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Amending Cultures of Selenium-resistant Bacteria with Dimethyl Selenone

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A possible biological intermediate in the reduction and methylation of selenium oxyanions, dimethyl selenone, was synthesized, and the first experiments involving the amendment of selenium resistant bacterial cultures with this compound are reported. The amount of volatile, reduced selenium-containing species released from these cultures into the headspace is significantly more than that produced in analogous experiments involving sodium selenate amended cultures. Dimethyl selenone is reduced in the presence of dimethyl sulfide and dimethyl disulfide in a complex growth medium, trypticase soy broth with 0.1% nitrate. This reduction occurs whether or not the reduced sulfur compounds are biologically produced.

Keywords: Amendment, biomethylation, bioremediation, dimethyl selenone, headspace analysis, fluorine-induced chemiluminescence

INTRODUCTION

The first clearly described mechanism for the biological reduction and alkylation of selenium oxyanions such as those of selenious acid, selenite and selenate was proposed in the 1940s. Work with analogous arsenic compounds led to the proposition that these oxyanions proceed through a series of intermediates in microbial cultures that ultimately lead to dimethyl selenide in the case of selenium oxyanions or trimethyl arsine in the case of arsenic.[1,2] Though none of the intermediates had been detected as produced by microorganisms (in molds of *Scopulariopsis brevicaulis*), when some of these suggested arsenic intermediates were added to bread molds containing these microbes, $(CH_3)_3As$ was qualitatively detected,[3] tentatively confirming the proposed mechanism.

Challenger and coworkers investigated the proposed selenium mechanism by examining cultures amended with a possible intermediate, methane seleninic acid (among others), and in these experiments they qualitatively detected CH_3SeCH_3 as it was produced using mercury chloride (and other) derivatives.[4,5] Challenger noted in the 1945 review of this work that dimethyl selenone, an intermediate in the selenium scheme, had not been prepared up to that point.[1]

It was another few decades before more information was available concerning the intermediates in the reduction and methylation of selenium. Beginning in the mid-1970s and through the late 1980s, research indicated that a compound detected in the headspace above microbial cultures in the laboratory[6,7] and environments in the field[8,9] might be dimethyl selenone; however, this has recently been called into question based on boiling point, mass spectral and chromatographic data. Instead, this volatile unknown is probably dimethyl selenenyl sulfide, $(CH_3)_2SeS$, which may be created as a biological, disproportionation or exchange product in the culture or headspace of these systems.[10]

Although the detection of dimethyl selenone as an intermediate produced in the reduction and alkylation of oxidized selenium salts has yet to be convincingly accomplished,[10] this does not mean that it does not in fact act as a chemical intermediate in this process. The work described here was designed to pursue this possibility from a different angle than the detection of $(CH_3)_2SeO_2$ as it is produced by bacterial cultures. Instead of trying to detect this species, as an *in situ* intermediate in microbial reduction and methylation, we have synthesized dimethyl selenone, added it to growing, selenium-resistant cultures of *Pseudomonas fluorescens* bacteria (known producers of alkylated selenium compounds), and examined the headspace for reduced selenium species that are normally created by these cultures. This is the first report of experiments involving selenium resistant bacterial cultures purposely exposed to

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dimethyl selenone. These semi-quantitative results shed light on the possibility that Challenger did indeed postulate correctly the path from an oxidized salt to a more volatile, reduced, alkylated product. Experiments were also carried out to determine whether dimethyl selenone could be reduced by other chemical species produced in anaerobic bacterial cultures.

EXPERIMENTAL

Materials

Sodium selenate and selenite were purchased from Sigma Chemical Co., St. Louis, MO, USA; dimethyl selenide (DMSe) from Strem Chemicals Inc., Newburyport, MA, USA; and 3-chloroperoxybenzoic acid (50–60%), methylene dichloride, methanol, acetonitrile and diethyl ether from Aldrich Chemical Co., Milwaukee, WI, USA. Materials for the culture media included trypticase soy broth purchased from Difco Laboratories, Detroit, MI, USA, supplemented with 0.1% potassium nitrate (Aldrich). Other chemicals and purchased chromatographic standards included dimethyl diselenide (DMDSe; Strem), dimethyl sulfide (DMS, Aldrich), and dimethyl disulfide (DMDS, Aldrich); all were used as received.

