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Bacterial Methylation of Selenium

Wen-Ing Hwang

BACTERIAL METHYLATION OF SELENIUM

BY

WEN-ING HWANG

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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.

Major adviser Date

Head, Chemistry Department Date

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

For several decades, excessive levels of selenium in feeds have been known to cause various toxic symptoms in livestock. Excessive selenium levels in feeds are the result of plants growing on soils which contain significant levels of available selenium. The natural cycling process by which selenium becomes available appears to involve various chemical weathering processes. In addition, microbial action may play either a direct or indirect role in rendering selenium soluble (1).

The extent to which microorganisms are involved in the natural cycling processes has been subject to considerable discussion. It has been demonstrated that mold can convert selenate or selenite to a volatile substance with a garlic-like odor. The substance has been identified as dimethyl selenide (2). Other workers (3,4) showed that the addition of selenite or selenate to soils resulted in the volatilization or loss of selenium. The volatilization was shown to be due to microbiological action, but the identity of the organisms was not determined.

Unpublished work from South Dakota State University has indicated that bacteria might also be involved in the methylation of selenium. Recently, other workers (5) have isolated one coryneform bacterium which can convert inorganic selenium to dimethyl selenide. This is not necessarily surprising since bacteria have been shown to be involved in the methylation of mercury and arsenic (6,7). Since little is known

concerning the actual involvement of bacteria in the natural cycling of selenium, this study was undertaken for the following purposes:

1. To isolate and identify soil bacteria which are capable of converting inorganic selenium to volatile compounds.
2. To identify the volatile selenium compound(s) generated by the isolated bacteria.

LITERATURE REVIEW

In the 1930's, selenium was identified as a potent toxic substance for cattle and other livestock (8). This focused attention on the biochemical properties of selenium compounds. The toxicity of selenium has been discussed in several reviews (1,9,10).

Simultaneously, work began accumulating which showed the element might have other biological effects. A few higher plants, such as Astragalus, accumulate relatively large amounts of selenium. Some of the Astragalus species, when cultured in the laboratory in solution or moist sand, exhibited markedly improved growth in response to the addition of selenium (11).

In 1957, selenium was identified as the essential component of a dietary factor that protected rats from severe necrotic degeneration of the liver (12), this finding led to the recognition by animal nutritionists that several important livestock diseases were actually selenium deficiency syndromes (13). The element has now been approved for addition to the diets of swine and poultry (14).

Until recently, little was known about the specific role or roles of selenium. It is becoming increasingly evident that animals, bacteria, and possibly higher plants all require trace amounts of selenium. When available in the proper amounts, the selenium is incorporated in a highly specific fashion into certain functional proteins of the cell (15). Three enzyme-catalyzed reactions have been shown to require the participation of a selenium containing protein (15);

these are the reactions catalyzed by formate dehydrogenase of bacteria, glycine reductase of clostridia, and glutathione peroxidase of erythrocytes.

As early as 1934, Challenger and North (16) showed that two strains of Scopulariopsis brevicaulis could convert selenite or selenate to a volatile selenium compound, which the authors assumed to be dimethyl selenide (DMSe). In later work (2), the identity of the volatile product was confirmed to be DMSe. Challenger (17) presented a pathway for the formation of dimethyl selenide from selenite by mold (see Fig. 1.).

It has been reported that fungi of several genera are able to form dimethyl selenide. Challenger et al. (2) reported that Scopulariopsis brevicaulis and Aspergillus niger could produce dimethyl selenide from selenate. Fleming and Alexander (18) also demonstrated that a strain of Penicillium could convert inorganic selenium to a volatile form identified as dimethyl selenide.

Recently, one coryneform bacterium was found which can convert inorganic selenium to dimethyl selenide (5). In later work, Doran and Alexander (19) reported that the conversion of selenite and selenate to volatile products was enhanced if soil was amended with organic materials. The authors also suggested that the microbial formation of dimethyl selenide was widespread.

In higher plants, certain Astragalus species which are called "accumulator plants" and may require selenium for their growth, have been shown to accumulate very high levels of selenium (11,20)

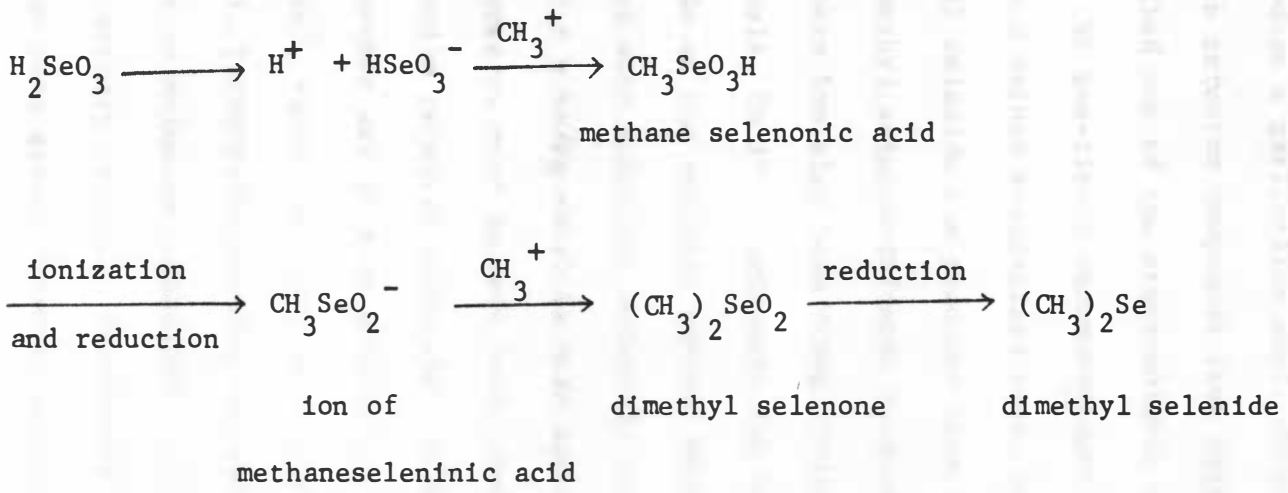


Fig. 1. Pathway for the formation of DMSe from selenite.

and produce a garlic-like odor. Christine et al. (21) isolated the volatile selenium compounds from Astragalus racemosus (Pursh.), and identified one of the ether-soluble compounds as dimethyl diselenide (DMDSe) by gas-liquid chromatography.

In a rather specialized case, Lewis et al. (22) found that dimethyl selenide was produced from cabbage leaves by enzymatic cleavage of Se-methyl selenomethionine selenonium salt.

There has also been great interest in the methylation of selenium by animals. In 1952, McConnell and Portman (23) identified dimethyl selenide as the volatile product which was produced when rats were injected with selenite. McConnell and Portman (24) showed the toxicity to be 1.6 g Se/Kg which is very low compared to selenite (3.5 mg Se/Kg). Consequently, most workers have considered methylation to be the method of selenium detoxification used by animals.

Another methylation product of animals is trimethylselenonium ion, $(CH_3)_3Se^+$, which was found as a urinary metabolite of selenium in 1969 (25,26). Trimethylselenonium ion appeared to be the main excretory product of selenium metabolism, since it routinely accounted for 30-50% of the urinary selenium, regardless of whether high or low doses of selenium were given. Trimethylselenonium ion may also represent another example of a methylated detoxification compound of selenium, Obermeyer et al. (27) showed its toxicity to be 49.4 mg Se/Kg which is much less toxic than selenite.

Ganther (28) performed a study on the enzymic synthesis of dimethyl selenide from sodium selenite in mouse liver extracts, the probable methyl donor for this process was shown to be S-adenosyl-L-methionine, and the system had a specific requirement for glutathione. Reduced triphosphopyridine nucleotide (TPNH), coenzyme A, adenosine 5'-triphosphate (ATP), and magnesium were also required for optimal activity.

Recently, Hsieh and Ganther (29) have postulated a pathway for the biosynthesis of hydrogen selenide and other selenides from selenite (see Fig. 2.). They suggested that under anaerobic conditions, glutathione reductase or excess glutathione effectively reduced glutathione selenopersulfide (GSSeH) to the -2 oxidation state. But it is still uncertain if this will occur under aerobic conditions.

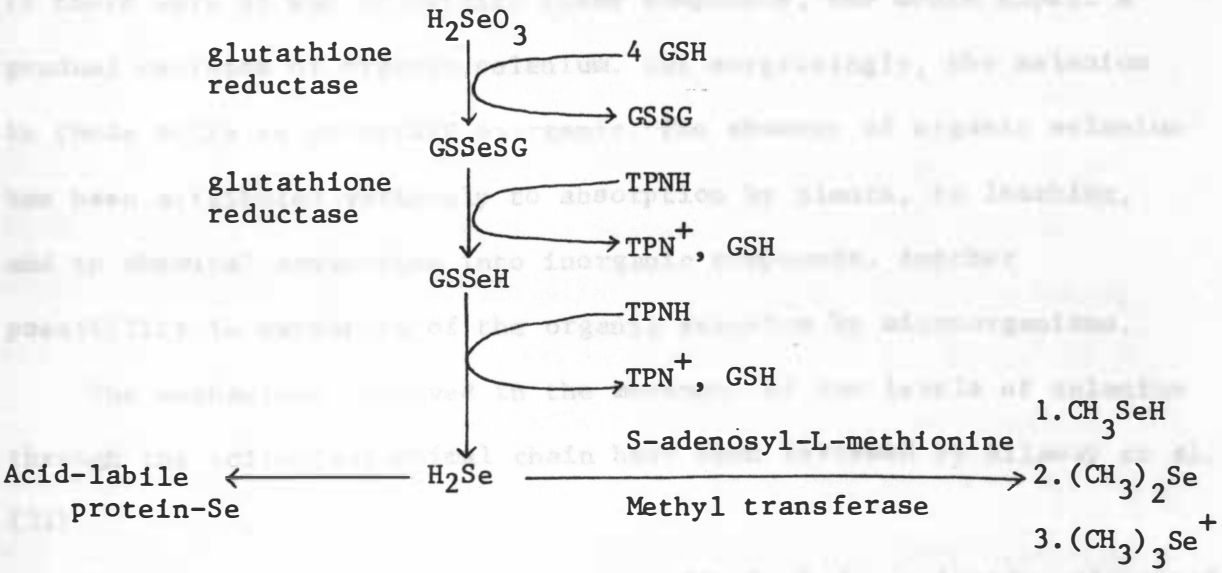


Fig. 2. Pathway for biosynthesis of hydrogen selenide and other selenides from selenite.

Methylation of selenium appears to be an important process in the detoxification of selenium as far as animals are concerned. The role of methylation in the overall cycling of selenium in nature is unclear, but a short review of possible involvement will be given.

Shrift (30) reviewed the selenium cycle in nature. He suggested that selenium may have a biological cycle similar to those of carbon, nitrogen, and sulphur. Within each there occur organisms which reduce the most oxidized form of the element, and other organisms which complete the cycle by oxidizing the reduced element to its initial state. He also pointed out that selenium accumulator plants, always associated with semi-arid seleniferous soils, biosynthesize organic selenium compounds such as Se-methylselenocysteine and selenocystathionine, and release these compounds into the soil when they decay. If there were no way to oxidize these compounds, one would expect a gradual increase of organic selenium. But surprisingly, the selenium in these soils is primarily inorganic. The absence of organic selenium has been attributed variously to absorption by plants, to leaching, and to chemical conversion into inorganic compounds. Another possibility is oxidation of the organic selenium by microorganisms.

The mechanisms involved in the movement of low levels of selenium through the soil-plant-animal chain have been reviewed by Allaway et al. (31).

The forms of selenium present in soils include: selenide, elemental selenium, selenite, selenate and various organic forms. The selenium

from selenide or elemental forms has not been considered as an important source for plant uptake (32). Selenite is found under oxidizing conditions, but is readily immobilized by either precipitation in ferric iron complexes or adsorption on sesquioxides (33). Alkaline conditions favor the formation of selenates.

Selenite and selenate are both taken up by the roots of plants. Within the plant these forms of selenium are reduced to the -2 oxidation state, and the Se^{-2} is incorporated into soluble amino acids, protein-bound amino acids, or both. Allaway et al. (31) stated in their review that in most studies of the soluble selenoamino acid in plants, seleno-cystathionine and/or seleno-glutathione has been noted, although these have usually been present in minor amounts in the non-accumulator plants. These seleno compounds will be released into the soil when the plants decay.

Under natural conditions, the movement of low levels of selenium from plants to animals is primarily a movement of protein-bound seleno-methionine, with lesser amounts of selenates, selenites and other organic selenium compounds. The major excretory product of selenium metabolism in animals is trimethylselenonium ion in the urine (25,26).

Actually, besides the soil-plant-animal chain, microorganisms are very important in the selenium cycle. As stated earlier, Challenger and North (16) reported that two strains of Scopulariopsis brevicaulis can convert selenite or selenate to a substance with a garlic-like odor. They concluded that the volatile product was dimethyl selenide.

Abu-Erreish et al. (4) reported that ^{75}Se added as selenite or as selenate to a mixture of soils was converted to a volatile form. Autoclaving the soil or using a nitrogen atmosphere inhibited Se evolution almost completely, indicating the involvement of microorganisms.

Recently, Francis et al. (34) demonstrated that dimethyl selenide was produced from soils after the addition of selenite and glucose. No DMSe was produced aerobically in the selenium-rich clay soil if it received glucose but no selenite. Later, Doran and Alexander (19) found that selenium was volatilized from soils amended with elemental Se, selenite, selenate, trimethylselenonium chloride, selenomethionine, and selenocystine when incubated in air or anaerobically. In a study in which Lima loam soil was incubated in air with various inorganic (selenium element, selenite and selenate) and organic forms (selenocystine, selenomethionine and trimethylselenonium chloride), dimethyl selenide was isolated as a volatile product. The authors also isolated a methionine-utilizing pseudomonad from soil, which converted selenomethionine to dimethyl diselenide (DMDSe) when incubated in air.

From the facts mentioned above, it appeared that the processes in soil which converted organic or inorganic selenium to volatile compounds were wholly or largely the result of microbial action. Doran and Alexander (19) suggested that the microbial formation of DMSe was widespread.

Although plants and animals do release DMSe or DMDSe, it probably does not result in a significant contribution to atmospheric selenium.

However, the microbial metabolism of organic selenium compounds, originating from plant and animal residues, could result in release of considerable quantities of volatile selenium to the atmosphere. After a period of time, DMSe may change to another form and return to the earth in rain water. Therefore, the conversion of volatile selenium compounds from inorganic and organic forms of selenium by microorganisms may play an important role in the cycling of the element.

This study was undertaken to examine further the role that bacteria might play in the natural cycling of selenium. Specifically, the objectives are to identify soil bacteria which are capable of converting selenite to volatile compounds, and to identify the volatile selenium compound(s) generated by the isolated bacteria.

