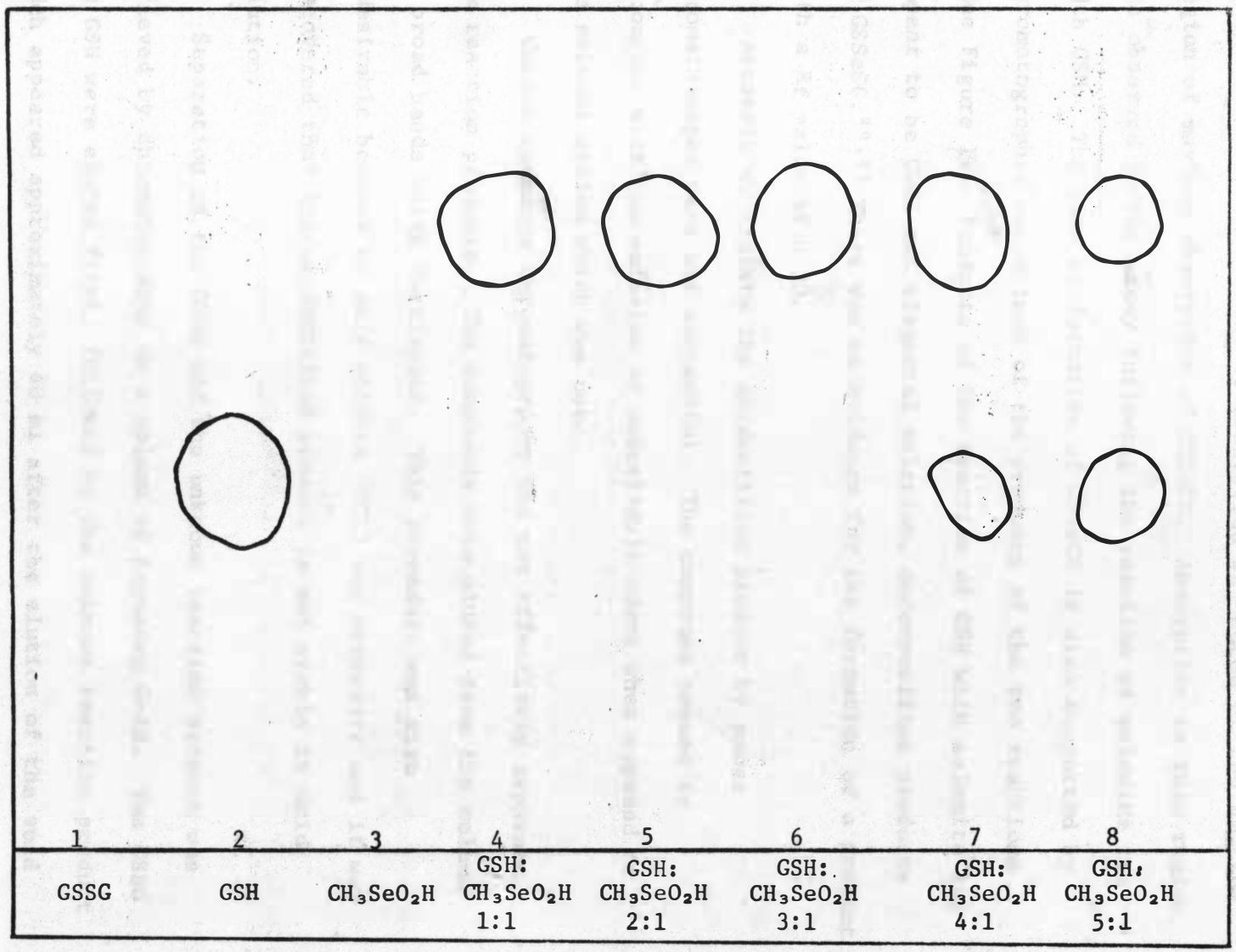

1
2
3
4
5
6
7
8
GSSG
GSH
CH_3SeO_2H
GSH: CH_3SeO_2H 1:1
GSH: CH_3SeO_2H 2:1
GSH: CH_3SeO_2H 3:1
GSH: CH_3SeO_2H 4:1
GSH: CH_3SeO_2H 5:1

Figure III

Paper chromatography of different mole ratios of reduced glutathione and methylseleninic acid. Detection with FMA (10^{-4}M) dissolved in pH 6.8 phosphate buffer.


