Delineating the Distribution of Selenium in Bacterial Cultures That Reduce And Methylate Oxyanions of This Toxic Metalloid

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by

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Delineating the Distribution of Selenium in Bacterial Cultures That Reduce And Methylate Oxyanions of This Toxic Metalloid

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Abstract

JIANG, WENBIAO, Delineating the Distribution of Selenium in Bacterial Cultures That Reduce and Methylate Oxyanions of This Toxic Metalloid. Master of Science (Chemistry), December, 1993, Sam Houston State University, Huntsville, Texas.

Purpose

This research was undertaken for the purpose of developing a method and using this method to analyze the distribution of selenium species in bacterial cultures which reduce and methylate oxyanions of this toxic metalloid.

Method

The analysis methods developed were based on atomic absorption spectroscopy for selenium at 196.0 nm and colorimetric method for selenium using 3,3'-diaminobezedine reagent.

Pseudomonas fluorescens K27 bacteria was used for this research and tryptic soy broth containing 0.1% potassium nitrate was used for growth of the bacteria. Selenite, selenate and elemental selenium amended bacterial cultures grown in aerobic and anaerobic conditions were analyzed in this work.

Findings

The analytical procedures developed for selenium delineation were found useful in this research: the results of spike addition method show the suitability of the adopted wet ashing procedure using nitric acid for the

biological precipitate and elemental Se; the matrix effect investigation using standard addition and serial dilution methods indicated that the matrix effect of these biological samples were not significant to the atomic absorption spectroscopy for Se analysis. The spectrophotometric measurement of selenite by 3,3'-diaminobezedine reagent after quantitative reduction of selenate to selenite could be used to differentiate the selenite and selenate in the bacterial cultures. The comparison between atomic absorption spectroscopy and colorimetric method for Se confirmed this analytical procedure was suitable for this research.

The selenium distribution of the bacterial culture analyzed by this pathway indicated: the only cultures that were found to produce volatile Se and elemental Se in our experiments contained live aerobic or anaerobic bacteria. Selenite amended cultures grown aerobically and anaerobically produced more elemental Se than similar selenate amended cultures. Selenate poisoned cultures grown aerobically and anaerobically produced more volatile Se than similar selenite poisoned cultures. Selenite was oxidized to selenate by atmospheric O_2 in sterile tryptic soy broth media containing 0.1% potassium nitrate treated aerobically, though the amount is relatively small.

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Thesis Director

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I would also thank Mr. Steve L. McCarty in our research group who is my great friend and provided great support to my work.

My thesis is dedicated to my family.

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Chapter 1
Introduction

Selenium in the Environment

Selenium (Se) and its compounds have been recognized as inorganic carcinogens of concern in the spectrum of identified environmental pollutants, based on observation of proved toxic effects and relative accessibility. From a public health standpoint, selenium holds a rather unique place among the elements because of small differences between concentrations which are essential and those that are toxic to animals and human beings. Movement of these toxic elements through the geocycle and their biological methylation in the environment to volatile selenium species further complicates the problem.

As selenium is of interest as a potential environmental toxicant, lots of attention has been focused on the detailed understanding of biomethylation and transformation of selenium. This methylation of toxic elements is an important transformation because it often leads to a change in both mobility and toxicity of the element. With selenium, biomethylation represents the conversion of a non-volatile precursor to volatile products such as dimethyl selenide and dimethyl diselenide, which are less toxic than other forms of non-volatile selenium species (Spallholz, 1994). Furthermore, the formation of volatile compounds may play an important role in the cycling of this element in the biosphere.

Many investigations of this transformation have been carried out, and several pathways have been proposed for reduction and methylation of selenium salts. For example, Doran (1982) suggested that the methylation of inorganic selenium may first involve reduction to the elemental Se and then to selenide form, which is subsequently methylated to form dimethyl selenide (Figure 1).

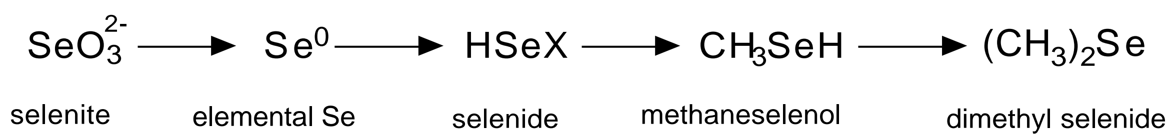


Figure 1. Proposed pathway for reduction and methylation of selenium salts. Adapted from Doran, 1982.

The conversion of oxyanions such as selenate and selenite to volatile methylated species such as dimethyl selenide and dimethyl diselenide by plants, microorganism, and mammals has been well documented in the literature. Also many workers have found elemental Se in bacterial cultures doped with oxidized selenium salts (Francis *et al.*, 1974; Doran and Alexander, 1977). Our bacterial cultures doped with selenite and selenate also yielded some red elemental selenium precipitate.

In this research we tried to analyze the distribution of selenium species quantitatively in bacterial cultures which reduce and methylate oxyanions of this toxic element, and further more, we attempted to understand the pathway of the remediation process of Se by *Pseudomonas fluorescens* K27, a selenium resistant bacterial strain which was isolated from Kesterson Reservoir in the San Joaquin Valley of central California.

The selenium resistant bacteria *Pseudomonas fluorescens* K27 can grow in either anaerobic or aerobic conditions. When grown in the presence of selenate or selenite oxyanions, volatile selenium species such as dimethyl selenide and dimethyl diselenide were found in the headspace of the K27 bacterial culture (Chasteen *et al.*, 1990); we have duplicated this work many times in our lab using the fluorine-induced chemiluminescence detector and capillary gas chromatography. Also some amounts of red precipitate appear after 24 hours growth in these culture. We believe this solid is elemental selenium.

The distribution of Se with different physical states and valence states is show in Figure 2.

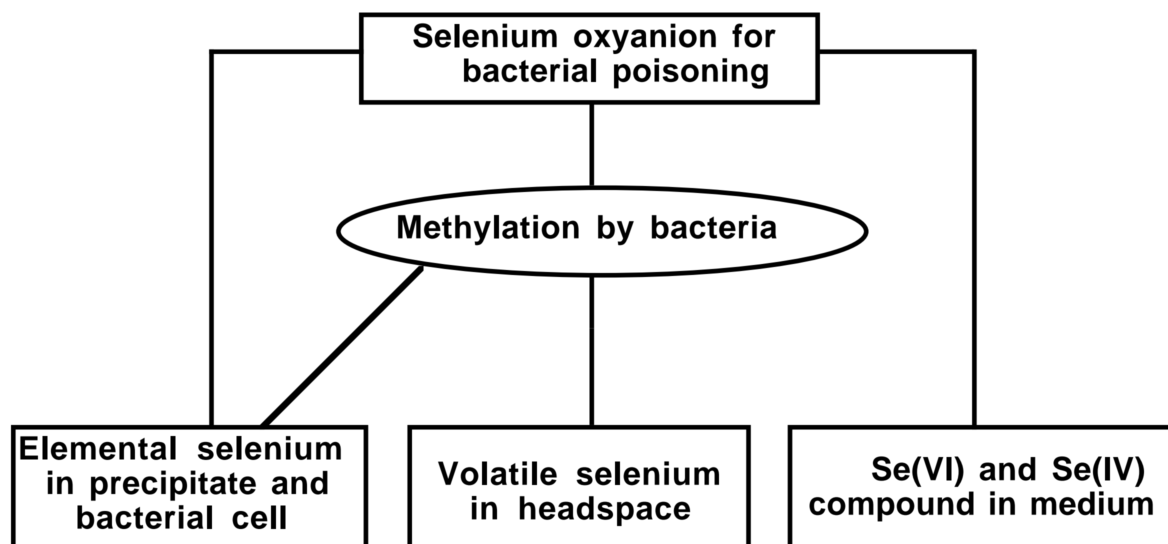


Figure 2. The distribution of selenium species after bacterial remediation.

The methylated selenium compounds such as dimethyl selenide and dimethyl diselenide escape to the headspace because of their volatility and relative insolubility in aqueous media. One of the other reduced products of this bioremediation process is elemental selenium, a solid which can be found in the precipitate of poisoned bacterial cultures of K27. The rest of selenium oxyanions are still in the medium after this uncompleted methylation process.