MATERIALS AND METHODS

Media

Nutrient agar plates containing 20 ppm Se were prepared by autoclaving 1000 ml nutrient agar medium (Difco). After the medium had cooled, 10 ml of a solution containing 2000 ppm Se as sodium selenite were added. The medium was then poured into petri dishes, and allowed to solidify. The plates were stored in a refrigerator until used.

A synthetic medium was prepared from two stock solutions. Solution A contained 30 gm Na_2HPO_4, 15 gm KH_2PO_4 and 20 gm glucose dissolved in 200 ml of distilled water. Solution B contained 5 gm NH_4Cl, 2.5 gm NaCl and 2.05 gm $MgSO_4 \cdot 7\ H_2O$ dissolved in 50 ml of distilled water. Forty ml of solution A and 10 ml of solution B were mixed and filtered through a sterile bacterial filter, the filtrate was added aseptically to 950 ml of sterile distilled water to make 1000 ml medium. Ten ml of a solution containing 2000 ppm Se as sodium selenite were added to obtain the 20 ppm selenium medium.

Throughout this study, Difco products (Difco Laboratories Incorporated, Detroit, Michigan) were used as the biochemical test media.

Source and isolation of bacteria

Soil samples from three locations were studied as sources of selenium-volatilizing bacteria.

1. The sample labeled "Soil" was the surface 152 mm of a well formed soil derived from the Mobridge member of the Pierre formation which is known to be highly seleniferous.
2. The sample labeled "M" was partially weathered material from the Mobridge member of the Pierre formation in an area where Astragalus racemosus grows.
3. The sample labeled "A" was taken from the region where Astragalus racemosus plants grow.
4. Culture No. 1 was previously isolated in this laboratory, but never identified.

Procedures for isolation of the bacteria were as follows:

1. For each soil sample, 5 gm were dispersed in 100 ml of sterile water giving $5x10^{-2}$ g/ml. Series dilutions were made to obtain $5x10^{-4}$ and $5x10^{-6}$ g/ml suspensions.
2. One ml of all dilutions of the soil suspension were spread over nutrient agar plates containing 20 ppm Se.
3. All inoculated plates were incubated at 32°C. After 24 hours, plates were selected which contained discrete colonies. Inoculates from each colony were transferred to new selenium plates.
4. The plates, to which the various types of colonies were transferred, were incubated for 24 or 48 hours. They were then

examined for garlic-like odor. The colonies on plates in which a garlic-like odor was detected, usually turned to reddish-brown after 24 or 48 hours.

5. Colonies from plates in which the odor of volatile selenium was detected were transferred several times to new nutrient agar plates until a pure culture was obtained. The culture was considered pure when only one type of organism could be detected under the microscope after completion of the Gram stain. The cultures were periodically transferred and stored on nutrient agar. In the event the purity of the culture was questioned, new transfers were made to determine that the cultures could still produce volatile selenium from plates containing sodium selenite.

Collection of volatile selenium compounds produced from bacteria.

The synthetic medium described earlier was used in all of the studies which follow.

Some of the defined medium (5 ml) was transferred aseptically into sterile capped tubes, these tubes were inoculated with a culture from nutrient agar slants and incubated at 32°C in a Model 50 Shaker Bath (Precision Scientific Company). The cultures were usually incubated for 18-24 hours, and were used to inoculate large flasks of medium for the production of volatile products. The entire contents of the culture tubes were aseptically transferred into a 4 liter flask containing 1 liter of the defined medium which contained 20 ppm Se as selenite. One ml of a stock solution of $^{75}SeO_3^{-2}$ was also added to facilitate quantitation and identification of volatile selenium compounds. The medium-containing flask was then attached to a series of traps as shown in Fig. 3.

The cold trap and dry ice-ethanol trap were used to remove excess moisture. The trap for volatile selenium products consisted of a tube packed with alternate layers of activated charcoal and glass wool. Six or seven layers of charcoal (0.5 gm per layer) were usually used. This type of trap was shown to be effective in trapping volatile selenium products by Lewis et al. (35). Air was forced through the entire apparatus at a rate of 400 ml/min.

Volatile products were collected during a five day incubation period. At the end of this time, the charcoal layers were sequentially removed, and the radioactivity was determined with a Packard Tri-Carb

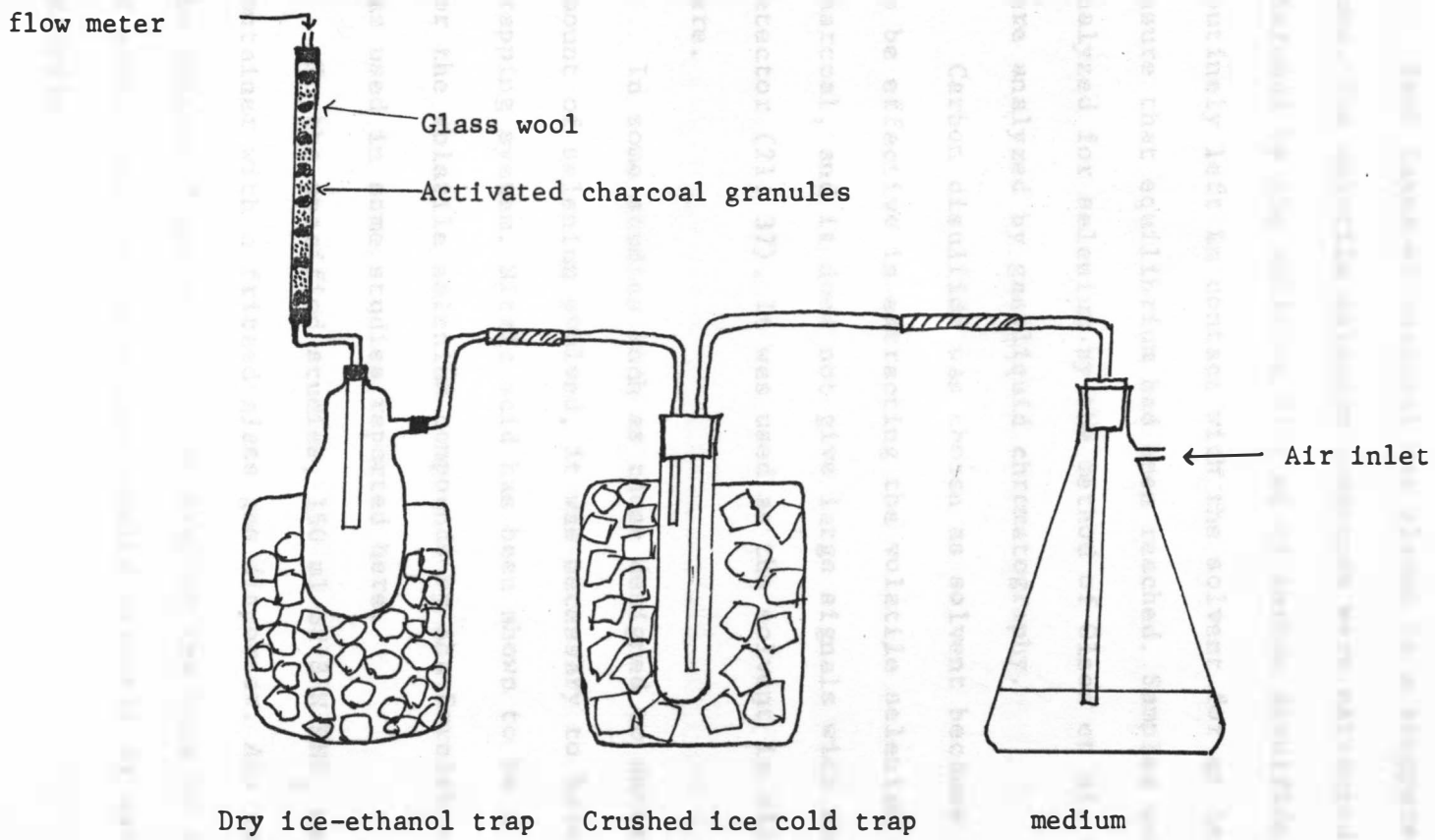


Fig. 3. Apparatus used for the collection of volatile selenium compounds generated from bacteria.

Scintillation Counter.

Each layer of charcoal was placed in a stoppered 15 ml centrifuge tube. The volatile selenium compounds were extracted from activated charcoal by the addition of 2 ml of carbon disulfide. Samples were routinely left in contact with the solvent for at least 2 hours to ensure that equilibrium had been reached. Samples were then taken and analyzed for selenium by the method of Olson et al. (36). Other aliquots were analyzed by gas-liquid chromatography.

Carbon disulfide was chosen as solvent because it has been shown to be effective in extracting the volatile selenium compounds from charcoal, and it does not give large signals with the hydrogen flame detector (21, 37). It was used as the solvent in all studies reported here.

In some studies such as those designed to determine the total amount of selenium evolved, it was necessary to have a very efficient trapping system. Nitric acid has been shown to be a good trapping agent for the volatile selenium compounds by Abu-Erreish et al. (4), and it was used in some studies reported here.

In the specified studies, 150 ml of 8 N HNO_3 were placed in a container with a fritted glass gas disperser. Air was forced through the culture flask and into the trap at the rate of 400 ml/min. Aliquots of nitric acid solution were counted directly or used for selenium analysis.

Identification of the volatile selenium compounds.

a. Gas chromatography studies

Dimethyl selenide (CH_3SeCH_3) and dimethyl diselenide ($CH_3SeSeCH_3$) were prepared, for chromatographic and gas chromatography-mass spectrometric standards, in our laboratory following the method of Bird and Challenger (38).

The analytical gas chromatograph used for the separation of the organic selenium compounds was a Varian Aerograph Series 2400 with a hydrogen flame ionization detector connected to a Sargent-Welch Recorder Model SRG. The column used to fractionate the selenium compounds was a 10 ft x 1/8 in., 20% Carbowax 20M on 60/80 Chromosorb W, coated with hexamethyldisilizane (HMDS). Nitrogen was used as carrier gas, at a flow rate of 30 ml/min. The flow rates were measured with a soap bubble flowmeter at the column exit. The column temperature was 120°C, and the injector and detector temperatures were 230 and 225°C respectively. The column conditions were similar to those described by Christine and Johnson (37).

b. Mass spectrometry studies

Liquid samples were analyzed with a Finnigan Model 3000 peak Identifier GLC/MS equipped with a Gohlke separator. The column was similar to that used in gas chromatography studies, with a helium carrier gas flow rate of 30 ml/min. The column temperature was programed to rise from 120 to 160°C at a rate of 2°C/min. The injector temperature was 180°C.

Volatile selenium compounds generated from different selenium sources by the bacterium in culture No. 1.

Various selenium compounds other than selenite were used as substrates to determine if volatile selenium could be produced by the bacterium in culture No. 1.

Sodium selenite (Na_2SeO_3) and potassium selenate (K_2SeO_4) were synthesized as described by Trelease and Beath (39). Trimethylselenonium chloride (TMSeCl) was synthesized as described by Obermeyer et al. (27). DL-selenomethionine (A grade, Calbiochem. San Diego, Calif.) and DL-selenocystine (A grade, Calbiochem. San Diego, Calif.) were used as purchased.

Only one bacterial isolate was used in these studies. The old culture from previous work, called culture No. 1 was used to inoculate tubes containing 5 ml of defined medium. These were incubated at 32°C in a Shaker Bath for 18 hours, and then used to inoculate the test media. The test media were prepared by adding 2 ml of a stock solution, containing 2000 ppm Se of the various selenium sources, to 200 ml of the defined medium in a 1 liter flask. Each test flask was then inoculated with 5 ml of an 18 hour culture, and incubated at room temperature. The flask was connected in series with a charcoal trap (containing one layer of 2 gm of charcoal) and a nitric acid trap to completely trap all selenium products. Two ml of carbon disulfide were used to extract the volatile products from charcoal. For the selenium analysis, 0.1 ml of carbon disulfide was used, while 2 ml aliquots nitric acid were used.

RESULTS AND DISCUSSION

Isolation and identification of the bacteria.

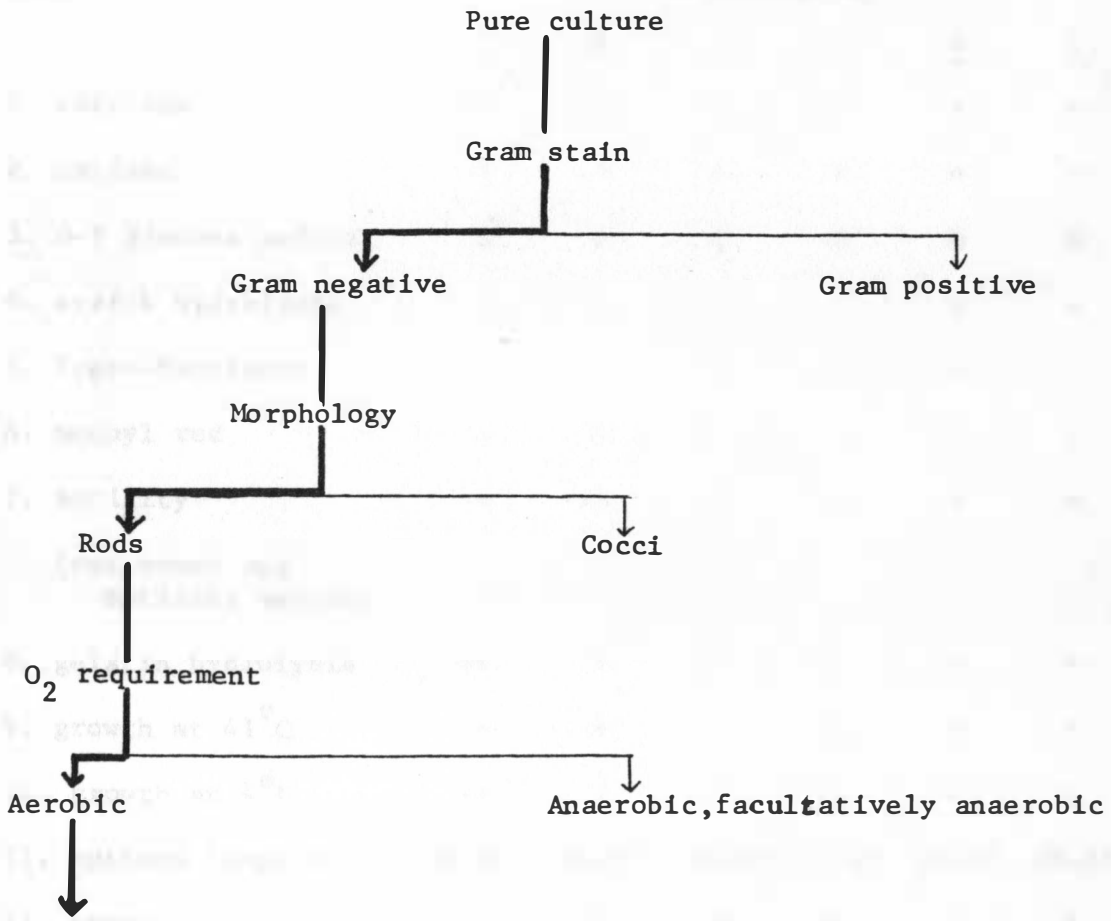
This work resulted in the isolation of five organisms. No. 2, 3 and 4 were isolated from the soil labeled as "Soil", No. 5 and 6 were isolated from the soil labeled as "M". However, no organism was isolated from the soil labeled as "A". Culture No. 1 was isolated previously in this laboratory, but never identified. All six organisms were capable of converting sodium selenite to volatile selenium compounds.