1
2
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GSSG
GSH
CH_3SeO_2H
GSH: CH_3SeO_2H 1:1
GSH: CH_3SeO_2H 2:1
GSH: CH_3SeO_2H 3:1
GSH: CH_3SeO_2H 4:1
GSH: CH_3SeO_2H 5:1

evidence for this was that no absorption was noted at 260 nm, the region of maximum absorption of GSSeSG. Absorption in this region was observed in the study following the reaction of selenite ion with GSH. The lack of formation of GSSeGS is also supported by chromatographic comparison of the products of the two reactions (See Figure IV). Products of the reaction of GSH with selenite ion appear to be GSSG and elemental selenium, decomposition products of GSSeSG.[20,21] There was no evidence for the formation of a product with a Rf value of 0.30.

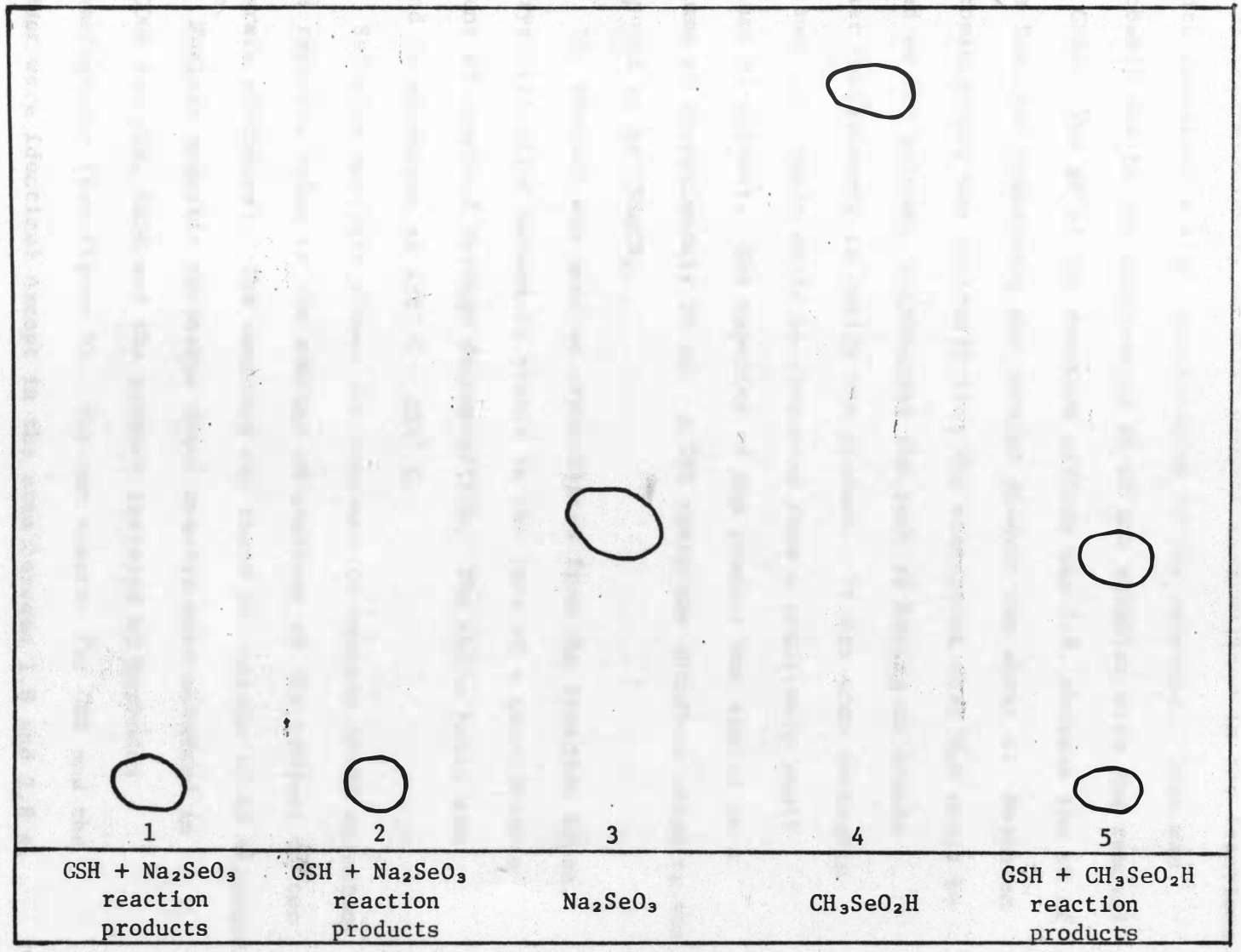
Attempts to isolate the unidentified product by paper chromatography were not successful. The compound seemed to decompose with the emission of undesirable odors when exposed to the solvent system which was used.