Methanethiol and dimethyl selenenyl sulfide were prepared and the retention time of these compounds determined as reported elsewhere.[10] Dimethyl trisulfide (DMTS) was prepared following the method of Milligan and coworkers,[11] and the retention time determined with a gas injection from the headspace above a diluted DMTS/acetonitrile solution.

Synthesis of dimethyl selenone

The synthetic method of Krief and coworkers[12] was followed (with small modifications) for the preparation of $(CH_3)_2SeO_2$. Approximately 11 g of 3-chloroperoxybenzoic acid (2.5 mol equiv. based on 50% purity) dissolved in 5 ml of methylene chloride was added to 1.41 g (0.013 mol) of dimethyl selenide that had been dissolved in 20 ml of methylene dichloride. This reaction mixture was stirred for 2 h then evaporated to dryness. The by-product of the reaction, 3-chlorobenzoic acid, was separated from the crude dimethyl selenone by repeated extractions with ether. The acid dissolved into the ether layer leaving $(CH_3)_2SeO_2$ behind. In the final extraction, crude dimethyl selenone was separated from ether by vacuum filtration. The product was recrystallized twice from HPLC grade methanol.

Table 1 Chromatographic and detector conditions

Gas chromatograph	
Carrier	He, 1 ml min^{-1}
Injector	275 °C
Temperature program	−20 °C for 1 min (LN_2 cryogen)
	−20 to 20 °C at 8 °C min^{-1}
	20–200 °C at 15 °C min^{-1}
Injection modes: splitless	—
split	Split ratio 1/65
Detector	
Transfer line	214 °C
Reaction cell pressure	<1 Torr
Source of F_2	AC discharge of SF_6 reagent gas
SF_6 Flow	~1 sccm

Chromatographic procedures

Table 1 lists the chromatographic and detector conditions used in this work. The gas chromatograph (GC) used for this work was a Hewlett-Packard model 5890 Series II with a 30 m, 0.32 mm i.d. capillary column. The column had a SE-54 1.0 µm, relatively non-polar chromatographic film (Alltech Associates Inc., Deerfield, IL, USA). The detector system used was a Sievers Research Sulfur Chemiluminescence Detector Model 300. The analog signal from the detector was processed by a Hewlett Packard 3396 Series II integrator.

Two 1.0 ml gas-tight syringes (Model A-2, Dynatech Precision Sampling Corp., Baton Rouge, LA, USA) were used alternately for sampling headspace gases. These syringes were cleaned between every injection by purging with nitrogen while being heated to 50 °C. Lab air was injected into the GC with a cleaned syringe between sample runs to confirm the cleaning process.

Retention times from gas samples of the headspace above chemical standards were used for identification of all of the compounds available from vendors except in the case of CH_3SH, CH_3SeSCH_3 and CH_3SSSCH_3 which were prepared and used as described. The compound eluting between dimethyl disulfide and dimethyl diselenide in this work is assumed to be dimethyl selenenyl sulfide and had a retention time identi-

cal to that of the gas phase product of a mixture of DMDS and DMDSe that produces CH_3SeSCH_3.[10] We have always found in our work with this chromatography that this peak is only seen in cultures amended with selenium species; however, a gas chromatography/mass spectrometry system (used in earlier work) was not available to additionally confirm the identity of this compound.

Freshly recrystallized dimethyl selenone was dissolved in acetonitrile and 1 μl of a 1 ng μl^{-1} solution ws analyzed by GC with the standard temperature program final temperature, described in Table 1, extended to 300 °C for five minutes. Because of the high melting point of this compound, larger masses of dimethyl selenone were not injected in order to protect the chromatographic column.

Culture growth and amendment

Pseudomonas fluorescens K27 is a selenium resistant bacteria isolated from the Kesterson Reservoir in the San Joaquin Valley, CA, USA.[13,10] This facultative anaerobe reduces and alkylates sulfur and selenium during anaerobic growth. Three different media were used to attempt to grow *Pseudomonas fluorescens* K27. The first two were aqueous minimal media containing only (1) salts, trace metals and glucose as a carbon source[14] and (2) salts, trace metals and succinate as the carbon source.[15] Both were supplemented with 1 g l^{-1} potassium nitrate as terminal electron acceptor. The growth of *Pseudomonas fluorescens* K27 on either of the minimal medium was not detectable when inoculated from live agar plates and subsequently incubated at 30 °C for four days. No further work was undertaken to grow K27 on minimal media.