The culture was judged to be pure when plates were obtained with all colonies of identical morphology, and when microscopic examination revealed only one type of organism. The pure cultures were then submitted to various morphological and biochemical tests to classify them. The tests are summarized in the following flow diagram (Fig. 4 and 6) and Table 1.

Summary of isolation procedure:

soil sample
↓
series dilution
↓
growth on 20 ppm Se nutrient agar
↓
detection of cultures producing garlic-like odor
↓
series transfer on nutrient agar plate
↓
pure culture

Fig. 4. Important characteristics of all six organisms.



Part 7 in Bergey's manual of determinative bacteriology,(8 th edition) contains the Gram negative aerobic rods and cocci, since all six organisms are aerobic Gram negative rods, it was determined that all six organisms belong to part 7 in Bergey's manual.

Table 1. Biochemical tests for all six organisms.

Test[a]	Cultures No.					
	1	2	3	4	5	6
1. catalase	++	+	+	+	+	+
2. oxidase	+	+	+	+	+	+
3. O-F glucose medium	O[b]	O	O	O	O	O
4. starch hydrolysis	-	-	-	-	-	-
5. Voges-Proskauer test	-	-	-	-	-	-
6. methyl red	-	-	-	-	-	-
7. motility (wet mount and motility medium)	+	+	+	+	+	+
8. gelatin hydrolysis	++	+	+	+	+	+
9. growth at 41°C	-	+	+	+	+	+
10. growth at 4°C	++	-	-	-	-	-
11. optimum temperature	30-32	35-37	35-37	35-37	35-37	35-37 (°C)
12. urease	-	+	+	+	+	+
13. nitrate reduction	+	+ (gas)	-	-	-	+ (gas)
14. Simmon's citrate	+	+	+	+	+	+
15. litmus milk	proteolysis digestion	-	-	-	-	-
16. glucose	+ (acid)	+	+	+	+	+
17. L-arabinose	+ (acid)	+	+	+	+	+
18. sorbitol	+ (acid)	+	+	+	+	+
19. lactose	+	-	+	+	+	+

Test	Cultures No.					
	1	2	3	4	5	6
20. sucrose	+	+	+	+	+	+
21. maltose	+	+	+	+	+	+
22. acetamide broth[c]	-	-	-	-	-	-
23. mannitol	+	+	+	+	+	+
24. trehalose	+++	+	++	++	++	+
25. meso-inositol	+++	+	+	+	+	+
26. Tech medium[d]	+	-	-	-	-	-
27. Pseudomonas P (test for pyocyanin)	-	-	-	-	-	-
28. King's B medium (test for fluorescence)	+ (yellow-green)	-	-	-	-	-
29. arginine dihydrolase[e]	+	-	-	-	-	-
30. flagella stain[f]	polar (1,2 or 3)					
31. requirement for enriched medium	all not required					
32. levan formation from sucrose	-	-	-	-	-	-
33. Indole produced	-	-	-	-	-	-

Note: a. For all biochemical tests except those specified, see reference 41 and 42.

b. The letter "O" indicates oxidative ; "F" indicates fermentative.

c. see reference 43.

d. see reference 44.

e. see reference 45 and 46.

f. see reference 47.

Most of the work has been concentrated on No. 1 culture, although the other cultures were also subjected to the biochemical tests. The organism from culture No. 1, when grown on nutrient agar, produced opaque, punctiform colonies which had slightly raised smooth surfaces with entire margins.

The organism was a gram negative short rod that had motility. When subjected to a simplified Leifson flagella stain, it was shown to possess one, two or three polar flagella (see Fig. 5). When cultured on King's medium (42), the organism formed a diffusible yellow-green pigment that fluoresced in ultraviolet light (wave length below 260 nm). These criteria along with the observations that the organism was oxidative, produced no indole or acetylmethylcarbinol, and required no enriched media, place it in the fluorescent group of the Pseudomonas genus.

Bergey's manual (pp. 220, Table 7.1 and 7.2) lists seven species in the fluorescent group. Certain of the biochemical characteristics, specifically lack of growth at 41 ^{o}C, lack of levan formation from sucrose, oxidase reaction, hydrolysis of gelatin but lack of hydrolysis of starch, and utilization of trehalose, meso-inositol and L-arabinose, make it appear the organism in culture No. 1 best fits with Pseudomonas fluorescens. This is summarized in Fig. 6.

Pseudomonas fluorescens are further subdivided into 4 biotypes and a group of miscellaneous strains. Because of the organism's response to certain tests, i.e. levan formation from sucrose and denitrification, it does not appear to fit with biotype I, II, III, and IV, the organism

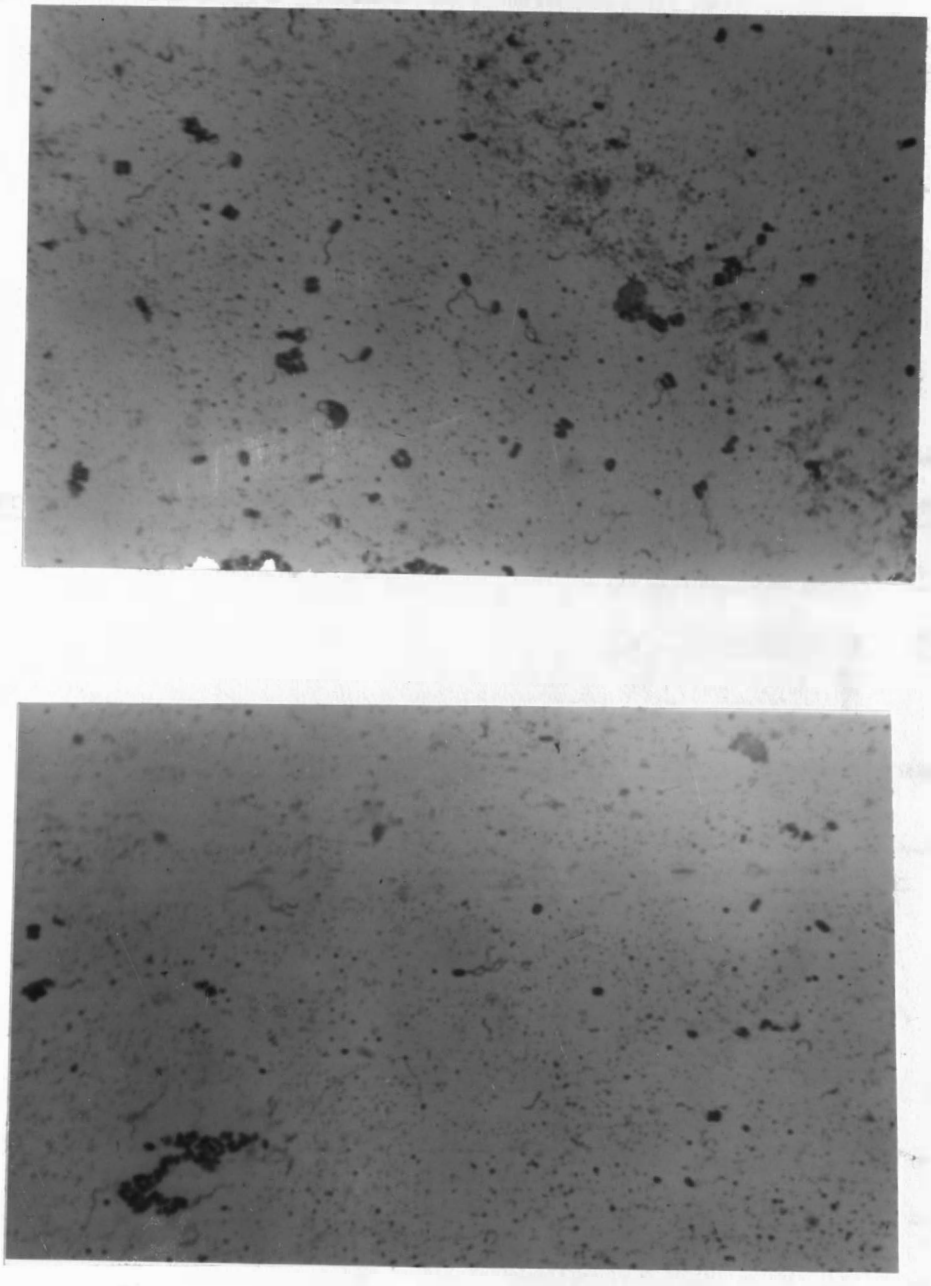
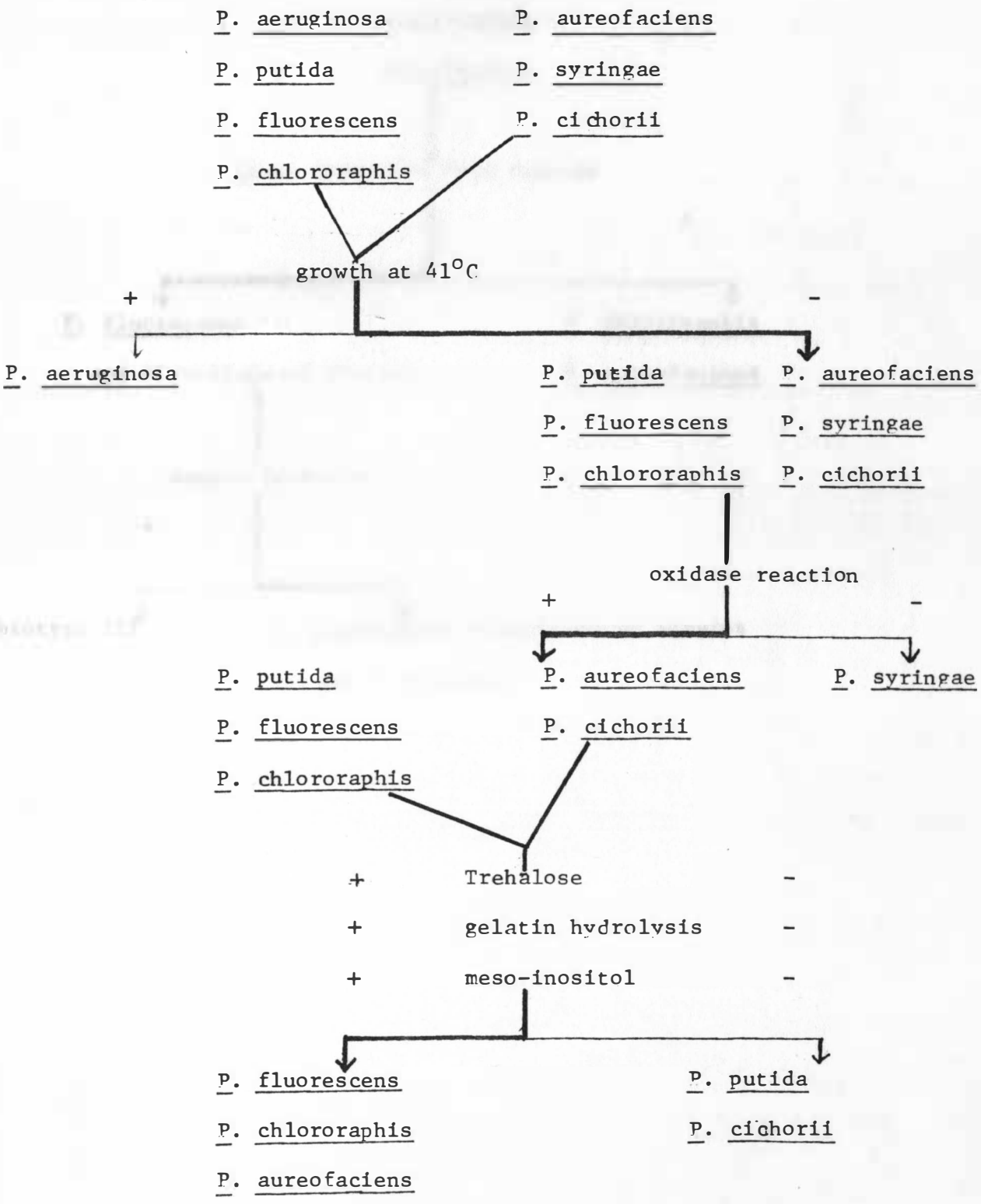
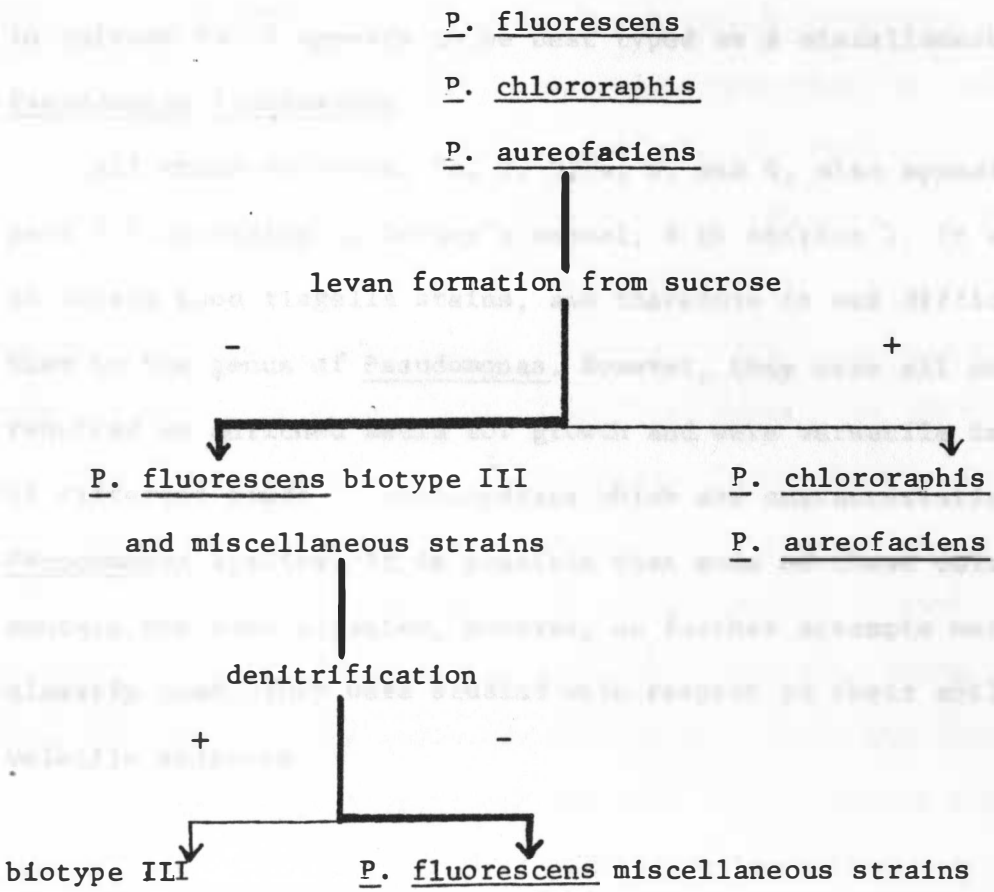


Fig. 5. Leifson's flagella stain of Culture No. 1.