Cation exchange chromatography did not effectively separate the reaction products. The compounds were eluted from the column in broad bands which overlapped. This procedure was also undesirable because an acid solvent (HCl) was necessary and it was discovered that the unidentified product is not stable in acid solution.

Separation of the GSSG and the unknown reaction product was achieved by chromatography on a column of Sephadex G-10. The GSSG and GSH were eluted first, followed by the unknown reaction product which appeared approximately 40 ml after the elution of the void volume. Some overlap was observed, but a majority of the fractions

Figure IV

Paper chromatography comparing the reaction products of reduced glutathione and methylseleninic acid with the reaction products of reduced glutathione and sodium selenite. Chromatograms 1 and 5 were detected with FMA (10^{-4}M) dissolved in 1.5 N NaOH. Chromatograms 2, 3 and 4 detected with 3% H_2O_2 and 0.1 M $SnCl_2$.


1
2
3
4
5
GSH + Na_2SeO_3 reaction products
GSH + Na_2SeO_3 reaction products
Na_2SeO_3
CH_3SeO_2H
GSH + CH_3SeO_2H reaction products

containing the unknown product were uncontaminated as shown by paper chromatography. A white product crystallized in the fractions which contained a high concentration of the compound. This was probably due to the increase in pH of the solution with the removal of GSSG. The pH of the reaction mixture was 2.8, whereas the pH of the fraction containing the unknown product was about 4. Sephadex chromatography was desirable from the standpoint that H_2O could be used as the solvent, eliminating the task of having to remove other contaminants to purify the product. It was also desirable because the sample could be recovered from a relatively small volume of solvent. The majority of the product was eluted in a volume of approximately 20 ml. A 25% yield was obtained assuming the compound to be $GSSeCH_3$.

The product was used as crystallized from the fraction tubes. Recrystallization seemed to result in the loss of a considerable amount of compound through decomposition. The white solid was found to decompose at 188° C - 192° C.

Selenium analysis showed the compound to contain 19.6% selenium. (The reported value is the average of analyses of the product of two separate syntheses). The compound was found to contain 10.4% nitrogen.

Nuclear magnetic resonance (nmr) spectra were obtained in CF_3COOH for GSH, GSSG and the product isolated by Sephadex chromatography (See Figure V). The nmr spectra for GSH and the product were identical except in the area between 1.8 and 2.8 δ. The isolated product showed a singlet at 2.0 δ in CF_3COOH, which