The third medium used (hereafter referred to as TSN) was prepared with 10 g l^{-1} trypticase soy broth with 1 g l^{-1} potassium nitrate. This media had been used before for growing this microbe.[16] Fifteen ml glass test tubes containing 5 ml sterile media were inoculated with bacteria from an agar plate. The tubes were sealed with Teflon® lined septa in screw caps with holes. Cultures were incubated for 24 h at 30 °C and then known amounts of either sodium selenate or dimethyl selenone were added in 5 ml of filter-sterilized media and then the tubes resealed. This brought the final volume of cultures in the sample tubes to 10 ml. The headspace of the tubes was sampled 24 h after being amended. Procedures using dimethyl selenone to amend bacterial cultures were performed on the same day that dimethyl selenone was recrystallized. Media blanks with only sterilized TSN media and bacterial culture blanks with only sterilized media inoculated with *Pseudomonas fluorescens* K27 were run with every experiment as controls.

Experiments with TSN media were carried out using two different types of media sterilization: high temperature autoclaving (120°C/16 psi for 30 min) and sterile filtering (through 0.22 μm pore filters, MSI, Westboro, MA, USA). All tubes, caps and septa were autoclaved before use.

Alkylated selenium/sulfur redox experiments

Experiments designed to determine the possibility of selenate, selenite or dimethyl selenone reduction by reduced sulfur compounds were carried out in the following manner. Three solutions, each containing only one of these selenium compounds, were made at a concentration of 10 mM in TSN medium. One nanogram each of dimethyl sulfide and dimethyl disulfide were added to each of these solutions from diluted stock solutions. The final volume of each solution was 10 ml. (A 1 ng addition of the sulfur species was chosen because it produced DMS and DMDS peak areas roughly comparable to those routinely seeen in healthy K27 cultures). Each mixture was then filter-sterilized and placed into the identical sampling tubes described above for the cultures, sealed with septa and screw caps, and kept at 30 °C in a thermostatic water bath for 24 h before the headspace was sampled.

Headspace analysis

One ml of the headspace from the sample tubes was removed using a gas tight syringe and immediately injected into the hot injector of the GC. Cryogenic trapping of the chemical species occurred at the head of the column in the −20 °C liquid nitrogen cooled oven for 1 min before the temperature ramp began.

For samples with high concentrations of headspace gases, known volumes less than 1.0 ml were removed and injected into the GC. These smaller volumes were required to avoid overloading the capillary column; however, all of the results reported were multiplied by the correct factor to produce peak areas normalized to a headspace sample size of 1.0 ml, i.e. 1.0 ml of gas removed from a sample's headspace and injected.

One ml of the headspace gas above dimethyl selenone crystals kept in a closed gas vial at room temperature was analyzed by GC with the temperature program extended to 300 °C.

RESULTS AND DISCUSSION

Characterization and detector response of synthesized dimethyl selenone

Dimethyl selenone was successfully synthesized and was characterized by melting point,[17] NMR, FTIR[18] and elemental analysis.

GC injections of dimethyl selenone in acetonitrile yielded no chromatographic peaks using an injected dimethyl selenone mass (1 ng) approximately 100 times the nominal detection limit for this family of selenium containing compounds, about 10 pg on-column. One ml of the headspace gas above solid $(CH_3)_2SeO_2$ also gave no response. This again confirms that dimethyl selenone does not elute between dimethyl selenide and dimethyl diselenide in non-polar chromatographic systems as had been suggested.[7–9] In fact, as noted elsewhere,[10] the melting and boiling points of this compound probably preclude its elution from these sorts of chromatographic conditions.