Fig. 6. Identification schemes for Culture No. 1.




P. fluorescens
P. chlororaphis
P. aureofaciens
levan formation from sucrose
-
+
P. fluorescens biotype III
and miscellaneous strains
P. chlororaphis
P. aureofaciens
denitrification
+
-
biotype III
P. fluorescens miscellaneous strains

(No. 1 culture)

in culture No. 1 appears to be best typed as a miscellaneous strain of Pseudomonas fluorescens.

All other cultures, No. 2, 3, 4, 5, and 6, also appear to fit in part 7 (according to Bergey's manual, 8 th edition). It was not possible to obtain good flagella stains, and therefore it was difficult to refer them to the genus of Pseudomonas. However, they were all oxidative, required no enriched media for growth and were versatile in their use of different kinds of carbohydrate which are characteristics of the Pseudomonas species. It is possible that some of these cultures may contain the same organism, however, no further attempts were made to classify them. They were studied with respect to their ability to produce volatile selenium.

Efficiency of the system for trapping volatile selenium products.

A preliminary study was conducted to determine the efficiency of charcoal as an adsorbent for volatile selenium compounds. It was necessary to know if the compounds would be adsorbed by a small amount of charcoal so that sufficient concentration could be achieved to make analysis by gas chromatography possible. A flask containing 1000 ml of defined medium to which $^{75}SeO_3^{-2}$ had been added, was inoculated with culture No. 6 as described in the Materials and Methods section. The volatile products were passed through a trap containing 7 layers (0.5 gm per layer) of charcoal. At the end of the incubation period, each layer was counted and the results are shown in Table 2. It can be seen that most of the radioactivity was trapped in the first two layers. Since at least a portion of the selenium volatilized by the bacteria was strongly adsorbed, charcoal should provide a suitable system for collecting the products for later identification.

Other workers (35) have reported the use of charcoal for the adsorption of volatile selenium products. Christine et al. (21) also studied the efficiency of various solvents in removing the selenium compounds from the charcoal. Carbon disulfide was shown to be a good solvent and it was used in this study since it would give very little interference in the gas chromatography and mass spectrometry studies. A study was conducted to confirm that CS_2 would truly extract the adsorbed selenium from charcoal. The data in Table 3 show that 45% of the adsorbed selenium was extracted from the charcoal in two hours.

Table 2. Recovery of ^{75}Se volatilized by culture No. 6 in the various layers of the charcoal trap.

(not corrected for background)

layer	CPM (count per min.)
1st	53,159
2nd	1,467
3rd	204
4th	112
5th	120
6th	111
7th	121
background	116

Table 3. Efficiency of carbon disulfide in extracting volatile selenium compounds from charcoal.

Original charcoal	217,306 CPM
2 ml of carbon disulfide after 2 hours extraction	98,139 CPM (45%)
Charcoal after 2 hours extraction	121,655 CPM

On this basis, carbon disulfide was assumed to be an adequate solvent for the gas chromatography studies which follow.

Since the possibility existed that charcoal would not trap all of the volatile selenium products, a comparison was made with trapping systems which included nitric acid. For this study, flasks containing 200 ml of medium were inoculated with culture No. 1 and incubated at room temperature for 5 days. Table 4 shows the results of these studies. Again the charcoal appeared to be quite efficient. About 78% of the total activity trapped was found in the first two layers. In this instance, however, some of the product did carry over to the third and fourth layers. The efficiency of the HNO_3 trap was very similar to that of charcoal with respect to recovery (see the 2nd column). However, when the traps were connected in series, 0.5% of the total volatile selenium passed through the charcoal and was trapped in the acid (see the third column). For total recovery studies, it appears that nitric acid would be the most suitable trap.

The data in Table 4 also show that for the three flasks of culture No. 1, an average of 27.6% of the selenium was volatilized. Total recovery of the added selenium ranged from 67.5% to 86.4%. The incomplete recoveries were probably due to several causes including incomplete trapping, precipitation of elemental selenium on the surface of the aeration train, and the nonhomogenous nature of the culture (cells which accumulated elemental selenium tended to settle out very rapidly).

Table 4. Recovery of volatile selenium produced by culture No.1 and collected by different traps for 5 days.

	Count/min. collected in trap system		
	Charcoal	Nitric acid	Charcoal + nitric acid in series
1st charcoal layer	118,318	-	130,939
2nd charcoal layer	58,259	-	70,326
3rd charcoal layer	32,175	-	27,540
4th charcoal layer	18,606	-	13,867
Nitric acid	-	179,860	12,444
Total CPM of volatile compound	227,358	179,860	255,116
Final medium	329,680	359,560	435,500
Original added CPM	799,500	799,500	799,500
% of volatile compd.	28.4%	22.5%	31.9%
Recovery	69.7%	67.5%	86.4%

Rate of selenium evolution by bacterial culture No. 1.

A study was conducted to determine the time required for maximum production of volatile selenium products by the bacteria. Three 1 liter flasks containing 200 ml of medium were inoculated as described in the Materials and Methods section. Air was forced through the culture flasks and into nitric acid traps. Samples were removed every 24 hours for selenium analysis. The data in Fig. 7 show that maximum selenium volatilization had almost occurred by 96 hours. Consequently, in all other studies, the collection period was routinely set at 120 hours. The total amount of selenium volatilized by the 3 sets of cultures averaged 47.3%. Total recoveries for the 3 sets were 83.9%, 74.9% and 71.8% respectively. It should be noted that the apparatus was not rinsed to remove any selenium adhering to the walls.

The amount of selenium volatilized was not as great in all instances as for the data reported in Fig. 7. In experiments where large amounts of media were inoculated, for the purpose of collecting significant amounts of product, the percent of the selenium volatilized appeared to be lower. The reasons for this are not understood.

Identification of volatile selenium compounds by gas chromatography.

In all the studies concerning the identification of volatile selenium compounds, charcoal was used as the trapping agent and carbon disulfide was used to extract the compounds. The standard compounds, dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe) showed good separation by gas chromatography (see Fig. 8a and 8b). Retention times

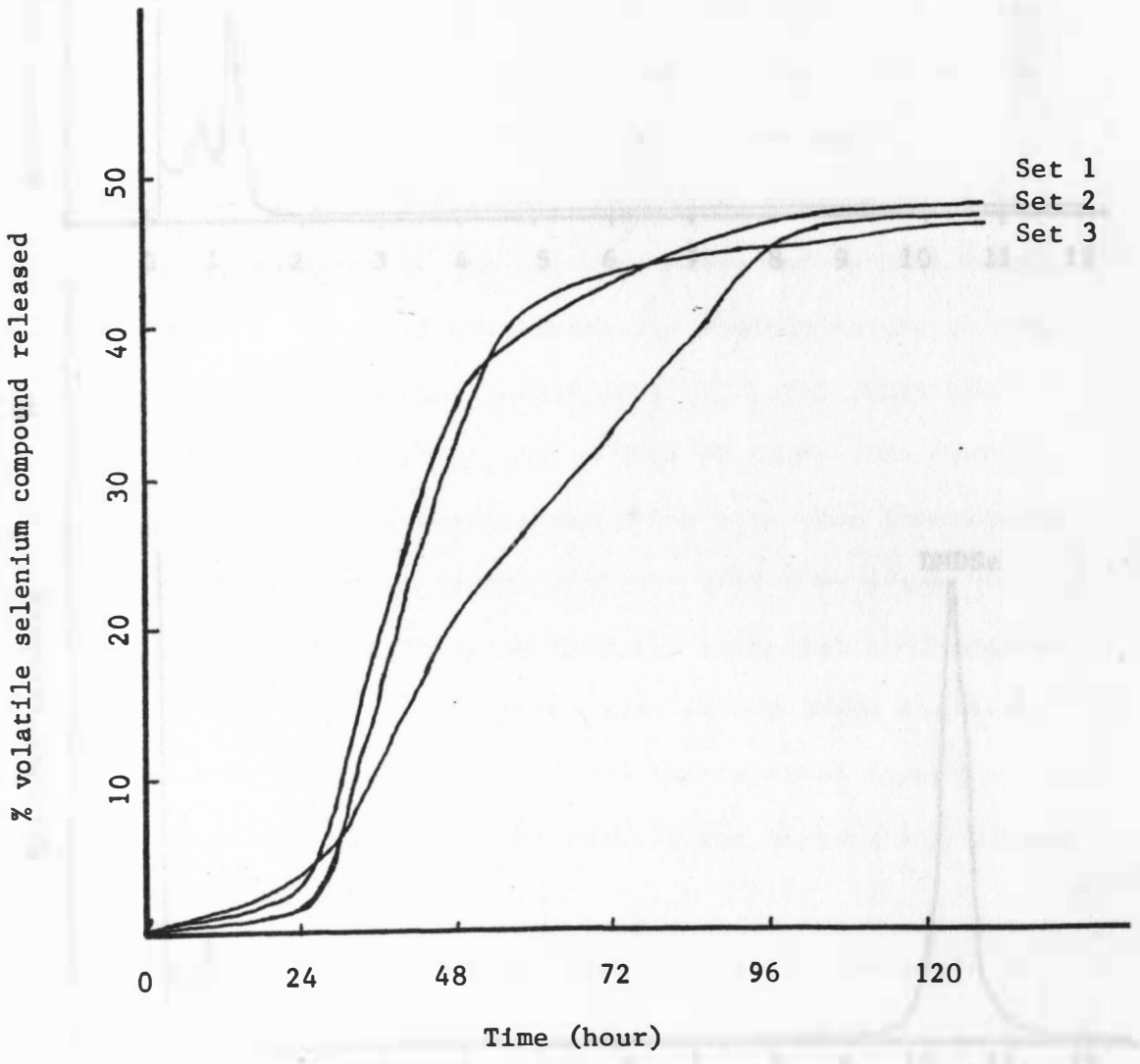


Fig. 7. Rate of production of volatile selenium by culture No. 1.

Fig. 8a.

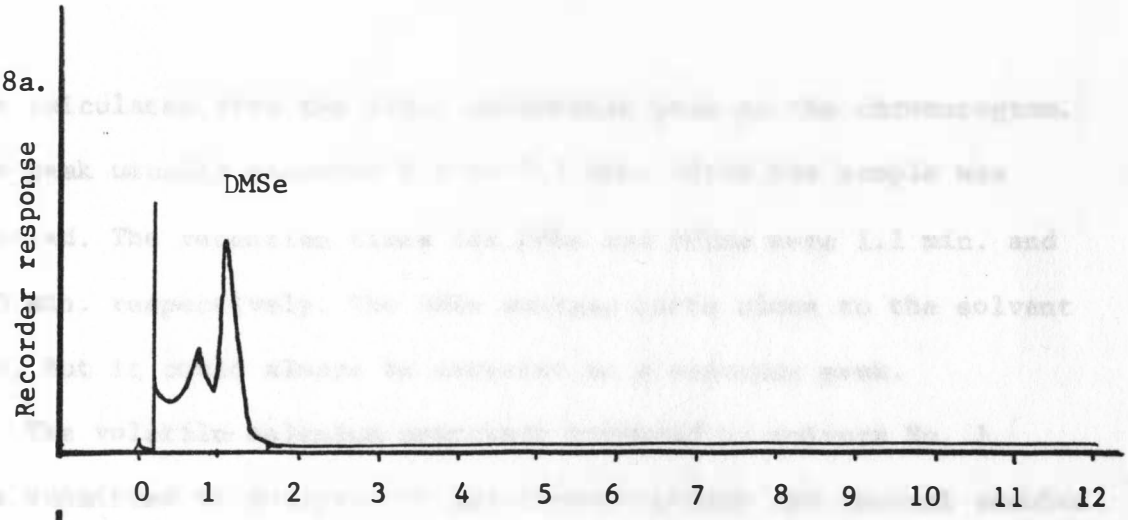


Fig. 8b.

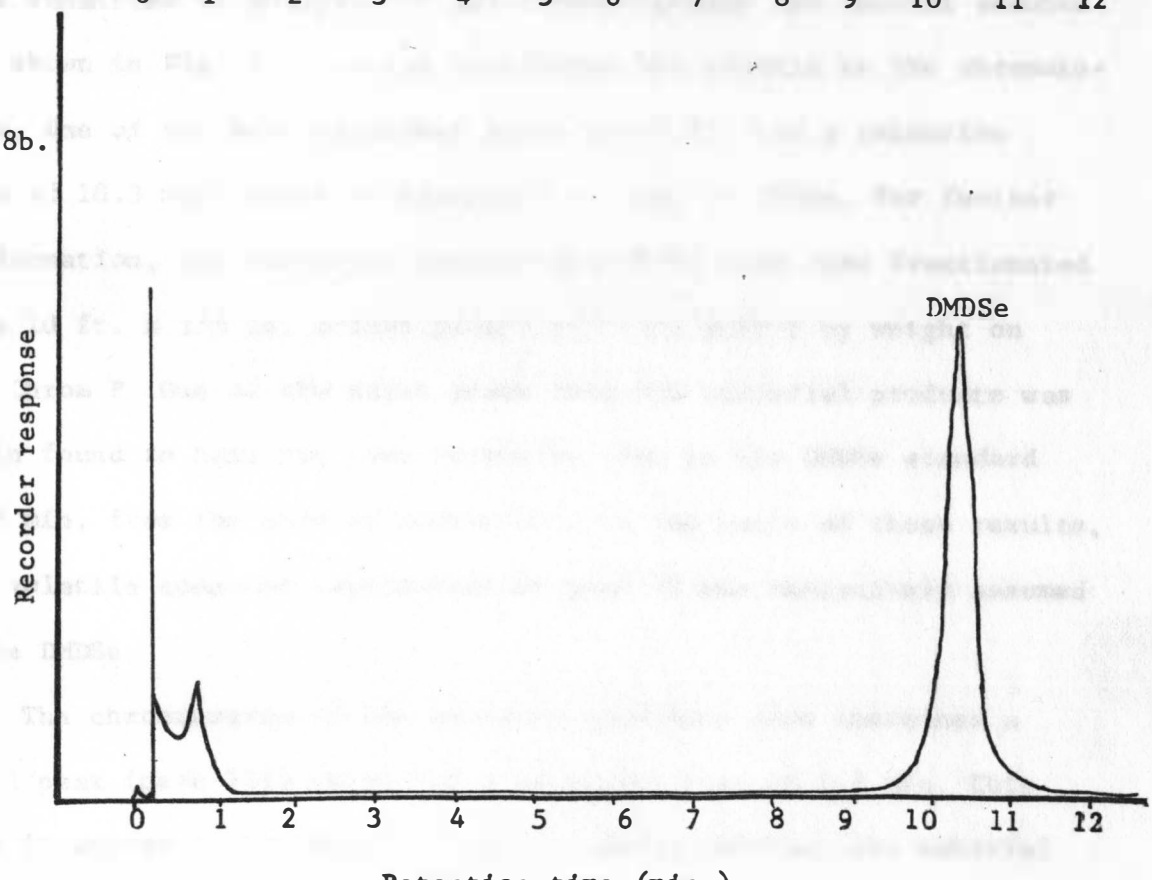


Fig. 8a. Gas chromatogram of dimethyl selenide. 1.76 µl DMSe (200 µg/ml).