Analytical Methods for Selenium Detection

Atomic Absorption Spectroscopy

One method of delineation of Se in this bacterial culture is using atomic absorption spectroscopy (AAS) to analyze the (chemically) digested elemental Se and selenium species within bacterial cells in precipitate and total selenium species within supernatant after separation by centrifugation. The spectrophotometric measurement of Se^{4+} at 420 nm by 3,3'-diamino-benzidine (DAB) can be used to measure the amount of Se^{4+} ion in the separated supernatant (Cheng, 1956). The amount of Se^{6+} ion is measured by the difference between the sum of Se^{6+} and Se^{4+} and that of Se^{4+} only, after the quantitative reduction of selenate to selenite by concentrated hydrochloric acid. In this study, a cross comparison was also made between AAS and spectrophotometric methods. The amount of volatile selenium species is assumed to be equal to the difference between the amount of original selenium added to the bacterial culture and the amount of selenium analyzed in precipitate and supernatant of the sample. The pathway of analysis is shown in Figure 3.

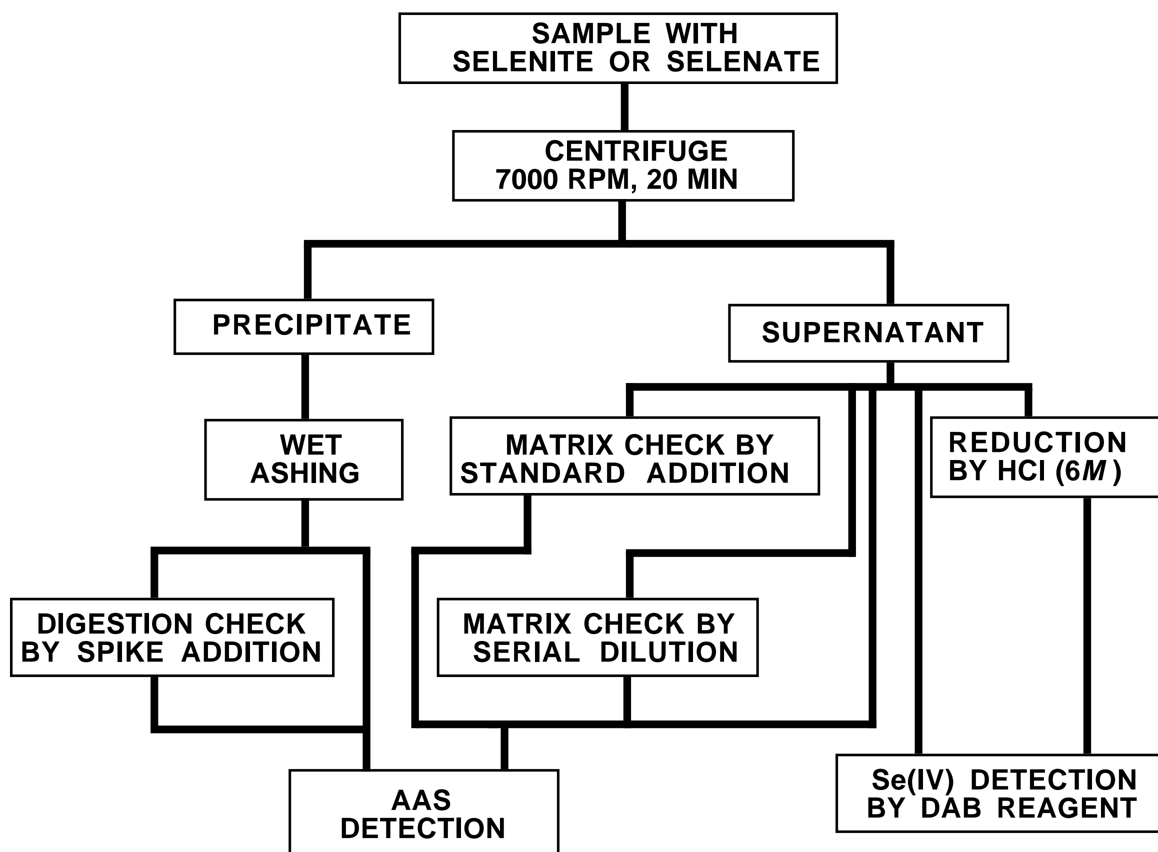


Figure 3. The pathway for the analysis of selenium distribution.

Selenium can be measured by atomic absorption spectroscopy in a flame at 196.0 nm. In AAS, the ground state atom absorbs light energy of a specific wavelength to enter the excited state. By measuring the amount of light absorbed, a quantitative determination of the amount of analytes can be made. It was found that the greatest sensitivity is given at 196.0 nm which is corresponding to energy required for the excitation of Se from lowest ^3P ground state to its first exited state (Rann and Hambly, 1965). The light used is a hollow cathode lamp, which is a low pressure gaseous discharge tube

with a hollow cathode made of the selenium element. A glow of discharge and the emission of a low-energy spectrum of the selenium element acts as the light source for the atomic absorption process. In this experiment, a multi-element lamp whose cathode contains more than Se was used (As-Se-Te). The multi-element lamp was used partly on the ground of economy and partly for its convenience.

The flame used in AAS has three functions: evaporate the solvent, decompose and dissociate the molecules, and provide ground state atoms for absorption of the exciting radiation. The general purpose air-acetylene flame appeared satisfactory for the selenium element determination in this work. A major disadvantage in working at wavelengths as short as 196.0 nm is the absorption of light by the flame gases which caused the lower sensitivity problem. The absorption varied considerably according to the operating conditions. For selenium, the transmission of a fuel-rich acetylene-air flame could diminish by 20% when water in the sample is sprayed into it; but, the effect is much less in the lean flames. A sensitivity of 2 ppm of selenium was accomplished in the blue-lean acetylene flame and the linear working range is up to 50 ppm. AAS is ideal for selenium species determination in bacterial medium because it can overcome the complication of the composition of the culture containing the bacterial and Se compounds.

Biological Sample Digestion by Wet Ashing

Components of the bacterial culture can be separated by centrifugation. The precipitate contains elemental selenium and bacte-

rial cells; the dissolved selenium compounds stay in the sample supernatant. It is necessary to digest the precipitate containing the elemental Se before the analysis by AAS. The most widely used digestion methods for the determination of selenium involve decomposition with acid or acid mixtures such as HNO_3, $HClO_4/HNO_3$, and $H_2SO_4/HClO_4$ (Adeloju *et al.,* 1984). The preference for these wet digestion methods is based on the reduced danger of losing selenium at the low temperatures at which they operate and the simplicity of the apparatus required for their operation. The methods of dry ashing, on the other hand, often result in great losses. The wet ashing procedure using HNO_3 with open container for bacterial culture samples is adequate for analytic methods based on AAS; a low matrix effect normally does not affect these methods (Adeloju *et al.,* 1984). A clear colorless solution obtained after digestion with HNO_3 procedure indicates that the digestion is complete. There is no significant difference between the results obtained for selenium by open digestion and those by closed digestion which utilized a similar acid or acid mixture (Adeloju *et al.*, 1983).

For the wet ashing method, a reasonably efficient digestion procedure is required that will adequately decompose the sample matrix while ensuring that little or no losses of the analytes occur. Also, there is a possible interference in AAS analysis caused by relatively large amount of acid used in our digestion process; on the other hand, we also considered the possible losing of selenium during the digestion because the oxidized product SeO_2 will sublime at 340 ^{0}C to 350 ^{0}C. The spike addition method was used to investigate the wet digestion procedure by HNO_3. Here, certain amounts of

black elemental selenium (commercially available) close to the amount of red elemental Se (commercially unavailable) in the precipitate were added into the digestion vessel as a spike. The samples with spikes undergo the digestion as well as the samples without spikes; and the blank sample with only the digestion reagent was also analyzed at the same time to check the interference caused by acid used in wet ashing to the AAS analysis. After all these samples are analyzed by AAS, the recovery was calculated by equation (1):

$$\text{RECOVERY} = \frac{\text{SAMPLE WITH SPIKE} - \text{SAMPLE WITHOUT SPIKE}}{\text{AMOUNT OF SPIKE}} \times 100\% \quad (1)$$

The recovery values showed the efficiency of the digestion method for this particular kind of sample and its suitability for the analysis by AAS.