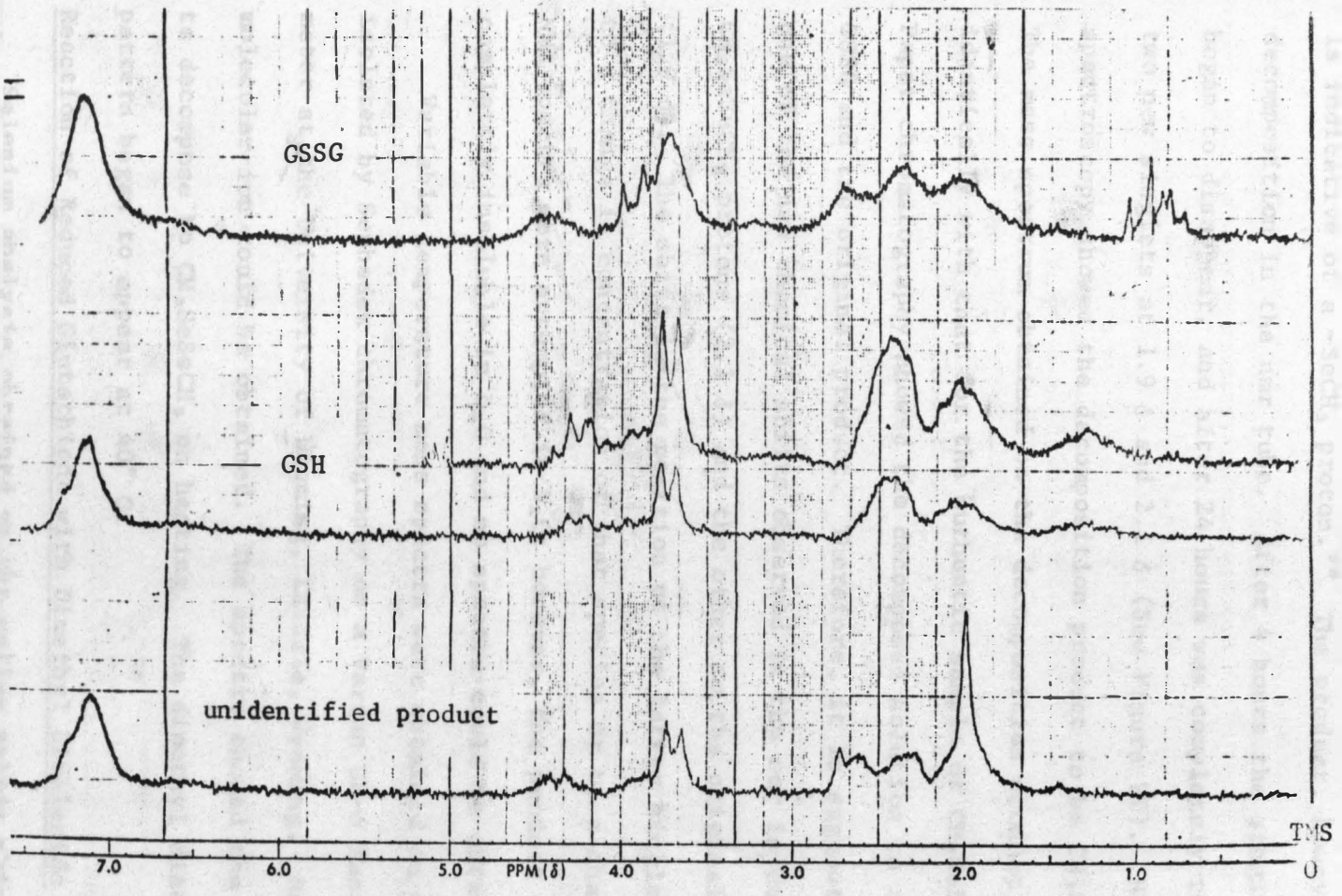


Figure V. Nuclear magnetic resonance spectra of oxidized glutathione, reduced glutathione, and the unidentified product in CF_3COOH.

is indicative of a $-SeCH_3$ proton.[38] The product, however, showed decomposition in the nmr tube. After 4 hours the singlet at 2.0 began to disappear, and after 24 hours was completely replaced by two new singlets at 1.9 δ and 2.1 δ (See Figure VI). Mass spectroscopy showed the decomposition product to be $CH_3SeSeCH_3$. The mass spectrum obtained on the decomposition product compared identically with that for the authentic sample of $CH_3SeSeCH_3$. Paper chromatography showed the decomposed solution to also contain GSSG and the original product. Therefore, it is suggested that one of the new chemical shifts observed in the nmr is due to the $CH_3SeSeCH_3$ protons (2.1 δ) and the other to the original product (1.9 δ). The shift in the position of the latter singlet may be due to a change in concentration of that species or to a change in pH. NMR spectra were attempted in D_2O; however, the product was completely insoluble in D_2O and no spectra could be obtained.