Fig. 8b. Gas chromatogram of dimethyl diselenide. 2.0 µl DMDSe (2mg/ml).

were calculated from the first detectable peak on the chromatogram. This peak usually occurred 0.6 to 0.7 min. after the sample was injected. The retention times for DMSe and DMDSe were 1.1 min. and 10.3 min. respectively. The DMSe emerged quite close to the solvent peak, but it could always be detected as a separate peak.

The volatile selenium compounds produced by culture No. 1 were submitted to analysis by gas chromatography and typical results are shown in Fig. 9a. Several substances are visible on the chromatogram. One of the most prominent peaks (peak VI) had a retention time of 10.3 min. which is identical to that of DMDSe. For further conformation, the bacterial product and DMDSe were also fractionated on a 10 ft. x 1/8 in. column packed with 10% EGSS-X by weight on Gas Chrom P. One of the major peaks from the bacterial products was again found to have the same retention time as the DMDSe standard (3.8 min. from the time of injection). On the basis of these results, the volatile compound represented by peak VI was tentatively assumed to be DMDSe.

The chromatogram of the bacterial products also contained a small peak (peak III) which had a retention time of 1.1 min. This made it appear to be DMSe. In order to establish that the material was indeed DMSe, further analyses were conducted in which 10 μg and 20 μg of the DMSe standard were added to 0.1 ml of the CS_2 extract. These mixtures were submitted to gas chromatography. Again Fig. 9a shows the chromatogram of the bacterial products. Fig. 9b and 9c show that there were proportionate increases in peak III

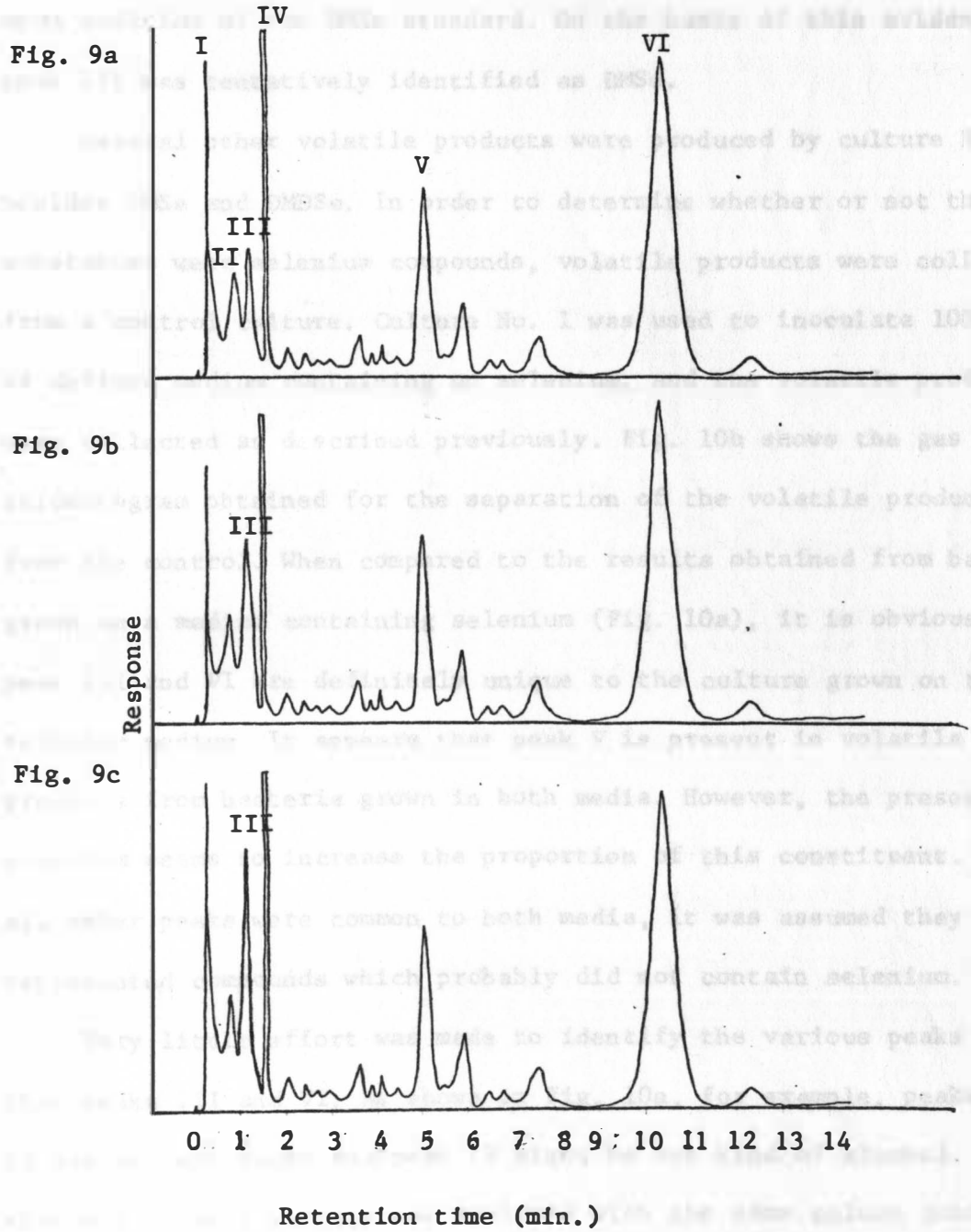


Fig. 9a. Gas Chromatogram of volatile compounds produced by culture No. 1. (1.4 µl)

Fig. 9b. 1.4 µl of a mixture of 0.1 ml extract + 10 µg DMSe.

Fig. 9c. 1.3 µl of a mixture of 0.1 ml extract + 20 µg DMSe.

upon addition of the DMSe standard. On the basis of this evidence, peak III was tentatively identified as DMSe.

Several other volatile products were produced by culture No. 1 besides DMSe and DMDSe. In order to determine whether or not these substances were selenium compounds, volatile products were collected from a control culture. Culture No. 1 was used to inoculate 1000 ml of defined medium containing no selenium, and the volatile products were collected as described previously. Fig. 10b shows the gas chromatogram obtained for the separation of the volatile products from the control. When compared to the results obtained from bacteria grown on a medium containing selenium (Fig. 10a), it is obvious that peak III and VI are definitely unique to the culture grown on the selenium medium. It appears that peak V is present in volatile products from bacteria grown in both media. However, the presence of selenium seems to increase the proportion of this constituent. Since all other peaks were common to both media, it was assumed they represented compounds which probably did not contain selenium.

Very little effort was made to identify the various peaks other than peaks III and VI. As shown in Fig. 10a, for example, peaks I and II are solvent peaks and peak IV might be one kind of alcohol. A standard alcohol mixture was analyzed with the same column conditions and one of the alcohols was found to have a retention time of 1.45 min. which is identical to peak IV. No further attempt was made to identify peak IV or the other minor peaks.

Fig. 10a.

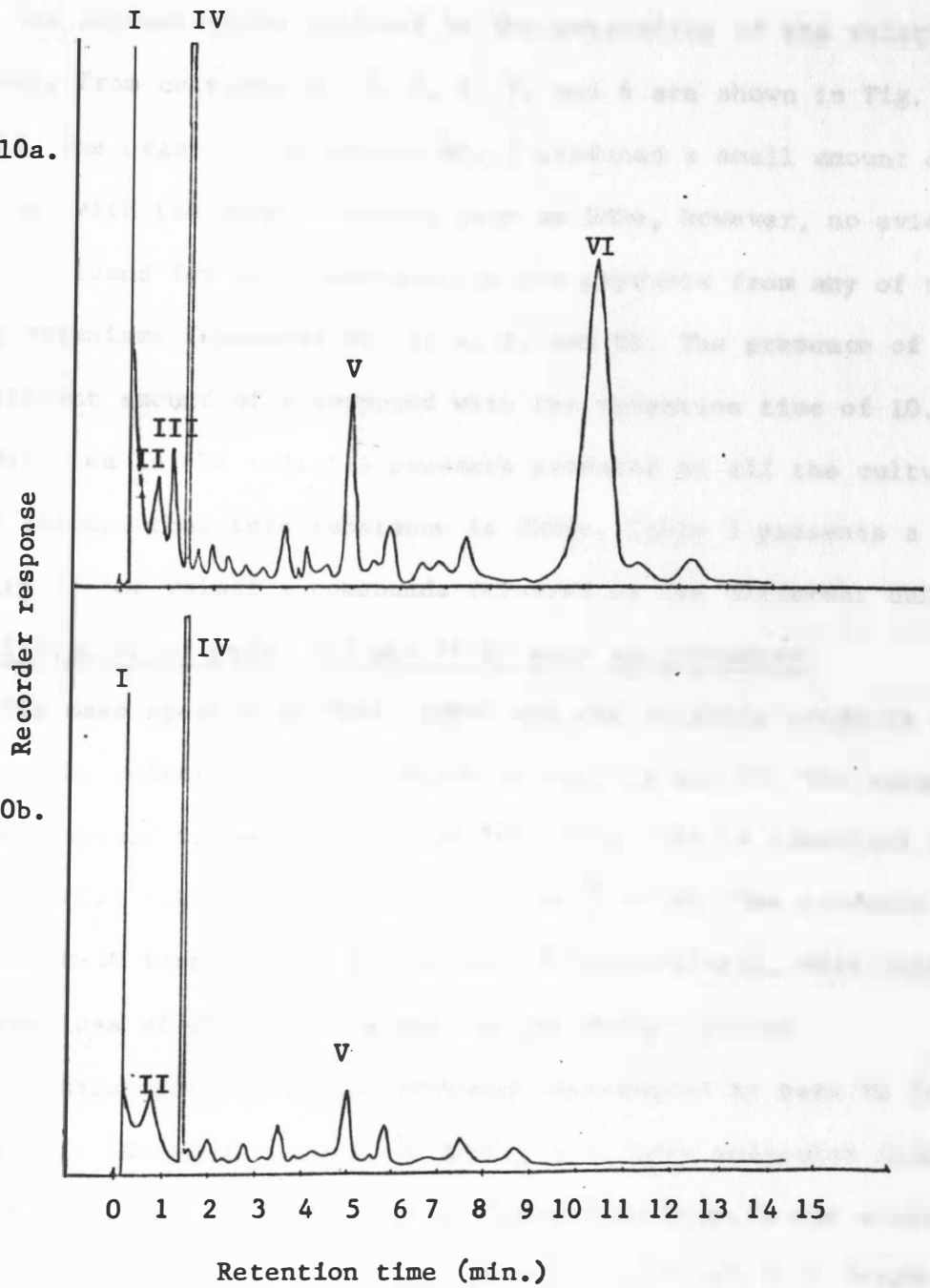


Fig. 10b.

Fig. 10a. Gas chromatogram of volatile compounds produced by culture No.1 grown on defined medium containing 20 ppm Se.

Fig. 10b. Gas chromatogram of volatile compounds produced by culture No.1 grown on defined medium without Se.

The chromatograms produced in the separation of the volatile products from cultures No. 2, 3, 4, 5, and 6 are shown in Fig. 11, 12 and 13. The organism in culture No. 2 produced a small amount of compound with the same retention time as DMSe, however, no evidence could be found for this substance in the products from any of the other organisms (cultures No. 3, 4, 5, and 6). The presence of a significant amount of a coupound with the retention time of 10.3 min. was detected in the volatile products produced by all the cultures. It is assumed that this substance is DMDSe. Table 5 presents a summary of the volatile compounds released by the different cultures.

Identification of peaks III and VI by mass spectrometry.

The mass spectra of DMSe, DMDSe and the volatile products produced by culture No. 1 are shown in Fig. 14 and 15. The spectrum of the compound represented by peak III (Fig. 14) is identical to that of DMSe; both have molecular ions at $\frac{m}{e} = 109$. The presence of M-15 and M-30 fragments at $\frac{m}{e} = 94$ and 79 respectively, were consistent with the loss of one methyl group and two methyl groups.

The mass spectrum of the compound represented by peak VI (see Fig. 15) is identical to that of DMDSe; both have molecular ions at $\frac{m}{e} = 188$. The isotopic abundance pattern showed that it was a selenium containing substance. The presence of M-15, M-30 and M-79 fragments at $\frac{m}{e} = 173$, 158 and 109 respectively, were consistent with the loss of one methyl group, two methyl groups, and a selenium atom. There was also a fragment pattern at $\frac{m}{e} = 94$ which was probably due to the cleavage between the two selenium atoms in DMDSe ($CH_3Se-SeCH_3$).

Fig. 11a.