Determination of Matrix Effects

The total amount of nonvolatile selenium species in sample supernatant can be determined by AAS directly. Before the analysis, the supernatant has to be diluted so that the analysis can be performed in the linear range of the AAS calibration curve. The medium used for these bacterial culture was tryptic soy broth with 0.1% KNO_3 (TSN). Though tryptic soy broth is a commercial product, the exact chemical composition can not be defined. It is made of nutrients such as the digested proteins from soy broth so that the energy, carbon, nitrogen, and sulfur requirements of the growing microorganism are available. Because of the undefined composition

of the TSN medium we used, it was necessary to investigate the possible matrix effect—an interference caused by the differences between the sample and a standard containing only the analysis element and, where appropriate, a solvent. This is an interference which actually influences the proportion of atoms in the flame available to absorb the resonance radiation and arises from chemical effects or physical effects which originate in the sample solution. Therefore, a serial dilution method and a standard addition method were used to check the matrix effect of the dilution of supernatants which are going to be analyzed by AAS.

For the serial dilution method, several dilutions of the sample supernatant with deionized water (DI water) were made with different dilution ratios. All of these dilutions were analyzed by AAS; the absorbance values were plotted against the dilution values. If a straight line is obtained, it indicates that there is no significant matrix effect. If the matrix effect is really significant, for example, compounds or radicals containing the element being measured are not broken down into individual atoms in the flame of AAS, a line with a downward curvature would be obtained because the interference caused by matrix effects is more apparent in more concentrated solution.

The second method of investigating the matrix effects is the standard addition method. This technique is used to overcome interference effects by matching standards with the samples, because the full composition of the samples is not known. The sample solution is divided into a number of aliquots—at least three are necessary. To all but one of these are added known increasing amounts of

the selenium standard. The solutions are analyzed by AAS and the absorbance readings plotted against the added concentration of Se. The standard addition line obtained in this procedure should be parallel to the true calibration line which passes through the origin. The extrapolated standard addition regression line will intersect the abscissa at a negative concentration value and the absolute value of the sample can be read off at this point. When using the standard addition method, only the addition of standard Se solution is needed. This has no effect on general composition, and no knowledge of the matrix composition is necessary. The concentration of Se in the supernatant obtained by this method is compared with that obtained by the true calibration line to show if the matrix effect influenced the AAS analysis.

Volatile Organoselenium

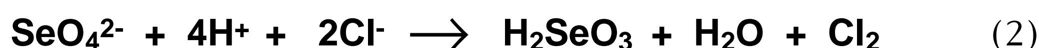
The amount of volatile selenium species reported in this work was assumed to be equal to the difference between the amount of Se element added into the bacterial culture and the sum of (1) elemental Se in precipitate and (2) the selenium compound in the supernatant measured by AAS. This assumption seems to be a good one based on the possible Se species known to be in these solutions based on our prior experience and research in the literature (for instance, see review in Spallholz, 1994).

Colorimetric Determination of Selenium

One of the disadvantages for AAS analysis is that it can determine the total amount of the element being measured in the sample,

but it can not determine the amount of this element with a specific valence number in the sample. However, speciation of Se with respect to oxidation state is important in assessing the bioavailability of this element and its toxicity. After the centrifugation process, if all the volatile selenium species such as dimethyl selenide and dimethyl diselenide are assumed to have been totally excluded from the bacterial culture, the only two chemical species of selenium dissolved in the supernatant will be SeO_4^{2-} and SeO_3^{2-}. One possible method to differentiate and approximately measure these oxyanions is based on the quantitative reduction of selenate to selenite by hot concentrated hydrochloric acid and the colorimetric measurement of Se(IV) by 3,3'-diaminobenzidine (DAB) reagent (Cheng, 1956).

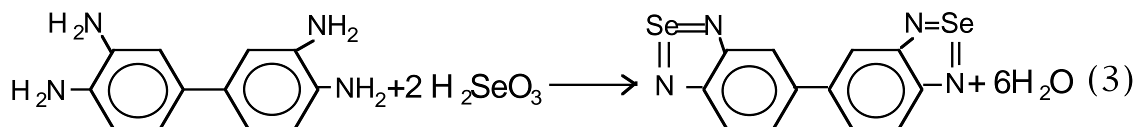
The reduction of selenate to selenite can be carried out in hot HCl (6M) in a closed system for 30 min. The important component of the reducing solution is the Cl^- ion and the Se^{6+} reduction can be carried out quantitatively to selenite. The reduction reaction is:



$$SeO_4^{2-} + 4H^+ + 2Cl^- \longrightarrow H_2SeO_3 + H_2O + Cl_2 \qquad (2)$$

The samples were heated in capped test tubes to avoid the loss of selenium through the formation of volatile compounds. The kinetic study of this reduction reaction by the differential pulse polarography (DPP) using a PARC polarographic analyzer with a PARC static mercury drop electrode indicated that in 6M HCl, 99.9% of the original SeO_4^{2-} was converted to SeO_3^{2-} after 30 min at 91 ^{0}C (Brimmer *et al.*, 1987). This confirms that the adopted procedure does result in quantitative conversion of selenate to selenite.

The sensitive method based on 3,3'-diaminobenzidine is the most widely used method for spectrophotometric measurement of Se^{4+}. This technique is based on the measurement of a yellow colored compound formed when DAB reagent reacts with Se^{4+} in acidic medium. It is believed the yellow compound to be a dipiazselenol:



and in 2M hydrochloric acid matrix, the maximum color development can be achieved in about 50 min (Cheng, 1956).

Within the pH range 5 to 10, the distribution coefficient of dipiazselenol between toluene and water is high, and one portion of toluene extracts practically all the selenium complex into the organic phase. The free reagent (DAB) is also extracted. Therefore, a reagent blank is used. The two absorption maxima of dipiazselenol occur at 340 nm and 420 nm; since the DAB absorbs strongly at 340 nm but negligibly at 420 nm, absorbances of the Se complex are measured at 420 nm. The extraction procedure makes this method more selective since the slightly colored sample will not interfere with the absorption. The limit of sensitivity of the method is 50 ppb with a 1 cm absorption cell (Cheng, 1956); the calibration curves follow Beer's Law over the range of 10 to 150 microgram of Se per 10 mL of toluene at 420 nm.

Waste Disposal

Because of the high toxicity of selenium compounds, all wastes after analysis were put in a glass container and kept in the vent hood of our lab. These aqueous wastes were evaporated to a minimum volume for further disposal. All of the toxic wastes generated in these experiments will ultimately be removed for disposal by a commercial waste hauler contracted by Sam Houston State University.

Chapter 2
Experimental Procedures

Apparatus and Reagents

Selenium standard for atomic absorption spectroscopy was obtained from Ricca Chemical Company (Arlington, TX USA). Sodium selenite, reagent grade, was obtained from Chemicals Procurement Laboratories Inc. (College Point, NY USA). Sodium selenate, 98%, was obtained from Strem Chemicals (Newburyport, MA USA). Tryptic soy broth was obtained from DIFCO Laboratories (Detroit, MI USA). 3,3'-diaminobenzidine, 99%, was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI USA). All reagents were used as received without further purification.

A Perkin Elmer 608 Atomic Absorption Spectrophotometer and a Perkin Elmer 552A UV/Vis Spectrophotometer (Norwalk, CT USA) were used for our experiments. The hollow cathode lamp (As-Se-Te) was obtained from Fisher Scientific (Pittsburgh, PA USA). A Sorvall RC-5 Superspeed Refrigerated Centrifuge (Dupont Instruments, Wilmington, DE USA) was used for centrifugation.

Experimental Procedures for Samples Poisoned by Selenium Oxyanions

Anaerobic Microbial Incubations

The bacteria we used in this experiment were *Pseudomonas fluorescens* K27, isolated from Kesterson Reservoir in central California. The K27 cultures were supplied by Ray Fall at University of

Colorado, Boulder. The medium used for this bacteria growth was tryptic soy broth with 0.1% KNO_3 (TSN). Although its exact chemical composition can not be defined, this is a widely used complex medium. Complex media are used when the medium composition is not important in bacterial growth experiments or, as in our case, where minimal media that successfully promote bacterial growth are not known and experiments aimed at finding a successful minimal medium failed (Zhang, 1993).

The selenium resistant K27 were grown at 30 ^{0}C in TSN medium containing 10 g/L tryptic soy broth and 1 g/L potassium nitrate as the electron acceptor. The media were sterilized by autoclave before inoculation with K27 bacteria, and the inoculated cultures were incubated at 30 ^{0}C in capped bottles overnight in a thermostatic water bath. After 24 hours of growth, the cultures were dosed with a desired amount of sodium selenate or sodium selenite from sterile filtered standards; then the poisoned cultures were incubated at 30 ^{0}C in water bath again for 24 hours or longer before further analysis.