Variable temperature mass spectra were obtained on the product isolated by Sephadex chromatography on a Varian CH-5 Mass Spectrometer at the University of Wyoming, Laramie, Wyoming. No molecular ion could be obtained. The spectra showed the compound to decompose to $CH_3SeSeCH_3$ on heating. The dimethyl diselenide pattern began to appear at 40° C.

Reaction of Reduced Glutathione with Dimethyl Diselenide

Selenium analysis obtained on the yellow solids which were obtained as described in the experimental section were all too low

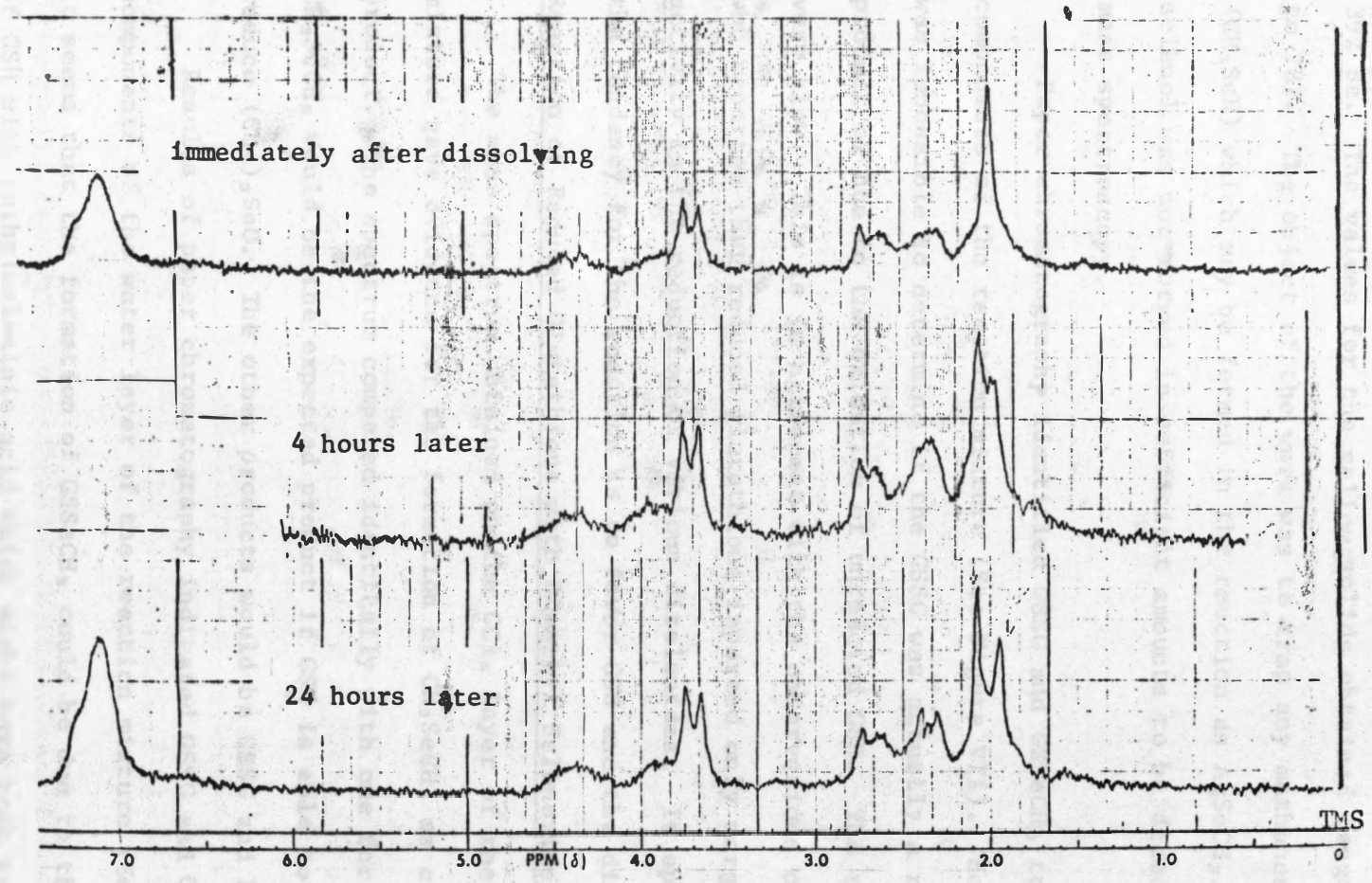


Figure VI. Nuclear magnetic resonance spectra showing the decomposition of the unidentified product in CF_3COOH.