Products of selenone amendment to bacterial cultures

The detection of the production of reduced, alkylated selenium compounds by *Pseudomonas fluorescens* is not novel and was indeed the reason why this organism was chosen for this work.[6,19,13,10] Our experience with this bacterium shows it to be able to reduce and methylate both selenate and selenite salts reproducibly when grown on a complex medium such as TSN. Previous work with this bacterium demonstrated that using TNS medium and a 30 °C culture temperature, this organism will grow into the stationary phase within a few hours.[20] Work in our laboratory, using optical density as a measure of culture growth, indicates that even when lag phase cultures are amended with as much as 10 mM sodium selenate (at 30 °C), arrival in the log phase is only slightly delayed and stationary phase growth is achieved within 6 h. All of the experiments described here were from cultures grown into a stationary phase, amended with selenate or dimethyl selenone and additional fresh medium and then incubated at 30 °C for 24 h before sampling. These, therefore, represent the analyses of headspace above stationary phase cultures.

The initial experiments with *Pseudomonas fluorescens* cultures amended with dimethyl selenone produced headspace concentrations of DMSe and DMDSe greater than we have ever seen in our work with selenium resistant microorganisms. All of our initial injections produced extremely overloaded chromatograms; therefore, it was necessary to determine a method of dilution in our static headspace sampling procedures that would allow us to roughly quantify the peak areas produced. Our subsequent experiments achieved a dilution of the amounts of sample injected on column by first injecting less than 1 ml of headspace (but never less than 0.1 ml, i.e. 10% of the volume of our gas syringe), and second, by using the split injection mode of our chromatogram and multiplying the integrated peak areas by the split ratio (see Table 1). As noted, these dilution factors were taken into account when calculating and comparing peak areas.

Table 2 is a listing of compound and normalized peak area data from seven different headspace sampling experiments: (1) TSN sterile media blank; (2) sterile TSN with 10 mM dimethyl selenone; (3) *P. fluorescens* K27 grown on TSN; (4) K27 grown on TSN and amended with 1 mM sodium selenate; (5) K27 on TSN amended with 1 mM dimethyl selenone; (6) sterile TSN + 10 mM sodium selenate + 1 ng DMS + 1 ng DMDS added; and (7) sterile TSN + 10 mM sodium selenite + 1 ng DMS + 1 ng DMDS added.

The detection of small amounts of methylated sulfur compounds in the headspace of autoclaved media blanks first led us to try growing K27 on minimal media containing only sulfate as a sulfur source. These cultures repeatedly failed to grow and ultimately we relied on the low temperature/filter-sterilization method as a means of eliminating reduced sulfur compounds in the blank headspaces. Headspace samples above sterile-filtered TSN, incubated for 24 h at 30 °C, showed no volatile sulfur species above our detection limits (see Table 2). The origin of these alkylated species in autoclaved TSN medium is not known but the sulfur containing amino acids present in trypticase soy broth are an obvious possible source. We found these volatile sulfur species using different batches of trypticase soy broth whenever autoclaving was used. An experiment in which

Table 2 Normalized integrated peak areas from the chromatographic analyses of headspaces samples containing volatile, reduced selenium and sulphur species

Sample	DMS	DMSe	DMDS	DMSeS	DMDSe	DMTS
Sterile TSN media	0	0	0	0	0	0
Sterile TSN media 10 mM $(CH_3)_2SeO_2$	0	0	0	0	0	0
K27 Blank	2×10^6	0	6×10^6	0	0	3×10^6
K27 on TSN media 1 mM SeO_4^{2-}	4×10^6	2×10^7	6×10^7	6×10^7	1×10^8	1×10^7
K27 on TSN media 1 mM $(CH_3)_2SeO_2$	3×10^9	1×10^{11}	6×10^8	2×10^{10}	1×10^{11}	0
Sterile TSN 10 mM SeO_4^{2-} 1 ng DMS 1 ng DMDS	5×10^7	0	2×10^7	0	0	2×10^6
Sterile TSN 10 mM SeO_3^{2-} 1 ng DMS 1 ng DMDS	7×10^7	0	2×10^7	0	0	0

TSN medium was held at a 120 °C/16 psi for 3 h (instead of 30 min) increased the concentration of these species in the medium headspace; therefore, thermal degradation does play a part in the appearances of reduced sulfur species in this autoclaved media.

The question of whether or not the reduction of dimethyl selenone was merely due to the degradation in or reaction with TSN medium components was answered by an experiment in which a sterile 10 mM $(CH_3)_2SeO_2$ TSN solution was treated as a blank without any bacteria added. Using autoclaved media, reduced selenium species were detected; however, after eliminating the reduced sulfur species produced by autoclaved-induced media decay, filter-sterilized dimethyl selenone amended TSN blanks showed no chalcogen containing peaks (see Table 2).