Sample Preparation and Poisoning

Sterilization was accomplished by autoclaving all culture bottles, caps, micropipet tips, TSN medium and other glassware for quantitative measurement. One mole/L aqueous solutions of sodium selenate and sodium selenite were prepared as stock solutions. The samples were prepared by micropipetting certain amounts of filter Se sterilized stock solution into known amounts of inoculated medium after 24 hours bacterial growth. For example: one hundred mL

of culture was poisoned with 1.01 mL of 1 M sodium selenate solution to get 10 mM selenate poisoned culture. The samples were then incubated at 30 ^{o}C in a water bath for 24 hours or longer before further analysis.

Sample Separation by Centrifuge

The samples were centrifuged at 7000 rpm for 20 min by the Sorvall RC-5 Superspeed Refrigerated Centrifuge to spin down the elemental selenium precipitate and cells at 10 ^{0}C. The precipitate was transferred to a 75 mL beaker, and clear supernatant was stored in the cooler at -2 ^{0}C to stop the bacterial growth and wait for further analysis.

Digestion of the Precipitate

To a 75 mL beaker containing the precipitate from the centrifugation process, 5 mL concentrated nitric acid was added; the beaker was placed on a hot plate, stirred continuously, and heated initially at medium rate for 5 min. Then the beaker was heated on maximum setting until nitrogen oxide fumes were given off for a short time and a white residue was left. The beaker was left to cool for about 2 min and digestion was repeated with an additional 2 mL concentrated nitric acid; this time it was heated until brown nitrogen oxide fumes almost ceased to appear. The beaker was cooled again for about 2 min and then 2 mL of 1:1 hydrochloric acid (37%) was added. The mixture was heated at a medium rate for 3 min. After that it was cooled to room temperature and made up to 25 mL or bigger volume with distilled DI water. These samples were analyzed by atomic absorption spectroscopy.

Investigation of Digestion Procedure by Spike Addition

One of the 24 hour selenite poisoned samples with 10 mM Na_2SeO_3 was used for analysis because of the relative large amount of red elemental selenium found in it. This sample was from Dr. Plishker's lab here at Sam Houston State University. It was swirled and separated into four portions all with the same volume. After separation by the centrifuge, approximately the same amount of elemental selenium was added into two of the precipitates. These two spike samples were taken through the digestion process together with the two other precipitate samples without the spike added. Two 75 mL beakers containing the same amount of acid for digestion were prepared for control. All six beakers were heated on one hot plate and processed by the same digestion process for the same time. After digestion, they were made up to the same volume by DI water. These samples were then analyzed by atomic absorption spectroscopy.

Matrix Effect Check by Standard Addition Method

Two of the bacterial samples poisoned with 10 mM selenate were used for this experiment. The supernatant obtained from centrifugation was diluted by a factor of 1/50 by DI water. The concentration of the selenium was first approximated by AAS analysis. Four separate equal fractions were then pipetted from the diluted supernatant and to three of them definite amounts of selenium were added. For example, the concentration of a diluted supernatant was 10.26 ppm measured from the AAS calibration line, then 3.76 mL, 8.14 mL and 13.76 mL of 100 ppm standard selenium solution were

added into three 50 mL diluted sample solutions. All three aliquots and one without Se addition were analyzed by AAS. The absorbance values were plotted against the added concentrations of selenium (0 ppm, 7 ppm, 14 ppm and 21 ppm), and a regression line was drawn through these four points. It intersected the abscissa on the negative side of concentration axis and the point of intersection indicated the absolute value of the concentration of selenium present in the diluted supernatant. The comparison of this value with the concentration of selenium by AAS obtained before showed the matrix effect of the 1/50 dilution of supernatant. This technique is shown in Figure 4.

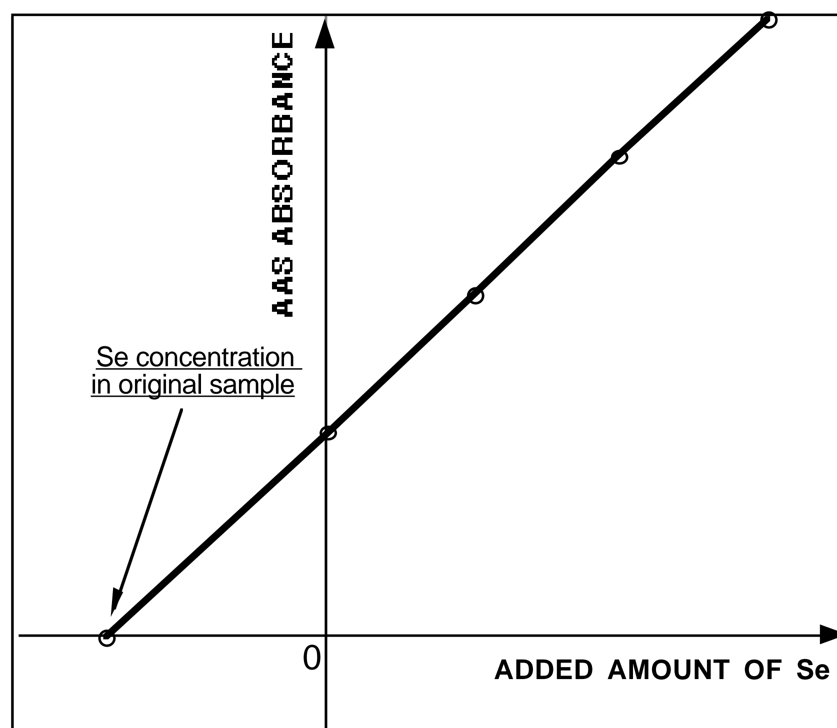


Figure 4. Graphical evaluation using the standard addition method.

Matrix Effect Check by Serial Dilution Method

One of the poisoned samples with 10 mM selenate was used for this experiment. The supernatant was first diluted 20 times by DI water, and then a 1/40 dilution was made from the 1/20 dilution; a 1/80 dilution was made from the 1/40 dilution, and so on. A total of five dilutions of supernatant were made with diluting range from 1/20 to 1/320. After atomic absorption analysis, the absorbance values were plotted against the diluting ratio to check the matrix effect. The reason that the dilution was started with 1/20 and not a less dilute sample is because a more concentrated dilution was out of the linear range of AAS calibration curve.

Atomic Absorption Detection of Selenium

The dilutions of supernatant, digestion solutions of precipitate, and other samples containing selenium species were analyzed by flame atomic absorption spectroscopy at wavelength 196.0 nm with slit width of 2.0 nm. The flame used was an air-acetylene oxidizing flame and the fuel to air ratio was 15 to 60 in order to achieve the lean and blue flame recommended for selenium element by the instrument's manufacturer. The light source was a Se-As-Te hollow cathode lamp. The linear working range of selenium is from 5 ppm to 50 ppm. Standard solutions in this range were diluted from a 1000 ppm Se standard solution for AAS, and DI water was used as a blank. The AAS instrument and lamp were turned on for at least 15 min to warm up before using. The wavelength setting, lamp source, linearity and height of burner head and the flow rate of air and acetylene were optimized every time before analysis. The inte-

gration time for each reading was 4 seconds. For each standard solution and sample, four to five absorption readings were taken and the average absorbance value was taken from them. When a considerable number of samples was being determined, after 10 to 12 samples, a blank solution (DI water) was aspirated and the auto zero button was pressed again.

Quantitative Reduction of Selenate to Selenite

Selenate was quantitatively converted to selenite by acidifying 7 mL of sample or standard selenate and selenite solution with 7 mL of concentrated (12 M) hydrochloric acid. The resulting solution was then placed in a capped test tube and heated in a water bath at 91 ^{o}C for 30 min. The test tube was placed in the bath so that the portion containing the sample (approximately 9.5 cm out of a total length of 11 cm) was totally immersed. The inner side of the screw cap was coated with Teflon® such that the Teflon was the only material besides glass that could come into contact with the sample. After the reaction, the samples were allowed to cool to room temperature and to wait for further spectrophotometric analysis.