to be meaningful here. The desired product, CH_3SeAg, contains 39% Se. The values for the yellow solids obtained ranged from 17% to 28%. The object of the work was to trap any methaneselenol (CH_3SeH) which may be formed in the reaction as $AgSeCH_3$. Methane selenol was not formed in sufficient amounts to be detected by mass spectroscopy.

Paper chromatography identified GSSG and $GSSeCH_3$ to be components of the reaction mixture (See Figure VII). However, it was impossible to determine if the GSSG was actually a reaction product or due to the oxidation of unreacted GSH. The reaction was very slow. This is in agreement with the observations of Gunther[39] who reported that reduced glutathione imparted only marginal activity in the reduction of various diselenides. It appears that the tendency for the reaction is to favor GSH and the diselenide.

Reaction of Reduced Glutathione with Dimethyl Selenoxide

The mass spectrum obtained on the CCl_4 layer of the reaction mixture gave evidence for the formation of CH_3SeCH_3 as a reaction product. The spectrum compared identically with one for CH_3SeCH_3. CH_3SeCH_3 would be the expected product if GSH is able to directly reduce $(CH_3)_2SeO$. The other products would be GSSG and H_2O.

Results of paper chromatography indicated GSSG and $GSSeCH_3$ were components of the water layer of the reaction mixture (See Figure VIII). It seems that the formation of $GSSeCH_3$ could be due to the reaction of GSH with methylseleninic acid which might have been present as