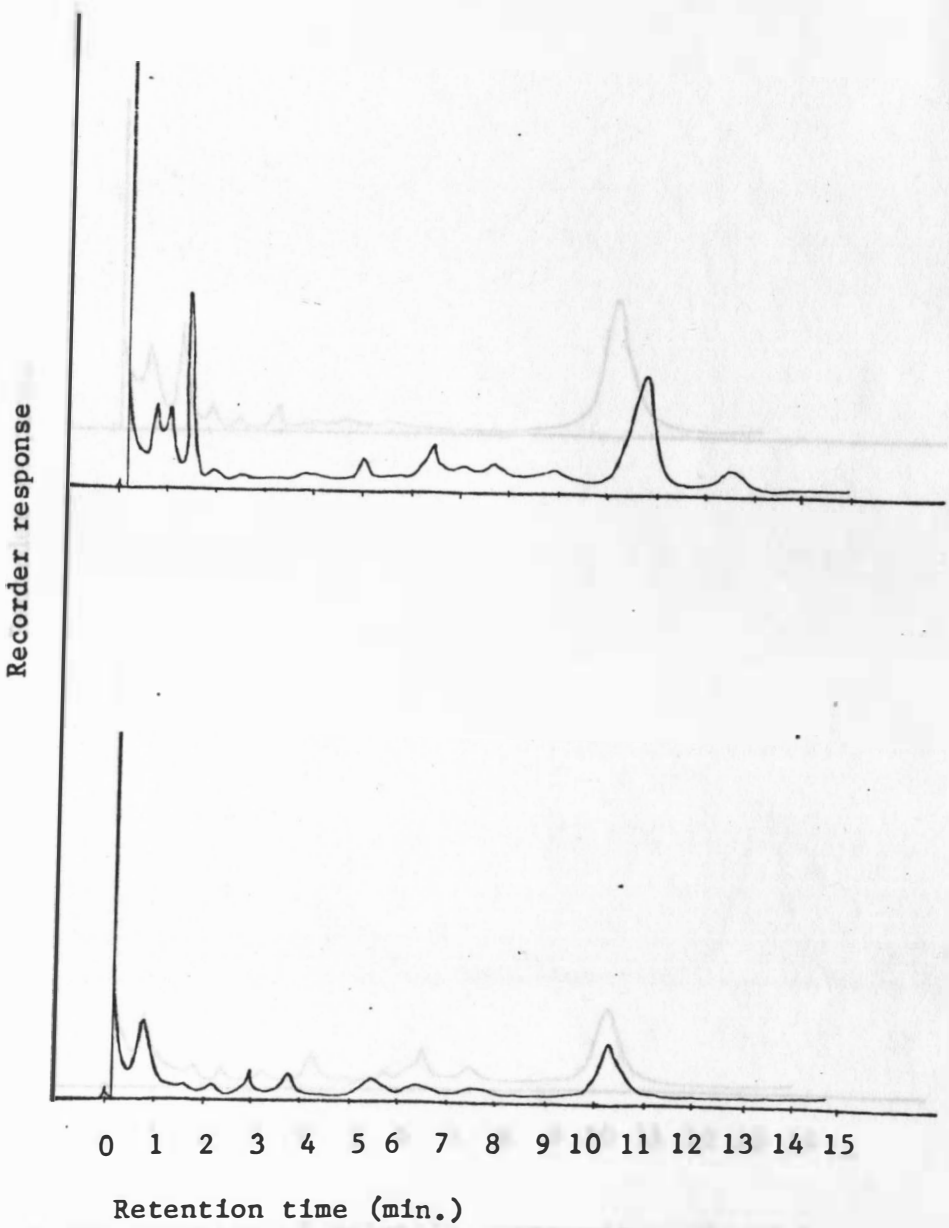


Fig. 11b.

Fig. 11a. Gas chromatogram of volatile compounds produced by culture No. 2.

Fig. 11b. Gas chromatogram of volatile compounds produced by culture No. 3.

Fig. 12a.

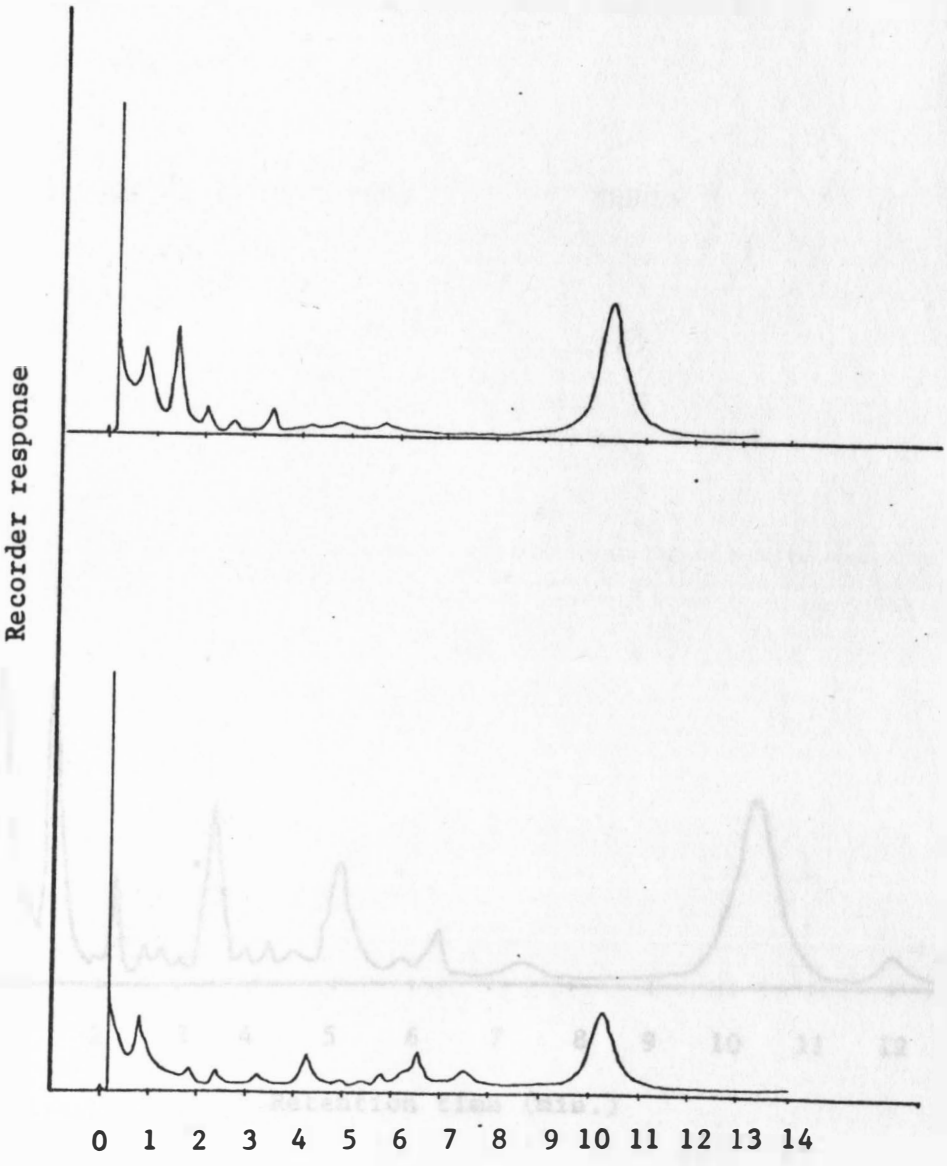


Fig. 12b.

Fig. 12a. Gas chromatogram of volatile compounds produced by culture No. 4.

Fig. 12b. Gas chromatogram of volatile compounds produced by culture No. 5.

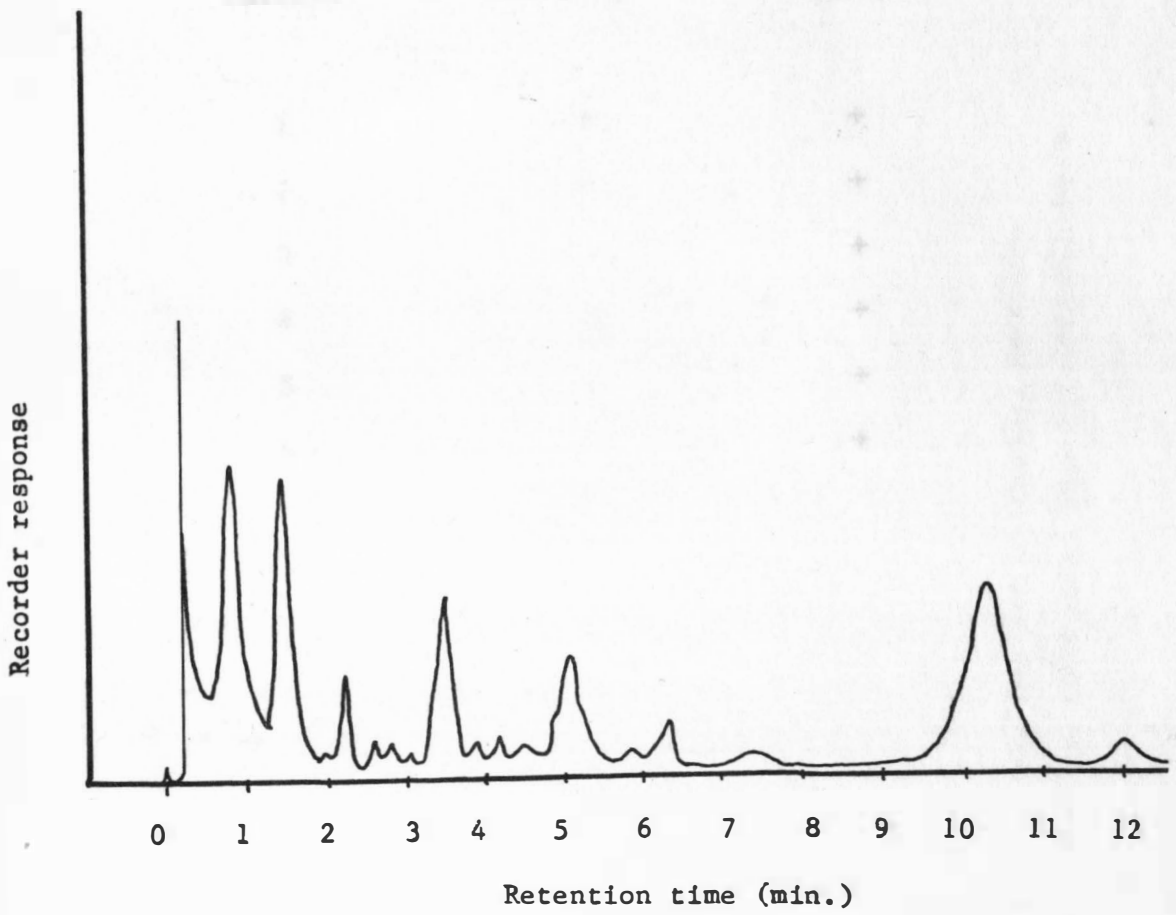


Fig. 13. Gas chromatogram of volatile compounds produced by culture No. 6.

Table 5. Summary of the volatile compounds released by different cultures.

Culture	DMSe	DMDSe
1	+	+
2	+	+
3	-	+
4	-	+
5	-	+
6	-	+

Fig. 14 a.

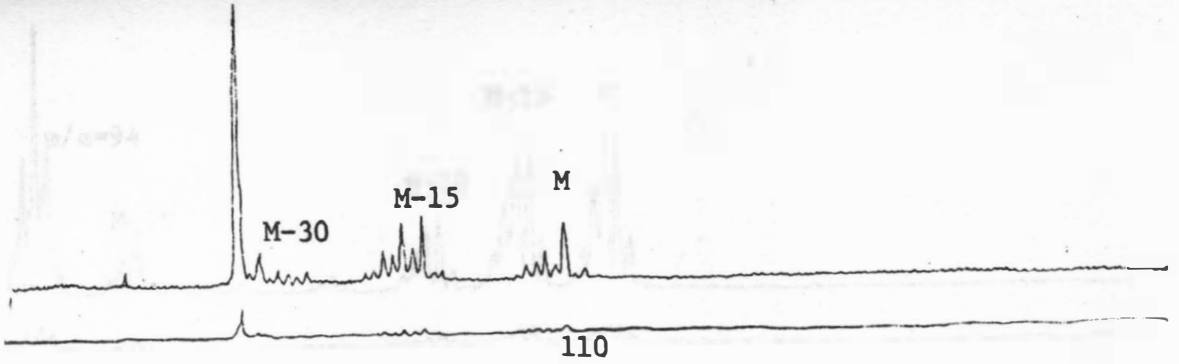


Fig. 14b.

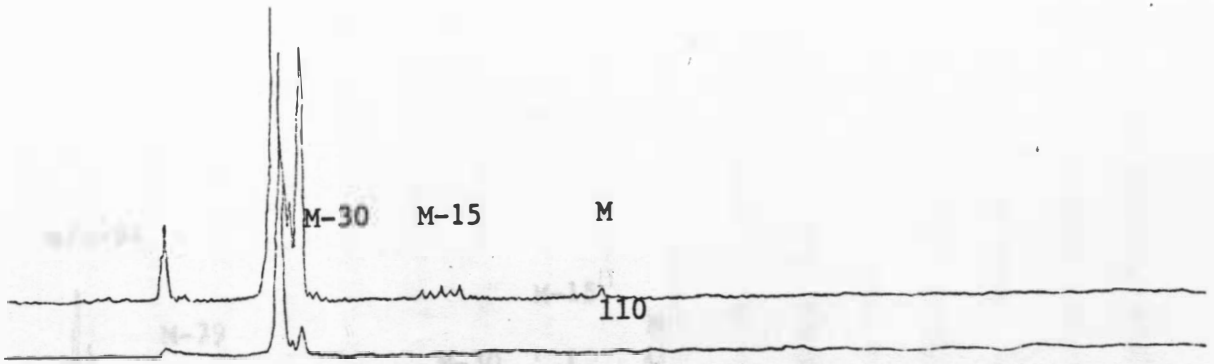


Fig. 14a. Mass spectrum of dimethyl selenide.

Fig. 14b. Mass spectrum of peak III from culture No. 1.

Fig. 15a.

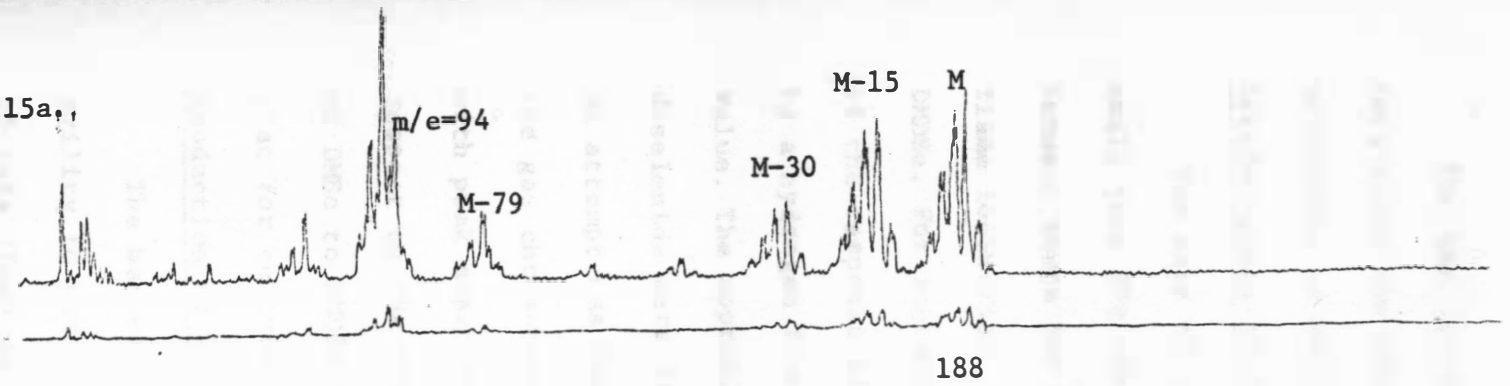


Fig. 15b.