Determination of Se^{4+} Ion by Spectrophotometric Detection

The reagent for selenium detection was 0.5% DAB hydrochloride solution prepared by dissolving 0.5 g of DAB with 10 mL 15% concentration HCl and 90 mL DI water. This solution was required to be made freshly just before the experiment and stored in a refrigerator at 0 ^{o}C (Cheng, 1956).

A solution containing 1 mg of Se^{4+} per mL was prepared by dissolving 0.2189 grams of sodium selenite in 100 mL of DI water. Other more diluted Se^{4+} standard solutions were made from the dilution of this standard. A solution containing 1 mg of Se^{6+} per mL was prepared by dissolving 0.2392 grams of sodium selenate in 100 mL of DI water. Other more diluted Se^{6+} standard solutions were made from the dilution of this standard using DI water as diluent.

The samples and standard solutions which were carried through the reduction process were placed in a 150 mL beaker and were diluted to 42 mL with DI water; the samples and standard solutions without reduction were amended with 7 mL concentrated HCl (12 M) and DI water to a final volume of 42 mL. This meant all samples were carried out in a matrix of 2 M HCl to achieve the maximum color development. Five mL of 0.5% DAB solution was added into the beaker containing sample and was allowed to stand for 60 min color development. Then the pH value was adjusted to 7 to 8 with 7 M ammonium hydroxide to ensure the total extraction of dipiazselenol by toluene. This solution was transferred to a 125 mL separatory funnel, supplemented with exactly 10 mL of toluene, and shaken vigorously for 30 seconds. The organic phase was saved and taken through centrifugation for 30 seconds. After separation, the absorbance at 420 nm was measured, using a reagent blank.

The calibration curve was produced by the absorbance readings of standard Se^{4+} solutions (excluding reduction process) versus their known concentrations. The linear range for this method was from concentrations of 10 to 140 micrograms of Se^{4+} per 10 mL of toluene in a 1 cm absorption cell. The spectrophotometer was turned on 15 min before the analysis to ensure a stable baseline.

Aerobic Growth of K27 on Minimal Medium

This sample was supplied by Dr. Plishker's Laboratory. The minimal medium (McEldowney and Fletcher, 1986) was made by dissolving 0.544 g KH_2PO_4, 0.2 g glucose, 0.38 g NH_4Cl, and adding 0.6 mL salt solution into 100 mL DI water. The pH value was 7.4. The salt solution used here was prepared by dissolving 10 g $MgSO_4 \cdot 7H_2O$, 1.0 g $MnCl_4 \cdot H_2O$, 0.4 g $FeSO_4 \cdot 7H_2O$ and 0.1 g $CuCl_2 \cdot 2H_2O$ in 1000 mL DI water.

The sterilized minimal medium was inoculated with *Pseudomonas fluorescens* K27. After 24 hours aerobic growth in a flask slightly covered with aluminum foil, the sample was poisoned with a sterilized sodium selenate solution, and the final concentration of selenate in bacterial culture was adjusted to 500 mM. After 24 hours' shaking in a water bath at 30 ^{0}C, the bacterial cultures were analyzed by AAS and spectrophotometric detection with DAB reagent according to experimental procedure for selenium oxyanion poisoned samples in this chapter. Here, the dilution ratio for supernatant obtained from centrifugation was increased to 1/1000 for AAS detection and 1/5000 for spectrophotometric detection because of the high concentration of selenium species present in the samples.

Amending Bacterial Cultures with Elemental Se

A bacterial culture, TSN medium inoculated with *Pseudomonas fluorescens* K27, was prepared according to the experimental procedure described earlier. After 24 hours growth, 200 mL bacterial culture was poisoned by 0.1579 g of black elemental Se, and 100 mL bacterial culture was poisoned by 0.0780 g of red elemental sele-

nium prepared John Coffman (personal communication, Bob Buchanan, Berkeley, CA, 1994). The method of making this red selenium allotrope is covered in the end of this section. Both allotropes of elemental selenium were not sterilized. The poisoned bacterial cultures were kept in a water bath at 30 ^{0}C for 24 hours. Before these samples were analyzed by AAS and spectrophotometric detection with DAB reagent, the headspace of the bacterial culture was analyzed by gas chromatography with sulfur chemiluminescence detection (GC/SCD) system. The instrumentation and experimental procedure used here were adopted from Limin Zhang's thesis (Sam Houston State University, 1993). After that, both these two samples were separated into three equal portions and analyzed according to the AAS and spectrophotometric experimental procedures described earlier.

Preparation of Red Elemental Se

The red elemental selenium used above was made by John Coffman in our laboratory (personal communication, Bob Buchanan, Berkeley, CA, 1994). First, 50 mL of 2 M ascorbic acid solution (laboratory grade reagent, manufacturer unknown) was mixed with 50 mL of 0.2 M NaOH solution to make 0.1 sodium ascorbate solution. Second, 25 mL of 0.1 M sodium selenite solution was added to this clear salt solution without stirring. The pH value was adjusted to 2.5 by adding concentrated hydrochloric acid, and the solution appeared red at that time. Third, this solution was centrifuged at 15000 rpm for 20 min. After separation, the red precipitate was washed by DI water two times, then was lyophilized by liquid nitro gen and collected.

Chapter 3
Data

Calibration Curves for Selenium Analysis

Both calibration curves used for AAS and spectrophotometric analysis of selenium were produced during every experimental period. One of the AAS calibration curves is shown in Figure 5, and one of the calibration curves for spectrophotometric analysis is in Figure 6. Error bars in Figure 5 represent the one standard deviation of four replicate readings.

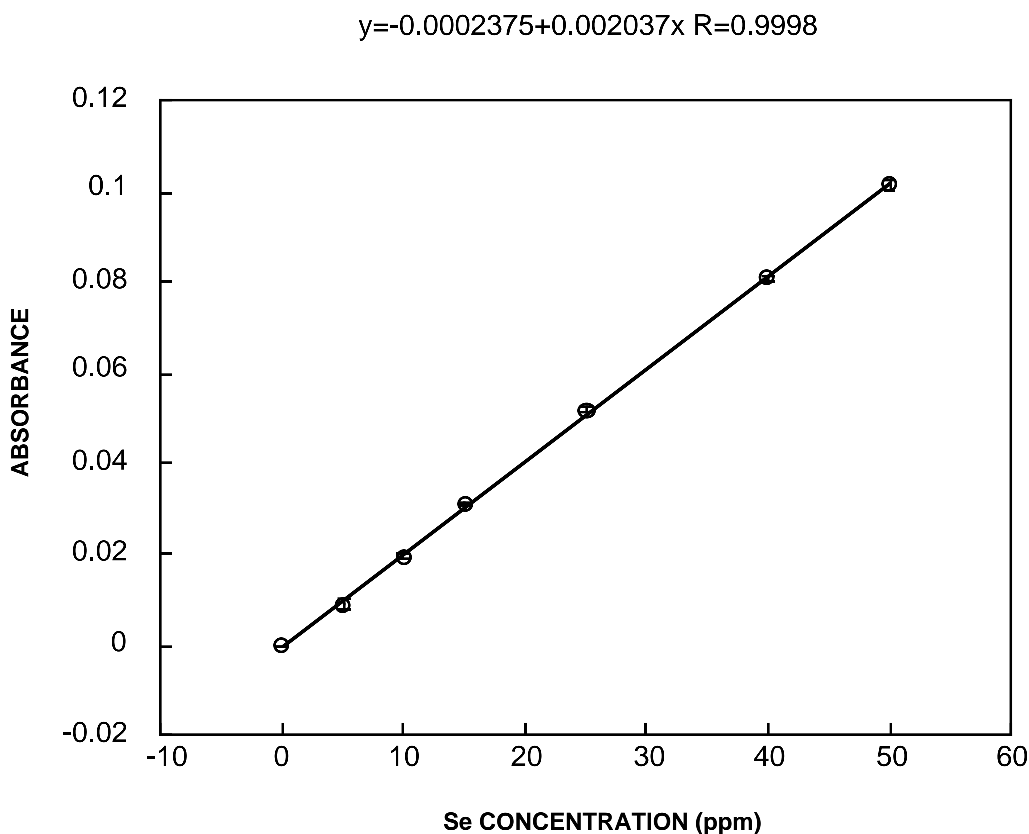


Figure 5. AAS calibration line for selenium.