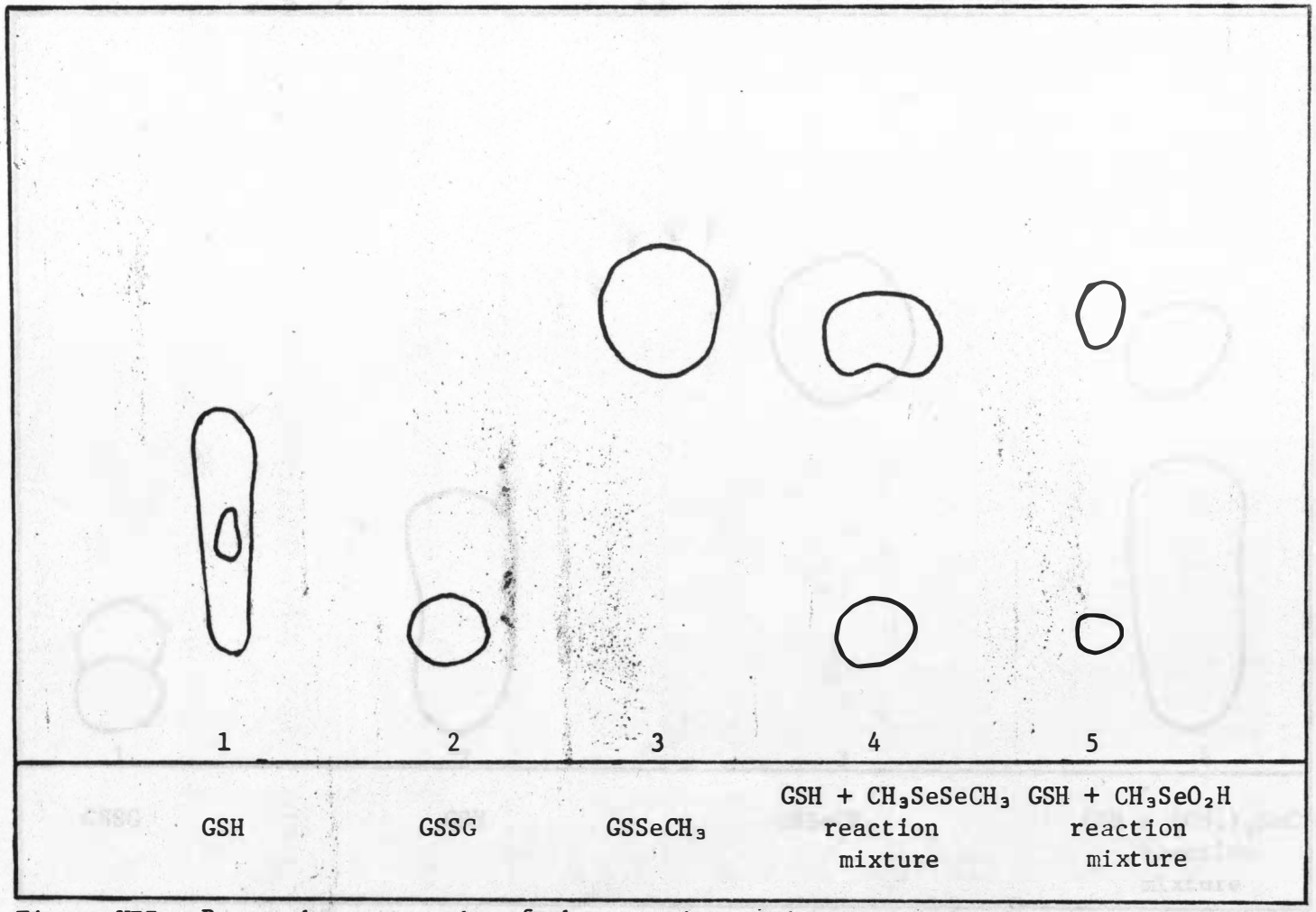


Figure VII. Paper chromatography of the reaction products of reduced glutathione and dimethyl diselenide. Detection with FMA (10^{-4}M) dissolved in 1.5 N NaOH.