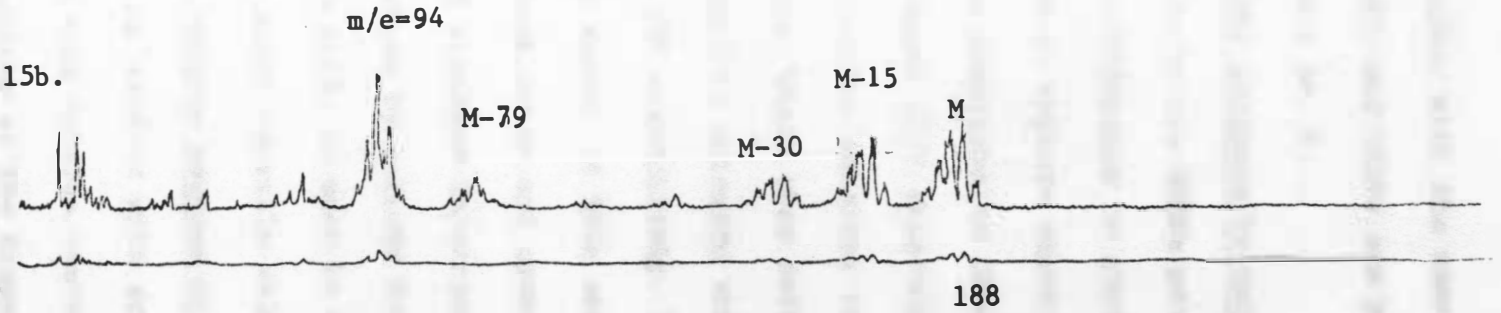


Fig. 15a. Mass spectrum of dimethyl diselenide.

Fig. 15b. Mass spectrum of peak VI from culture No. 1.

The gas chromatographic data together with the mass spectral data allow the conclusion that both DMSe and DMDSe are produced in the metabolism of sodium selenite by culture No. 1.

Determination of the ratio of DMDSe:DMSe produced by culture No. 1.

The size of the DMSe peak relative to the DMDSe peak was quite small (see Fig. 9a for instance). The difference in concentration between these two is even greater than it appears since the hydrogen flame ionization detector is much more sensitive to DMSe than to DMDSe. For instance, Christine and Johnson (37) determined the ratio of the response given by an electron capture detector to that given by a hydrogen flame ionization detector, which they defined as the ϕ value. The approximate ϕ value for dimethyl selenide and dimethyl diselenide were found to be 0.01 and 130 respectively. In this study, an attempt was made to quantitate the amount of DMSe and DMDSe from the gas chromatogram by determining peak areas and comparing them with peak areas obtained from several standard injections. With respect to the volatile products produced by culture No. 1, the ratio of DMSe to DMDSe was determined to be 1:18. It must be concluded that for culture No. 1, DMDSe is the major volatile selenium product.

Production of volatile selenium from various sources by culture No. 1.

The bacterium in culture No. 1 was studied with respect to its ability to produce volatile selenium from various sources. The purpose of this study was to determine the ability of the organism to utilize some of the forms which might be present in soil. Selenate is known to be the main form of soluble selenium in soils, the amino acids

would be added to soils by decomposition of plant material and finally, trimethylselenonium ion would be present from animal excretory products. All selenium sources were included in the media at a level of 20 ppm Se. Fig. 16, 17 and 18 show the chromatograms obtained in the separation of the volatile products and Table 6 summarizes the results. The organism was capable of converting selenite, selenate and selenocystine to both DMSe and DMDSe. It converted trimethylselenonium chloride only to DMSe and it appears that very little selenium could be released from selenomethionine (only a trace of DMSe could be detected). Under the conditions described, selenocystine appeared to be the best source for the production of volatile selenium.

As stated earlier, Doran and Alexander (19) isolated a methionine-utilizing pseudomonad from soil which converted selenomethionine to DMDSe. They indicated that this was somewhat unusual, and in their study they suggested that the microbial formation of DMSe was widespread. On the contrary, from our present research, it seems that the microbiological formation of DMDSe may be widespread. All six cultures which were studied converted selenite to DMDSe. Culture No. 1 identified as P. fluorescens was capable of forming DMSe but only in small amounts relative to DMDSe. Culture No. 2 also appeared to produce both products. It is possible the other organisms produced DMSe but at a level which was not detectable in our study. However, there can be no question that, for these organisms, DMDSe was the

Fig. 16a.

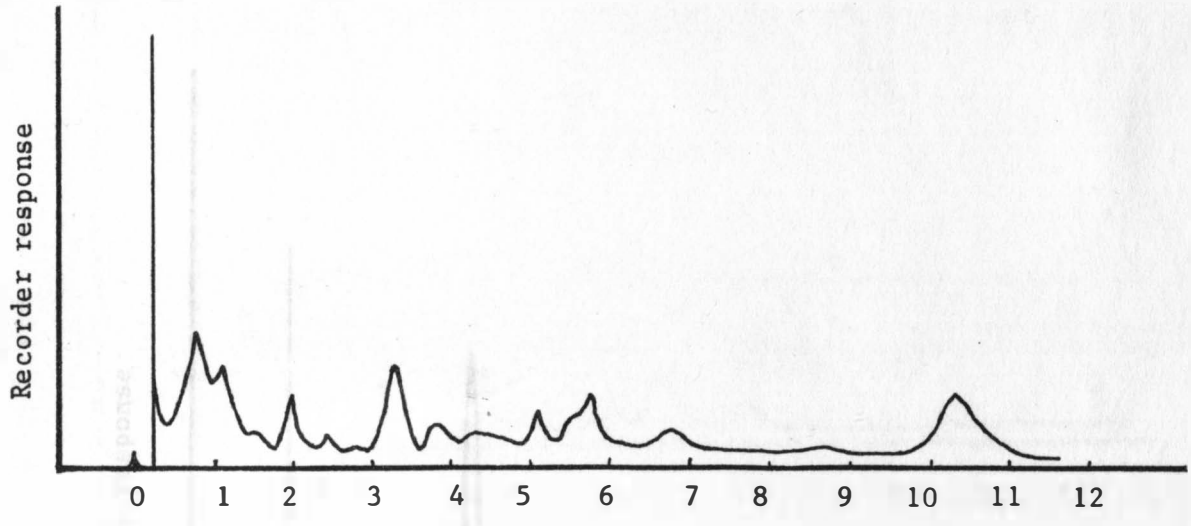


Fig. 16b.

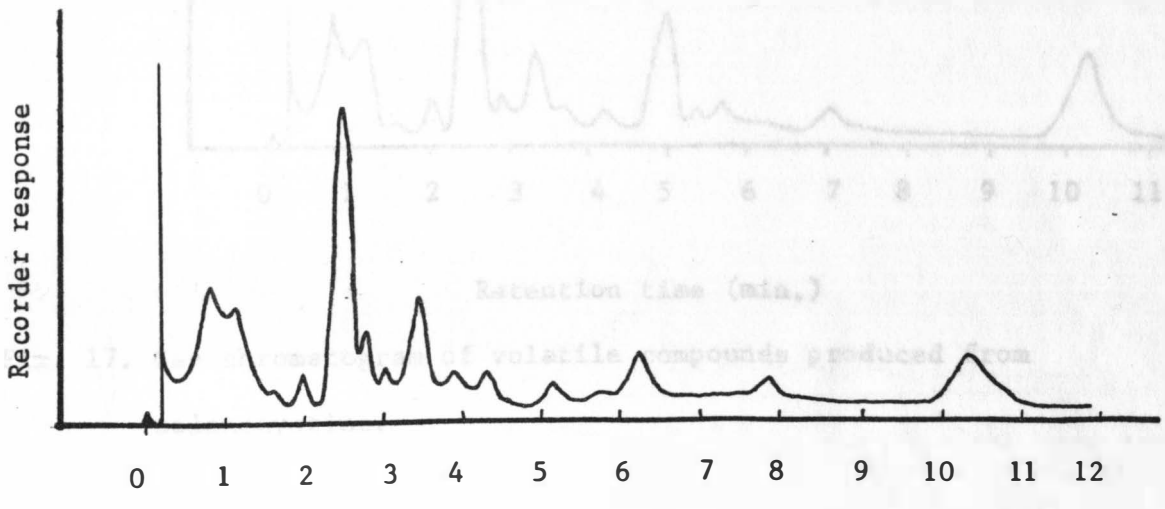


Fig. 16a. Gas chromatogram of volatile compounds produced from selenite.

Fig. 16b. Gas chromatogram of volatile compounds produced from selenate.

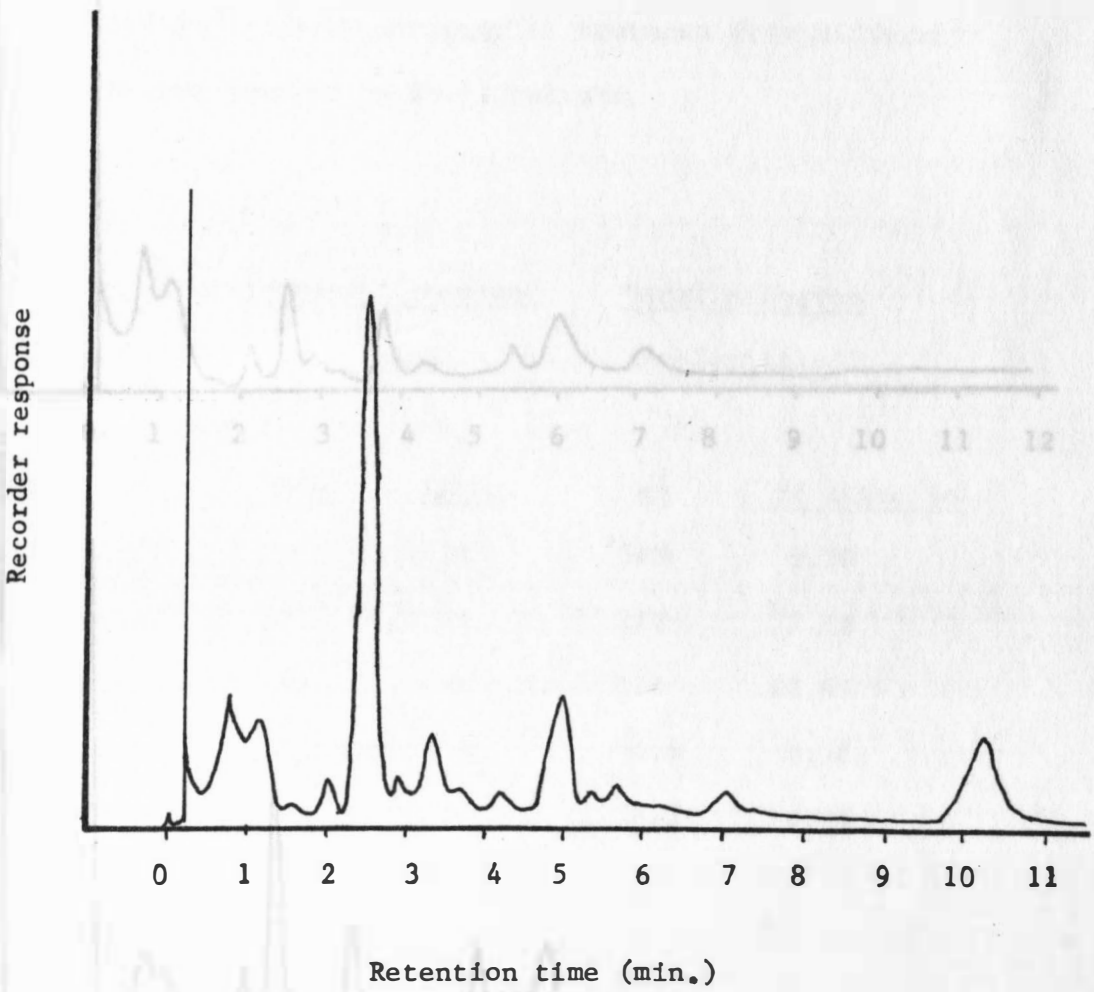


Fig. 17. Gas chromatogram of volatile compounds produced from selenocystine.

Fig. 18a.

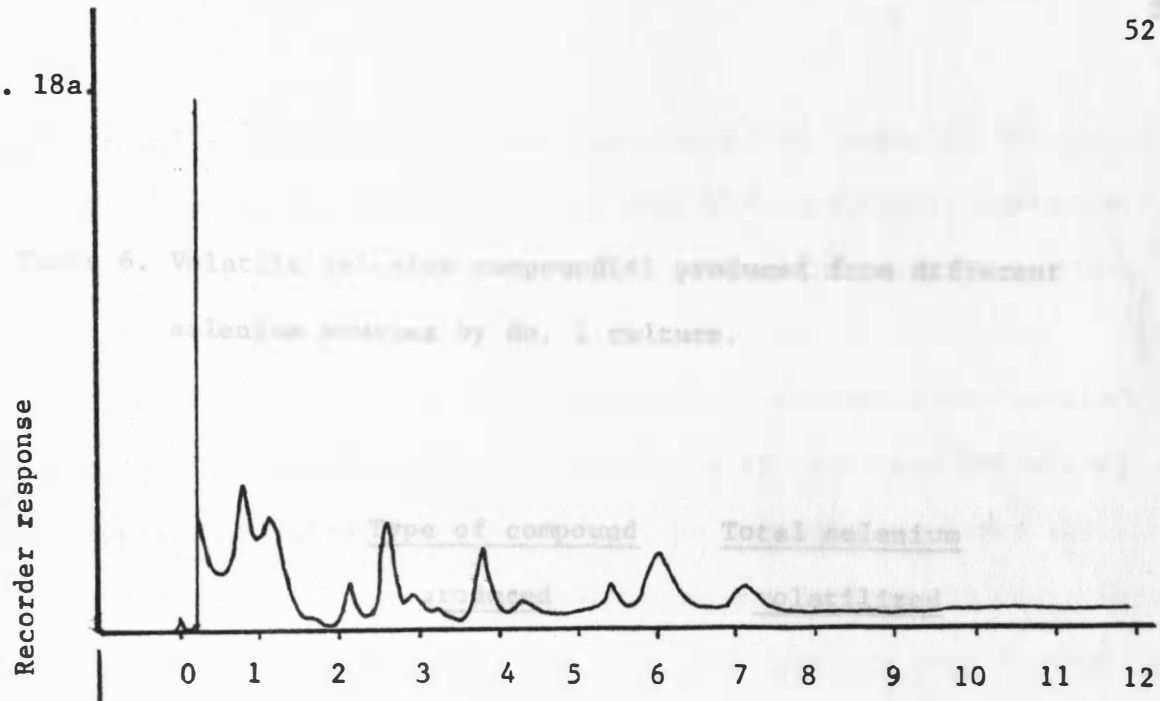


Fig. 18b.