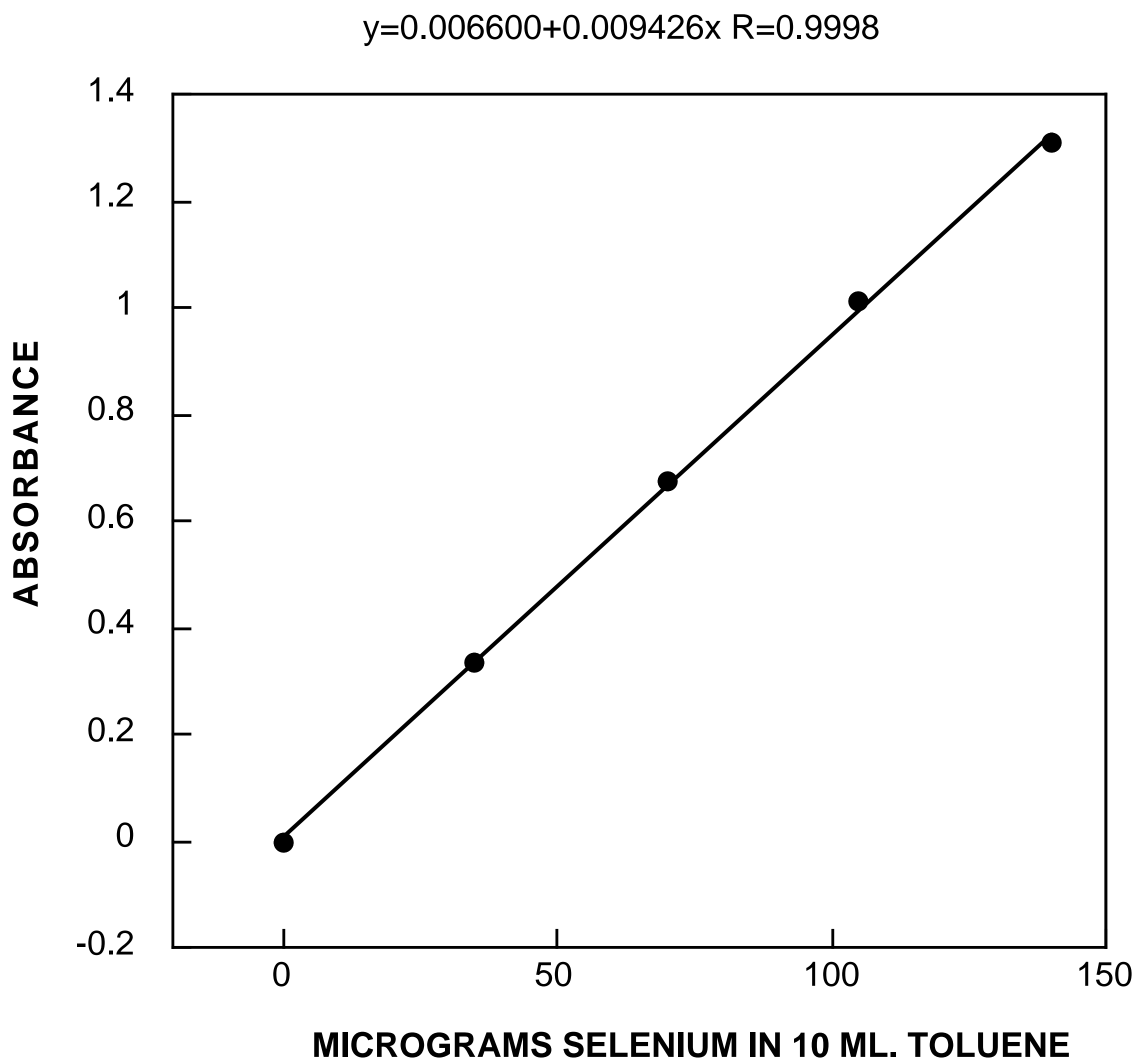


Figure 6. Calibration line for spectrophotometric analysis of selenite with DAB reagent.

Biological Sample Digestion Process Checked by Spike Addition

Four precipitate samples used here were obtained by separating one selenium poisoned sample into four equal portions and then going through the centrifugation process. The control samples here consisted of digestion acid equal to the concentration used in the other four biological samples. The recoveries were calculated by Equation (1). The results of this investigation are show in Table 1.

Table 1. The result of investigation on digestion procedure by spike addition. Zero g entries represent readings below the detection limit of approximately 5 X 10^{-5} grams.

	AMOUNT OF SPIKE	AMOUNT OF Se DETECTED BY AAS	RECOVERY
CONTROL #1	0 g	0 g	none
CONTROL #2	0 g	0 g	none
SAMPLE WITHOUT SPIKE #1	0 g	4.038X10^{-3} g	none
SAMPLE WITHOUT SPIKE #2	0 g	4.079X10^{-3} g	none
SAMPLE WITH SPIKE #1	0.0043 g	8.212X10^{-3} g	96.5%
SAMPLE WITH SPIKE #2	0.0034 g	7.804X10^{-3} g	110.2%

Determination of Matrix Effects

The graphic evaluation on matrix effect for dilutions of the supernatant using the standard addition method is shown in Figure 7, and its result is shown in Table 2. The graphic evaluation on matrix effect using serial dilution method is shown in Figure 8. Error bars represent the one standard deviation of four replicate readings.

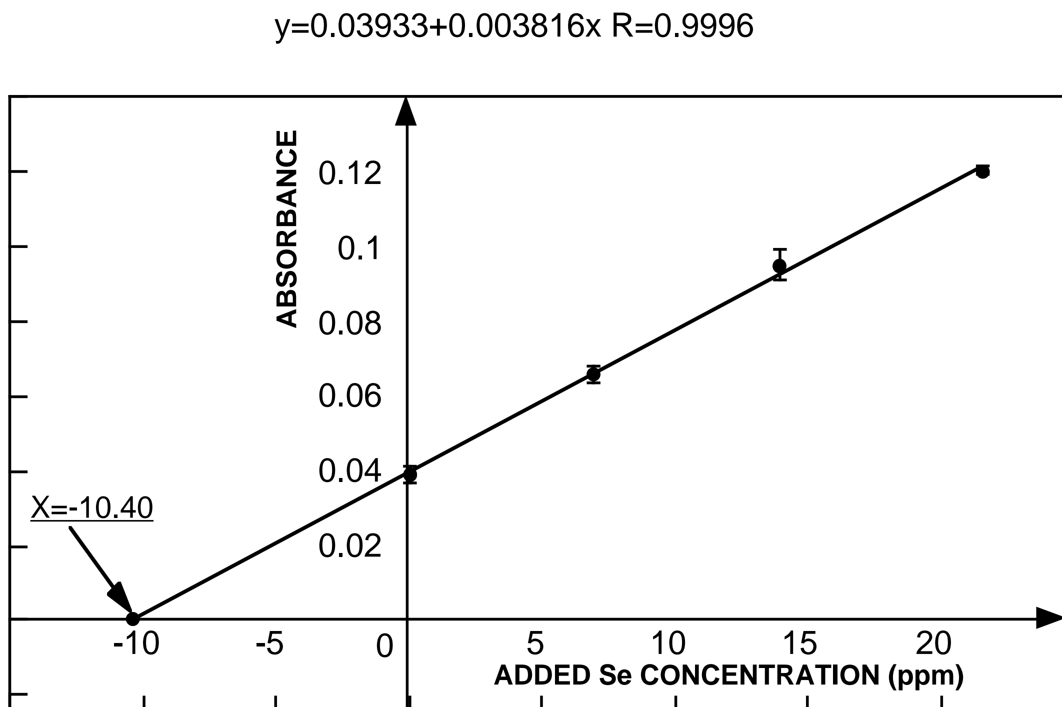


Figure 7. The matrix effect checked by standard addition method.

Table 2. The comparison between standard addition method and the method using AAS calibration curve only.