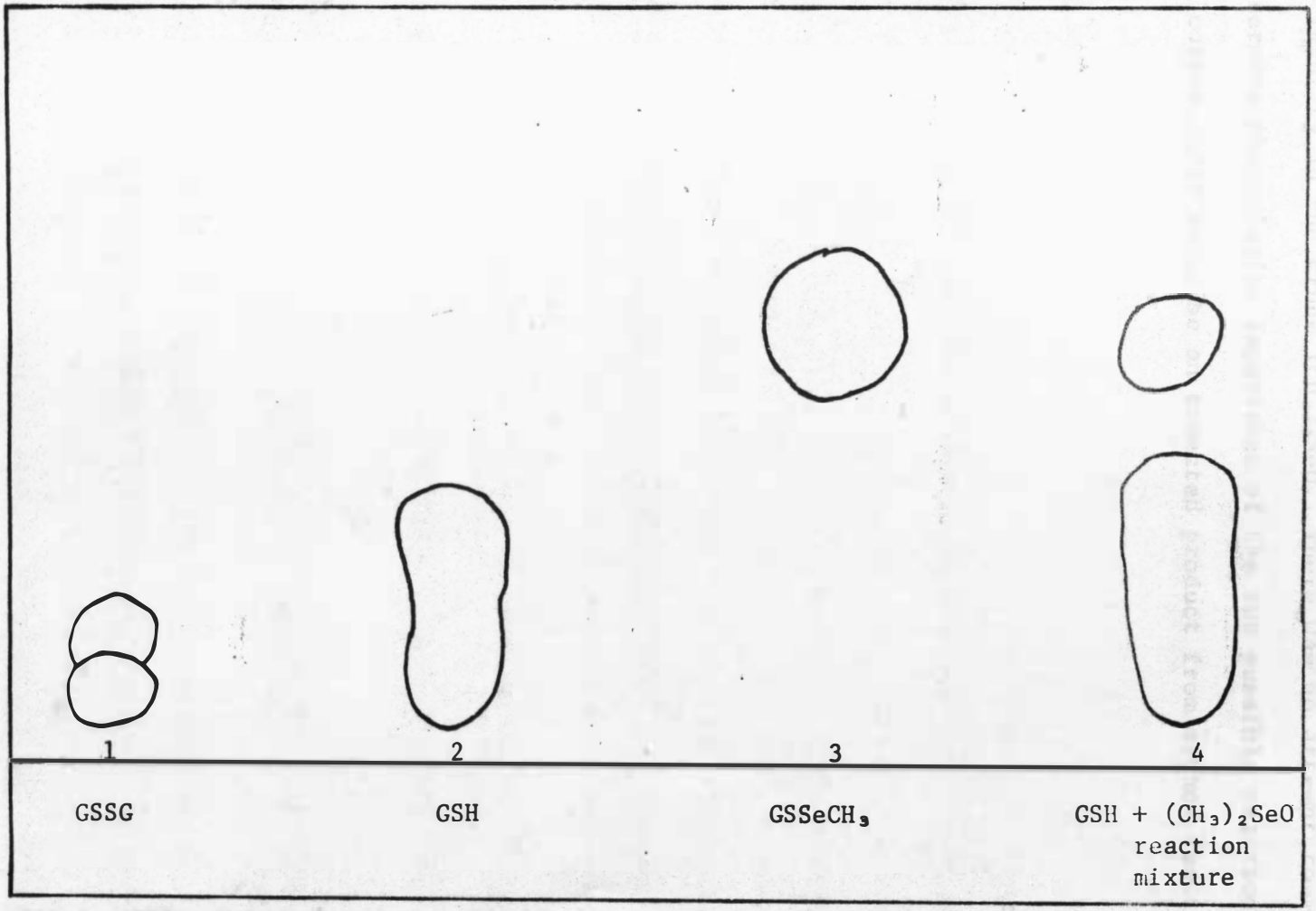


Figure VIII. Paper chromatography of the reaction products of reduced glutathione with dimethyl selenoxide. Detection with FMA (10^{-4}M) dissolved in 1.5 N NaOH.

an impurity in the crude $(CH_3)_2SeO$. There was no attempt to determine the relative importance of the two possible reactions. Of course, GSSG would be an expected product from either reaction.

CONCLUSIONS AND SUMMARY

On the basis of the results presented here, I would like to propose three conclusions. First, the reaction of reduced glutathione and methylseleninic acid suggested by Palmer and Olson[22] does indeed proceed as suggested:

$$3GSH + CH_3SeO_2H \longrightarrow GSSG + GSSeCH_3 + 2H_2O$$

There are several lines of evidence which support this conclusion. The data obtained from paper chromatography were consistent with the predicted molar ratio of 3 GSH to 1 CH_3SeO_2H. With respect to the products, the presence of GSSG in the reaction mixture was verified by paper chromatography. There are also several good points of evidence for the formation of the methylated derivative of glutathione, $GSSeCH_3$. First, selenium and nitrogen analyses are consistent with the theoretical values for $GSSeCH_3$:

	Theoretical	Found
% Se	19.72%	19.6%
% N	10.50%	10.4%

Secondly, the nmr data suggest that the methyl group is present. Finally, it has been shown that the product under question decomposes to $CH_3SeSeCH_3$ and GSSG. This is consistent for a substance with the structure of $GSSeCH_3$ since compounds of the general structure RSeSR' are known to disproportionate to the corresponding diselenides and disulfides,[40] according to the equation:

$$2RSeSR' \longrightarrow (RSe-)_2 + (R'S-)_2$$

Secondly, I would like to propose that the reaction suggested between reduced glutathione and dimethyl diselenide

$$GSH + CH_3SeSeCH_3 \longrightarrow GSSeCH_3 + CH_3SeH$$

is not likely to be a spontaneous biological reaction. The reaction is very slow and the CH_3SeH was never identified.

And, thirdly, although evidence is crude, it appears that the reaction of reduced glutathione with dimethyl selenoxide does proceed spontaneously:

$$2GSH + CH_3\underset{\underset{O}{\|}}{Se}CH_3 \longrightarrow CH_3SeCH_3 + GSSG + H_2O$$

The data presented here appear to support the metabolic scheme proposed by Palmer and Olson[22] (See Figure I). Two of the three proposed reactions of GSH with methylated selenium derivatives studied here can occur by nonenzymic reactions. Therefore, the production of volatile selenium from these substances by rats as reported,[33] may not be due to enzymic reactions, but may merely be due to spontaneous reactions such as those studied in this thesis.

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