Comparison between sodium selenate and dimethyl selenone amended cultures

Fig. 1 contains two chromatograms from headspace samples above K27 bacterial cultures samples incubated for the same amount of time after being amended. Chromatogram A was from the headspace above a K27 culture amended with 1 mM sodium selenate; the volume of gas sample injected (splitless) was 0.5 ml. Chromatogram B was from the headspace above a K27 culture with 1 mM dimethyl selenone added; the volume of sample injected (split) was 0.2 ml. The difference between these two chromatograms is most striking with regard to the size of the DMSe and DMDSe peaks. The selenone doped culture produced much larger amounts of these two species. This difference is even more pronounced when taking into account the difference in syringe volumes used and the fact that the volume of gas injected on-column from the selenone doped culture is 1/65 than of the selenate amended culture, ie. the dimethyl selenone chromatogram was from a split injection and the selenate chromatogram was splitless. In fact, the selenone chromatogram is still overloaded and the integrator probably underestimated the peak areas of the two selenium containing species. The ratio of on-column sample in chromatogram A to sample in chromatogram B is 162.5. Put another way, the chromatogram of the dimethyl selenone amended culture displayed much larger amounts of headspace selenides even when the headspace sample injected on-column was 1/162.5 of that from the selenate amended culture. Normalized data from similar chromatograms are listed in Table 2 for

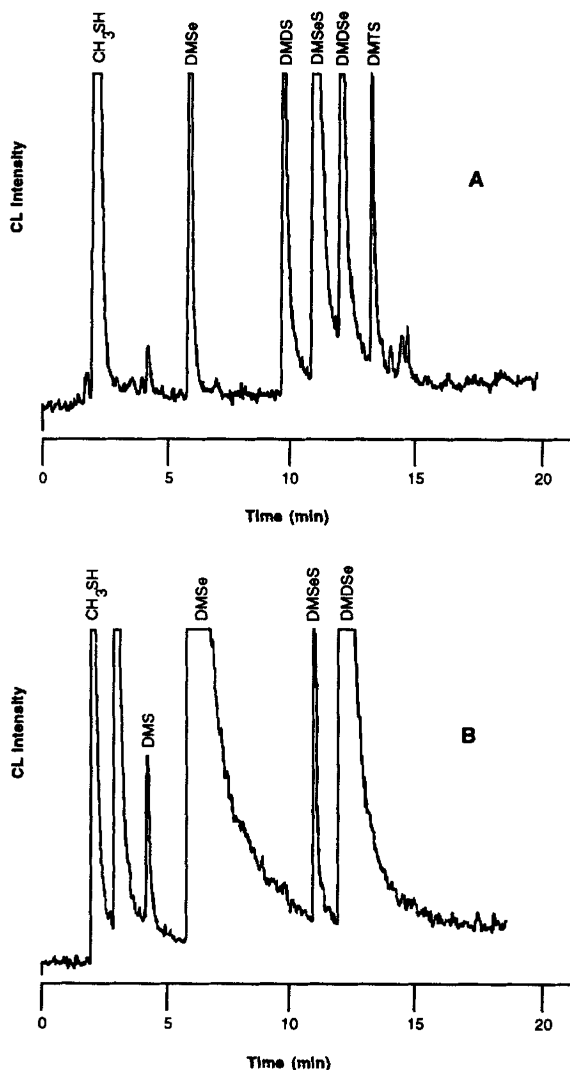

Figure 1 Chromatograms of the headspace above *Pseudomonas fluorescens* K27 amended with 1 mM sodium selenate (A) and 1 mM dimethyl selenone (B). The headspace sample for (B) has been diluted by a factor of 162.5 relative to (A). See text for dilution details.

K27 amended with Na_2SeO_4 and K27 amended with $(CH_3)_2SeO_2$. The ratio of the two dimethyl selenide peaks (selenone amended/selenate amended) is 5000 and for the diselenide peaks this ratio is over 1000.