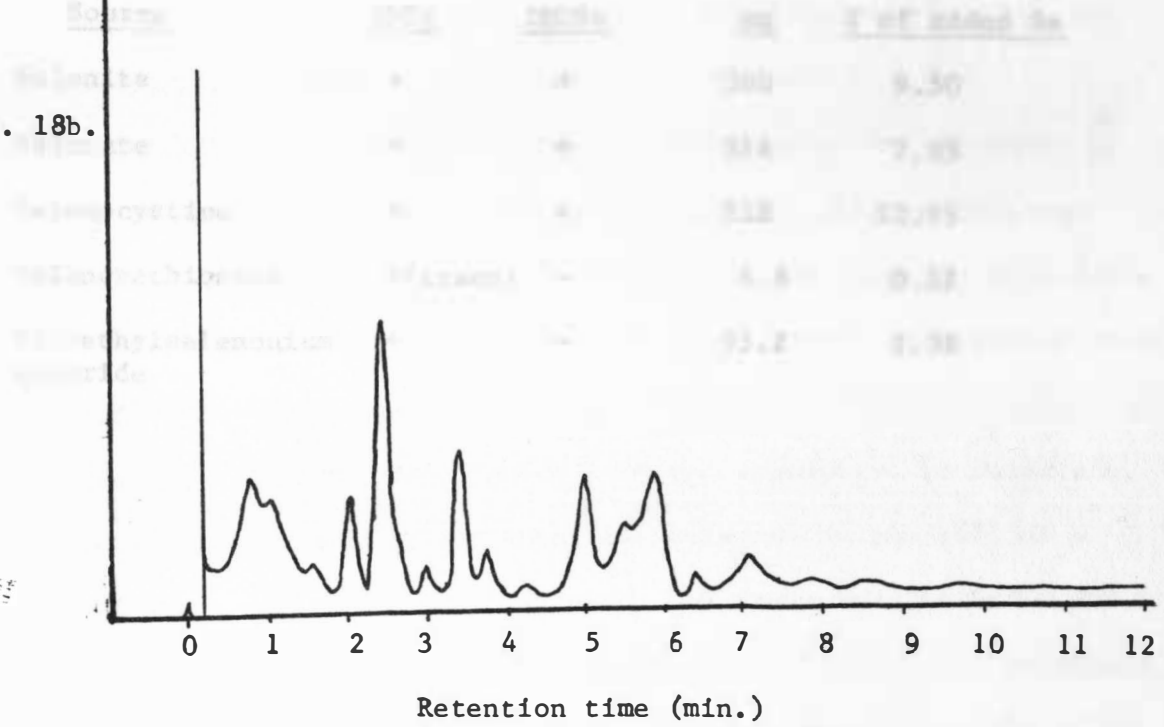


Fig. 18a. Gas chromatogram of volatile compounds produced from trimethylselenonium chloride.

Fig. 18b. Gas chromatogram of volatile compounds produced from selenomethionine.

Table 6. Volatile selenium compound(s) produced from different selenium sources by No. 1 culture.

	Type of compound produced		Total selenium volatilized	
Source	DMSe	DMDSe	µg	% of added Se
Selenite	+	+	380	9.50
Selenate	+	+	314	7.85
Selenocystine	+	+	518	12.95
Selenomethionine	+(trace)	-	4.8	0.12
Trimethylselenonium chloride	+	-	95.2	2.38

major selenium product. It is not surprizing that among the many species of microorganisms, one might find the capability to produce most any product, but this is the first work to indicate that DMDSe is a major product from bacteria.

It is interesting that Se-methyl selenocysteine is the dominant seleno-amino acid in the accumulator plants (48,49), and DMDSe has been reported a volatile compound from the accumulator plants (21). On the other hand, Se-methyl methionine is the dominant seleno-amino acid in the non accumulator plants (50), and DMSe has been reported as the major volatile product (9). DMSe has also been shown to be the major volatile product in animals.

The production of DMSe has been considered to be a highly effective detoxification mechanism in animals, because DMSe was shown to be about one-five hundredth as toxic as selenite in animals (24). Probably the formation DMDSe may be an effective detoxification mechanism for microorganisms.

The fate of the DMDSe released to the atmosphere is uncertain, but it can most likely be oxidized and returned to the soil as a part of the selenium cycle. This study has shown that bacteria are capable of converting natural forms inorganic selenium and selenium compounds originating from plant and animal residues to DMDSe, which may in turn be changed to another form after a period of time and return to the earth in rain water to complete the selenium cycle.

SUMMARY

The objectives of this work were to isolate bacteria capable of volatilizing selenium, and to identify the products which were produced. Six cultures which could volatilize selenium were isolated from the soil. All six cultures were found to fit into Part 7 of Bergey's manual. One of these was identified as Pseudomonas fluorescens. The other five cultures resembled Pseudomonas but were not definetly classified.

The organism identified as P. fluorescens converted 22.5 to 47.3% of the selenium present in the medium as selenite, to volatile forms. The volatile products were identified as dimethyl selenide and dimethyl diselenide by gas chromatography and mass spectrometry. These compounds were present in the ratio of 1:18. P. fluorescens was also capable of producing both products from selenate and selenocystine, but only dimethyl selenide was produced from selenomethionine and trimethyl-selenonium chloride.

All of the other five cultures were capable of producing dimethyl diselenide from selenite. Only one of these cultures (culture No. 2.) was able to produce dimethyl selenide in detectable amounts.

On the basis of this work, it can be concluded that many bacteria are capable of volatilizing selenium, and that dimethyl diselenide is the major volatile selenium product for several of these bacteria.

REFERENCES

1. Muth, O.H., J.E. Oldfield, and P.H. Weswig, 1967. Symposium: Selenium in biomedicine. The AVI Publishing Company, Inc., Westport, Conn.
2. Challenger, F., D.B. Lisle, and P.B. Dransfield, 1954. Studies on biological methylation. Part XIV. The formation of trimethylarsine and dimethyl selenide in mould cultures from methyl sources containing ^{14}C. J. Chem. Soc., pp. 1760-1771.
3. Ganje, T.J., and E.I. Whitehead, 1958. Evolution of volatile selenium from Pierre shale supplied with selenium-75 as selenite or selenate. Proc. S.D. Acad. Sci. 37:81-84.
4. Abu-Erreish, G.M., E.I. Whitehead, and O.E. Olson, 1968. Evolution of volatile selenium from soils. Soil Science 106:415-420.
5. Doran, J.W., 1976. Microbial transformations of selenium in soil and culture. Ph.D. dissertation, Cornell University, Ithaca, N.Y.
6. Wood, J.M., F.S. Kennedy and C.G. Rosen, 1968. Synthesis of methylmercury compounds by extracts of a methanogenic bacterium. Nature 220:173-174.
7. Chem. and Eng. News. 1971. July 19, pp. 29-33.
8. Franke, G.W., 1934. A toxicant occurring naturally in certain samples of plant foodstuffs. I. Results obtained in preliminary feeding trials. J. Nutr. 8:597-608.
9. Rosenfeld, I., and O.A. Beath, 1964. Selenium-geobotany, biochemistry, toxicity, and nutrition. Academic Press, New York.

10. National Academy of Sciences. 1976. Medical and biologic effects of environmental pollutants. Selenium. Washington, D.C.

11. Trelease, S.F., and H.M. Trelease, 1938. Selenium as a stimulating and possibly essential element for certain plants. Science 87:70

12. Schwarz, K, and C.M. Foltz, 1957. Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc. 79:3292-3293.

13. Nesheim, M.C., and M.L. Scott, 1961. Nutritional effects of selenium compounds in chicks and turkeys. Fed Proc. 20:674-678.

14. U.S. Department of Health, Education and Welfare. Food and Drug Administration. 1974. Selenium in animal feed. Fed. Reg. 39:1355.

15. Stadtman, T.C., 1974. Selenium biochemistry. Proteins containing selenium are essential components of certain bacteria and mammalian enzyme systems. Science 183:915-922.

16. Challenger, F., and H.E. North, 1934. The production of organo-metalloidal compounds by microorganisms. PartII. Dimethyl selenide. J. Chem. Soc. PP. 68-71.

17. Challenger, F., 1945. Biological methylation. Chem. Rev. 36:315-361.

18. Fleming, R.W., and M. Alexander, 1972. Dimethyl selenide and dimethyl telluride formation by a strain of Penicillium. Appl. Microbiol. 24:424-429.

19. Doran, J.W., and M.Alexander, 1976. Microbial formation of volatile selenium compounds in soil. Soil Sci. Soc. Am. J. 40:687-690.

20. Beath, O.A., H.F. Eppson, and C.S. Gilbert, 1937. Selenium distribution in and seasonal variation of type vegetation occurring on seleniferous soils. J.Am. Pharm. Assoc. 26:394-405.

21. Christine, S.E., C.J. Asher, and C.M. Johnson, 1968. Isolation of dimethyl diselenide and other volatile selenium compounds from Astragalus racemosus (Pursh.). Astralian J.Biol. Sci. 21:13-20.

22. Lewis, B.G., C.M. Johnson and T.C. Broyer, 1974. Volatile selenium in higher plants. The production of dimethyl selenide in cabbage leaves by enzymatic cleavage of Se-methyl selenomethionine selenonium salt. Plant and Soil 40:107-118.

23. McConnell, K.P., and O.W. Portman, 1952. Excretion of dimethyl selenide by the rat. J. Biol. Chem. 195:277-282.

24. McConnell, K.P., and O.W. Portman, 1952. Toxicity of dimethyl selenide in the rat and mouse. Proc. Soc. Exptl. Med. 79:230-231.

25. Byard, J.L., 1969. Trimethyl selenide, a urinary metabolite of selenite. Arch. Biochem. Biophys. 130:556-560.

26. Palmer, I.S., D.D. Fisher, A.W. Halverson, and O.E. Olson, 1969. Identification of a major selenium excretory product in rat urine. Biochim. Biophys. Acta. 177:336-342.

27. Obermeyer, B.D., I.S. Palmer, O.E. Olson, and A.W. Halverson, 1971. Toxicity of trimethylselenonium chloride in the rat with and without arsenite. Toxicol. Appl. Pharmacol. 20:135-146.

28. Ganther, H.E., 1966. Enzymic synthesis of dimethyl selenide from sodium selenite in mouse liver extracts. Biochemistry 5:1089-1098.

29. Hsieh, H.S., and H.E. Ganther, 1975. Acid-volatile selenium formation catalyzed by glutathione reductase. Biochemistry 14:1632-1636.

30. Shrift, A., 1964. A selenium cycle in nature? Nature 201:1304-1305.

31. Allaway, W.H., E.E. Cary, and C.F. Ehlig, 1967. The cycling of low levels of selenium in soils, plants, and animals. In Symposium: Selenium in biomedicine, edited by Muth, O.H., J.E. Oldfield, and P.H. Weswig, 1967. pp. 273-296.

32. Painter, E.P., 1941. The chemistry and toxicity of selenium compounds with special reference to the selenium problem. Chem. Rev. 28:179-213.

33. Cary, E.E., G.A. Wieczorek, and W.H. Allaway, 1967. Reactions of selenite-Se added to soils that produce low selenium forages. Soil Sci. Soc. Am. Proc. 31:21-26.

34. Francis, A.J., J.M. Duxbury, and M.Alexander, 1974. Evolution of dimethyl selenide from soils. Appl. Microbiol. 28:248-250.

35. Lewis, B.G., C.M. Johnson, and C.C. Delwiche, 1966. Release of volatile selenium compounds by platns. Collection procedures and preliminary observations. J. Agr. Food Chem. 14:638-640.

36. Olson, O.E., I.S. Palmer, and E.E. Cary, 1975. Modification of the official fluorometric method for selenium in plants. J. Assoc. Offic. Anal. Chem. 58:117-121.

37. Christine, S.E., and C.M. Johnson, 1966. The separation of some alkyl selenium compounds by gas chromatography. J. Chromatog. 21:202-206.

38. Bird, M.L., and F. Challenger, 1942. Potassium alkaneselenonates and other alkyl derivatives of selenium. J. Chem. Soc. 570-574.

39. Trelease, S.F., and O.A. Beath, 1949. Selenium. Champlain Printers, Burlington, Vermont. pp. 266.

40. Buchanan, R.E., and N.E. Gibbons, 1974. Bergey's manual of determinative bacteriology. Eighth edition. The Williams & Wilkins Co., Baltimore.

41. MacFaddin, J.F., 1976. Biochemical tests for identification of medical bacteria. The Williams & Wilkins Co., Baltimore.

42. Lennette, E.H., E.H. Spaulding, and J.P. Truant, 1974. Manual of clinical microbiology. Second edition. American Society for Microbiology. Washington, D.C.

43. Rand, M.C., A.E. Greenberg, and M.J. Taras, 1975. Standard methods for the examination of water and wastewater. 14 th edition. Amer. Pub. Health Assoc., Washington, D.C. pp. 899.

44. Taras, M.J., A.E. Greenberg, R.D. Hoak, and M.C. Rand, 1971. Standard methods for the examination of water and wastewater. 13 th edition. Amer. Pub. Health Assoc., Washington, D.C. pp. 655.

45. Blazevic, D.J., and G.M. Ederer, 1975. Principles of biochemical tests in diagnostic microbiology. A Wiley Biomedical Publication. New York. pp. 3-6.

46. Sherris, J.C., and J.G. Shoesmith, 1959. Tests for the rapid breakdown of arginine by bacteria:Their use in the identification of Pseudomonas. J. Gen. Microbiol. 21:389-396.

47. Clark, W.A., 1976. Notes. A simplified Leifson flagella stain. J.Clin. Microbiol. 3:632-634.

48. Shrift, A., and T.K. Virupaksha, 1963. Biosynthesis of Se-methyl selenocysteine from selenite in selenium-accumulating plants. Biochim. Biophys. Acta 71:483-485.

49. Shrift, A., and T.K. Virupaksha, 1965. Seleno-amino acids in selenium-accumuluating plants. Biochim. Biophys. Acta 100:65-75.

50. Virupaksha, T.K., and A.Shrift, 1965. Biochemical differences between selenium accumulator and non-accumulator Astragalus species. Biochim. Biophys. Acta 107:69-80.