CONCENTRATION MEASURED BY STANDARD ADDITION METHOD	10.40 ppm
CONCENTRATION MEASURED BY CALIBRATION CURVE	10.26 ppm

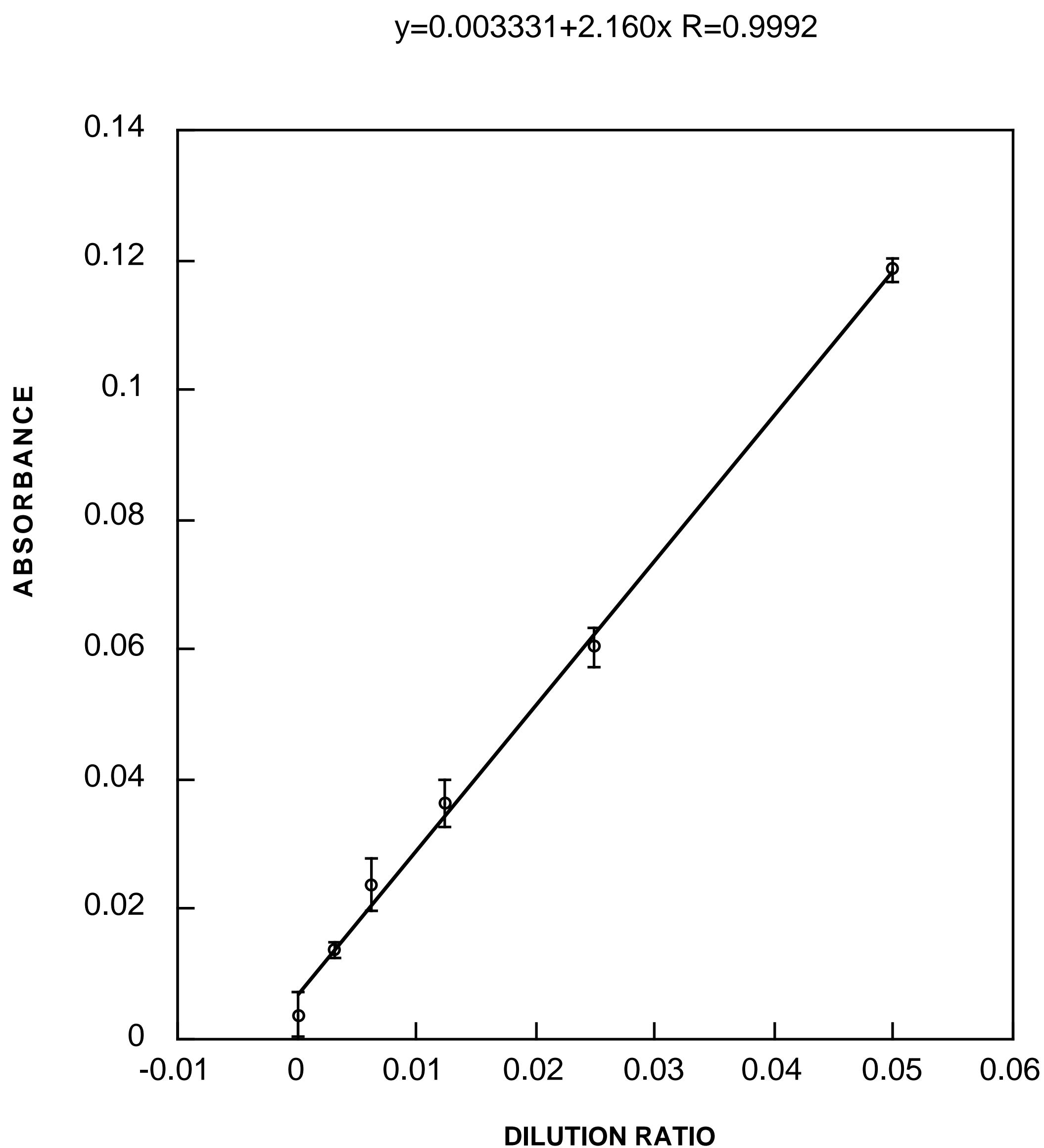


Figure 8. The graphic evaluation of matrix effect checked by serial dilution method.

Reduction of Selenate to Selenite by HCl and Spectrophotometric Measurement of Selenite with DAB Reagent

After original standard samples were taken through the reduction process with 6 M HCl, the total selenite was measured by spectrophotometric analysis with DAB reagent in 2 M HCl. The recoveries were calculated by equation (4):

$$\text{RECOVERY} = \frac{\text{TOTAL Se DETECTED AFTER REDUCTION}}{\text{TOTAL Se IN ORIGINAL SAMPLE}} \text{X100\%} \quad (4)$$

The results for averages of duplicated samples are shown in Table 3.

Table 3. Analytical results for selenite recovery by spectrophotometric analysis with DAB reagent after conversion of selenate to selenite in 6 M HCl. Zero µg entries represent that there was no selenate standard in the original samples.

ORIGINAL SAMPLE		TOTAL Se DETECTED (µg)	RECOVERY (%)
Se^{4+} (µg)	Se^{6+} (µg)		
35	0	35.54	101.5
70	0	70.18	100.3
35	35	72.09	103.0
140	0	138.11	98.7

The Comparison Between AAS and Colorimetric Measurement of Se

The amounts of Se for three different poisoned bacterial samples measured by AAS and colorimetric methods are presented in Table 4. The comparison between these two methods were evaluated by the ratio which was equal to the amount of Se measured by AAS divided by that measured using colorimetric method with DAB reagent.

Table 4. The comparison between AAS and colorimetric methods for selenium measurement.

	TOTAL Se MEASURED BY AAS	TOTAL Se MEASURE BY COLORIMETRIC METHOD	COMPARISON BETWEEN TWO METHODS (RATIO)
SAMPLE 1	8.042×10^{-2}g	7.979×10^{-2}g	1.007
SAMPLE 2	1.799×10^{-2}g	1.803×10^{-2}g	0.9978
SAMPLE 3	6.819×10^{-2}g	6.826×10^{-2}g	0.9990

Selenium Distribution

The results of all of the selenium distribution experiments for anaerobic and aerobic samples, including controls, are shown in Table 5. The percentage of added selenium that was volatilized was calculated by the difference between the added mass of selenium in control samples and that found in selenium poisoned samples through AAS and DAB analysis. Data for the single experiments involving the amendment of K27 cultures with two allotropic forms of selenium are in Table 6, and the data from the single experiment

completed using minimal medium are in Table 7. All data presented were the average results of triplicate samples.

Table 5. Results of selenium distribution in aerobic, anaerobic, and selenite and selenate poisoned K27 cultures and controls. All experiments detailed were for 24 hours growth after poisoning.

SAMPLE NAME	VOLATILE SELENIUM	Se^{4+}	Se^{6+}	ELEMENTAL SELENIUM
TSN+10 mM SeO_3^{2-} ANAEROBIC	0%	100%	0%	0%
TSN+K27+10 mM SeO_3^{2-} ANAEROBIC	0.05%	96.67%	0%	3.28%
TSN+10 mM SeO_3^{2-} AEROBIC	0%	93.57%	6.43%	0%
TSN+K27+10 mM SeO_3^{2-} AEROBIC	0.97%	96.16%	0%	2.87%
TSN+10 mM SeO_4^{2-} ANAEROBIC	0%	0%	100%	0%
TSN+K27+10mM SeO_4^{2-} ANAEROBIC	7.41%	0.23%	92.07%	0.29%
TSN+10 mM SeO_4^{2-} AEROBIC	0%	0%	100%	0%
TSN+K27+10 mM SeO_4^{2-} AEROBIC	7.89%	0.21%	91.59%	0.31%

Table 6. Results for delineation of TSN medium inoculated with K27 and poisoned with 10 mM red elemental Se and black elemental Se after 24 hours growth. Here, 10 mM added elemental Se is the nominal concentration and was calculated assuming that the elemental selenium dissolves in the culture. Actually it does not.

Sample	Volatile Se	Se^{4+}+Se^{6+}	Elemental Se	Recovery
TSN+K27+10 mM Se^0 (Red)	0	0	100%	92.9%
TSN+K27+10 mM Se^0 (Black)	0	0	100%	97.9%

Table 7. Results for delineation of selenium species in minimal medium, inoculated with K27, poisoned with 500 mM selenate after 24 hours aerobic growth and then analyzed after 24 hours further growth at 30 ^{0}C.

Sample	Volatile Se	Se4+	Se6+	Elemental Se
Minimum Medium *Pseudomonas fluorescens* K27 Amended w/500 mM Selenate	0.94%	0.89%	97.84%	0.33%

Chapter 4
Results and Discussion

Analytical Pathway for Selenium Delineation

Biological Precipitate Digestion

The wet ashing method using nitric acid appeared satisfactory for the biological precipitates containing red elemental selenium. From the data of spike addition experiments, we know that the average of the recoveries for duplicated samples with spikes was 103.4%; this value indicated there is little or no loss of selenium during the digestion with open containers. The clear solution obtained after adding hydrochloric acid into the digestion mixtures showed that the bacterial cells were totally destroyed and the elemental selenium completely oxidized to soluble selenium compound. This was an essential preparation and requirement for the following AAS analysis.