The large differences between these amounts of selenium compounds in the different headspace samples cannot simply be caused by variability between different cultures grown in different tubes. As a comparison, the percent relative standard deviation of DMSe and DMDSe peaks among K27 samples amended with 1 mM sodium selenate and analyzed as described was 118% and 79%, respectively ($n=4$). The high relative standard deviation between headspace constituents of replicate bacteriological cultures is not unexpected. These are not static systems; they are living assemblages involving complex interactions of temperature, media and growth rates. We think that the replicate variability reported for the unamended cultures is therefore not surprising. This is an unusual datum, unavailable in the literature as far as we can tell; however, that experiment was only reported in order to show the significance of the extreme differences in the headspace components' peak area ratios between cultures amended with the same concentrations of selenate or selenone (factors of 1000 to 5000 for DMSe and DMDSe, respectively). These would be ratios of 2 or 3 if culture variations were the only reason for the variance.

Sodium selenite amended K27 cultures produced slightly different results as far as which chalcogen containing peaks were most prominent; however, the magnitude of the reduced selenium species detected was comparable to the selenate experiments and again, much less than those of the selenone amended cultures.

Interaction between sulfides and dimethyl selenone in complex culture medium

The results of the above set of experiments suggested that the next step was to determine whether or not the reduction of dimethyl selenone could be brought about solely by a chemical reduction of $(CH_3)_2SeO_2$ by the reduced sulfur species always produced in K27 cultures in TSN (see K27 blank in Table 2); these sulfur species are presumably produced via reduction of sulfur containing amino acids in TSN. To this end, dimethyl selenone amended TSN solutions [10 mM $(CH_3)_2SeO_2$] were additionally doped with 1 ng DMS and 1 ng DMDS from standards and then filter-sterilized. These solutions were kept at 30 °C for 24 h as before. The resulting chromatogram, Fig. 2, shows that DMSe and DMDSe are both present in this headspace; however, the amounts released into the headspace were much less than those of bacterial cultures when taking into account the fact that this chromatogram comes from 1.0 ml headspace gas injected in splitless mode. It should be noted that the $(CH_3)_2SeO_2$ amount added to this sample was 10 times greater than that added to the sample

producing the chromatogram of Fig. 1(b) and that reduced sulfur species are still present in the headspace.

Finally, analogous experiments were carried out to determine whether or not DMS and DMDS had sufficient reducing power in TSN medium to reduce either selenate or selenite and produce alkylated selenium products into solution headspace. The chromatograms from these experiments showed no volatile, reduced selenium species in headspace—only the added DMS and DMDS were detected. These are the final two experiments listed in Table 2.

These data indicate that dimethyl selenone is relatively stable in sterile TSN medium and that this sterile chemical environment does not reduce $(CH_3)_2SeO_2$ when no reduced sulfur species are present. The presence of reduced sulfur species in sterile TNS solutions containing dimethyl selenone does produce dimethyl selenide and dimethyl diselenide (Fig. 2). The only source of selenium in these species is from the added selenone and the reduction is hence ultimately a function of the contribution of electrons from the reduced sulfur species to the selenium moiety of the selenone, though some of the TSN material may play a (catalytic) role. A decrease in the DMS and DMDS concentrations in culture headspace when selenone is added might therefore be expected to occur as these reduced sulfur compounds are consumed by reducing the more oxidized selenone. This would suggest that the reduction of dimethyl selenone is only an inorganic chemical interaction between $(CH_3)_2SeO_2$ and DMS and/or DMDS; however, the greater amount of volatile, reduced selenium species in live bacterial cultures as compared to those only amended with reduced sulfur species [Fig. 1(b) compared to Fig. 2] suggests that the presence of live bacteria do play a part in the production of reduced selenium species in these environments.

The final step in the reduction and methylation of selenium oxyanions may be a combination of biological reduction with electrons supplied by a metabolic process and an "inorganic" reduction by electrons supplied by reduced sulfur compounds, also microbially produced. This conclusion can be reached in the light of previous work with aerobic microbial systems amended with sodium tellurite and sodium selenite. In these experiments, volatile, reduced selenium and tellurium compounds were detected without volatile reduced sulfur species being present in the headspace.[16] In that system it appears that microbial reduction is the only source of electrons.

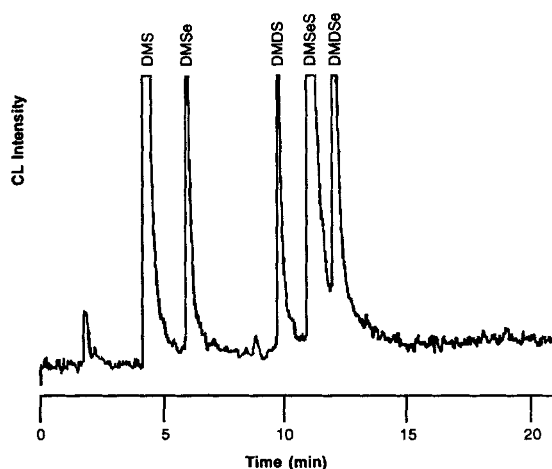

Figure 2 Chromatogram of headspace above sterile TSN medium with 10 mM dimethyl selenone and 1 ng dimethyl sulfide and 1 ng dimethyl disulfide added.

The headspace decrease of volatile sulfur species was, in fact, seen when comparing DMS and DMDS peak areas in 10 mM selenate and 10 mM selenone amended cultures in our experiments (data not shown). However, since this effect was not detected in cultures amended with concentrations below 10 mM, this may be solely due to the (higher) toxicity of dimethyl selenone as compared to sodium selenate. This might reflect the lower bacterial production of sulfides by a less healthy culture. We propose that if dimethyl selenone were a biological intermediate its creation might be the rate limiting step in the reduction and alkylation of oxyanions of selenium. As such, its concentration in bacterial cultures might never rise to that of our 10 mM $(CH_3)_2SeO_2$ amended cultures and therefore its toxicity might not severely affect culture growth.

We have also amended cultures of phototrophic non-sulfur purple bacteria[21] with dimethyl selenone. The preliminary results are similar to those with *Pseudomonas fluorescens* K27: the headspace analyses above these anaerobic bacteria (incubated in light) yield large amounts of dimethyl selenide and dimethyl diselenide. This suggests that the reduction of dimethyl selenone in anaerobic bacterial cultures is not limited to a bacterial genus, species or even particular strain (like K27, isolated from selenium rich environment) and is not limited to a particular physiological group of bacteria but occurs under chemotro-

phic as well as under phototrophic growth conditions.

CONCLUSIONS

Dimethyl selenone is relatively stable in sterile TSN medium. Dimethyl selenone does not produce a response in this chromatographic system at masses 100 times the detection limits for the other reduced alkylated selenium compounds determined in this work. This is expected because the high boiling point of $(CH_3)_2SeO_2$ probably precludes its elution from a gas chromatographic system at these temperatures.

Anaerobic bacterial cultures grown in this medium, when amended with dimethyl selenone, release volatile, reduced selenium compounds into the headspace in concentrations significantly greater than analogous experiments using sodium selenate or sodium selenite. However, since reduced sulfur species, specifically dimethyl sulfide and dimethyl disulfide also reduce dimethyl selenone in TSN medium, the production of DMSe or DMDSe cannot be clearly attributed to a strictly biological mechanism. In fact, it is probable that both biological and chemical reduction is occurring. Since these reduced sulfur species are themselves produced via microbial reduction and methylation in TSN media, the distinction between strictly chemical and biological reactions may be irrelevant from an energy standpont: whether the electrons that reduce oxyanions of selenium come directly from electron transport systems in bacterial membranes or from reduced sulfur intermediates, they have ultimately come from the reducing power of the organism, which thereby benefits by decreasing the concentration of toxic selenium species by the volatilization and removal of these components from the immediate environment. These microbes may also benefit by the regeneration of oxidized sulfur compounds which can then be used as an electron sink by these organisms.

With reference to the validity of amending bacteriological cultures in order to test the possible veracity of a proposed intermediate: this method was a mainstay of Challenger's research in the field of metalloidal metabolism fifty years ago, and in our opinion was the first step that had to be taken once dimethyl selenone became available to those working in this field. While it is obvious that the results of these experiments cannot be held out as confirmation of the existence of an intermediate, we have not done so; we only conclude that in the light of our results it does seem more probable that *if* dimethyl selenone were biologically created in bacterial cultures it would be easily reduced to selenides by other components in the culture, be they inorganic or biological.

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