From the data presented in Table 1, we can also conclude that the acid used for sample digestion appears to have no significant interference on AAS detection of selenium. One fault with this process is that the selenium species in the bacterial cells can not be determined separately from the elemental Se, and they were digested together with the elemental selenium; therefore, the measured percentage of Se^0 in analyzed cultures could be higher than the true value, although the red elemental selenium appeared to be the major part of the precipitate pellets. The actual chemical form and valence state of selenium within K27 bacterial cell is not known based on our knowledge now.

Matrix Effect of Supernatant

From the matrix effect investigation by the serial dilution method, it was determined that the matrix effect was not significant in AAS analyses of selenium in dilutions of supernatant with dilution ratios from 1/20 to 1/320. Our dilution ratios of samples with 10 mM selenate or selenite for direct AAS analysis were within this range. This conclusion was double checked by standard addition method. The concentration of a 1/50 dilution of the supernatant was measured by both standard addition method and the method using only the AAS calibration line. The two obtained concentrations were very close to each other, and this also confirmed that the matrix effect of this sample dilution was not significant for AAS measurement.

Based on these experimental results, our measurements of Se in supernatant were determined by direct AAS analysis of diluted supernatant with dilution ratio from 1/100 to 1/25, where the absorbances were within the linear the range of AAS calibration curve. After that, the dilution ratio was used to calculated total amount of Se in original supernatant.

Quantitative Reduction of Selenate to Selenite and Colorimetric Analysis of Selenite with DAB Reagent

Brimmer *et al.* (1987) reported that the selenate anion can be quantitatively reduced to selenite in 6 M HCl. The analytical method they used was differential pulse polarography using a PARC polarographic analyzer with a PARC static mercury drop electrode. Our measurement of selenite using spectrophotometric analysis with

DAB reagent after selenate standard conversion to selenite also obtained the same result; that is, this reduction is quantitative. The data presented in Table 3 confirmed that the adopted reduction process and colorimetric method were suitable for our work.

Comparison Between AAS and Colorimetric Measurement of Se

The concentration of total selenium in supernatant samples measured by AAS was very close to that obtained by colorimetric method after quantitative reduction of selenate to selenite process. This is shown by the ratios in Table 4 that are very close to one. This result indicated the suitability of these two methods for the selenium analysis in our biological samples.

Selenium Distribution in Poisoned Bacterial Sample

Bacterial Culture poisoned with Selenium Oxyanions

The selenium distribution of bacterial sample poisoned with 10 mM selenite or selenate is presented in Table 5. The data here show that both selenate and selenite can be partially reduced to red amorphous elemental selenium in *Pseudomonas fluorescens* K27 bacterial cultures; also at the same time in the same cultures, volatile selenium compounds were evolved. The volatile Se species have been determined in part to be dimethyl selenide, dimethyl selenenyl sulfide and dimethyl diselenide by Chasteen (1990) and Zhang (1993).

Reduction and methylation of selenium oxyanions occurred in both aerobic and anaerobic conditions; it turns out that the K27 bacteria used in our experiments are active in both aerobic and

anaerobic environments. Also it was noticed that similar amounts of red elemental Se were found in both anaerobic and aerobic bacterial cultures poisoned with selenate or selenite (3.28% and 2.87% conversion to Se^0 for cultures poisoned with selenite; 0.29% and 0.31% for cultures poisoned with selenate); this means that O_2 is not a key variable in the selenium oxyanions reduction to elemental Se, when nitrate is the terminal electron acceptor—as is the case of anaerobic growth in TSN medium. It was found that the selenite poisoned cultures produced about 10 times as much red elemental Se as that produced by selenate poisoned culture with the same growing time. This may indicate that selenite is more easily reduced to elemental Se than is selenate; however, the volatile selenium species evolved from selenate poisoned samples were much more than those evolved from selenite poisoned samples. The same results were observed when methylated selenium species such as dimethyl selenide and dimethyl diselenide in the headspace of similar bacterial cultures were analyzed by GC/SCD system (Steve McCarty, unpublished experimental results). The small amount of selenite found in selenate poisoned sample suggests that the reduction of selenate into elemental selenium is at least in some small part a two-step reaction, in which selenate is reduced to selenite and then to red amorphous elemental selenium, although this reduction may occur at a cell membrane surface where all six reduction electrons are available.

In 24 hours experiments using TSN medium, it was found that some selenite was oxidized to selenate in aerobic sterile culture but not in anaerobic ones; atmospheric O_2 probably did this. This maybe significant in longer aerobic experiments. No selenate was

reduced to selenite in aerobic or anaerobic sterile TSN medium without K27 bacteria inoculation. Finally, and most obviously, the only experiments where volatile Se or elemental Se were found were in live bacterial cultures.

The experimental results of minimal medium poisoned with 500 mM selenate after 24 hours aerobic growth shows that K27 grows well aerobically and is active in this minimal medium. About the same percentage of red elemental Se was found in this culture as those TSN media poisoned with 10 mM selenate in anaerobic and aerobic condition; but the absolute amount of red selenium produced aerobically here were much more. The percentage of the volatile selenium species evolved in 500 mM selenate (minimal media) culture was about seven times less than those of the 10 mM selenate TSN samples; even so the absolute amount of volatile Se produced in the minimal medium sample was still much higher. The amount of volatile Se produced in K27 bacterial culture was not proportional to the amount of Se poisoning this culture. That is, 500 mM poisoned aerobic cultures did not produce 50 times as much volatile Se as 10 mM doped cultures.

Bacterial Culture poisoned with Elemental Selenium

There were not any volatile methylated selenium species found in the headspace of K27 bacterial culture poisoned with either 10 mM black elemental Se or red elemental Se; also, no oxidized selenium species were found in the supernatant after centrifugation of the samples. This apparently indicates that the elemental selenium can not be methylated to some volatile compounds such as

dimethyl selenide and dimethyl diselenide, and the elemental selenium amending in samples was not biologically affected by K27 bacteria in the time of this experiment—24 hours. This may suggest that the elemental Se is probably not the intermediate step of the methylation process of selenium oxyanions by *Pseudomonas fluorescens* K27, or more probably that bacterial access to insoluble Se precludes their remediation of the elemental form.

Conclusions

1. The only cultures that produced volatile selenium and elemental selenium in our experiment contained live aerobic or anaerobic bacteria.

2. Selenate is stable in sterile TSN media of aerobic and anaerobic environments at 30 ^{0}C for a 24 hour period.

3. Selenite amended cultures grown aerobically and anaerobically produced more elemental selenium than similar selenate amended cultures.

4. Selenate amended cultures grown aerobically and anaerobically produced more volatile selenium than similar selenite amended culture. This volatile organoselenium was determined by the difference between the mass of added selenium and the mass of selenium determined by AAS and DAB analysis.

5. Selenite is oxidized to selenate by atmospheric O_2 in sterile TSN media treated aerobically but unshaken, though the amount is relatively small in a 24 hours experiment (about 6%).

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Appendix

Chemical Abstract Service Registry Numbers

Reagent Name	CAS Registry Number
Ammonium hydroxide	1336-21-6
Ammonium chloride	12125-02-9
Ascorbic acid	50-81-7
Cupric chloride	13468-85-4
3,3'-Diaminobenzidine	91-95-2
Ferrous sulfate	7782-63-0
Glucose	50-99-7
Hydrochloric acid	7647-01-0
Magnesium chloride	7786-30-3
Magnesium sulfate	10034-99-8
Nitric acid	13587-52-5
Potassium nitrate	7757-79-1
Potassium phosphate	7778-77-0
Selenium (powder)	7782-49-2
Sodium hydroxide	1310-73-2
Sodium selenate	13410-01-0
Sodium selenite	10102-18-8
Toluene	108-88-3

Vita

Wenbiao Jiang was born in Shanghai, P.R. China on Feb 25th, 1969. He graduated from Nanyang High School in Shanghai in 1987. Wenbiao received a Bachelor of Engineering degree of Environmental Engineering in Chemistry and Chemical Engineering Department from Shanghai University of Technology in July, 1991. In August 1992, he entered the chemistry graduate program at Sam Houston State University in Huntsville, Texas. He received his Master of Science degree in Chemistry Department in December, 1994.