Dimethylselenide and Dimethyltelluride Formation by a Strain of *Penicillium*

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Received for publication 20 June 1972

A strain of *Penicillium* which produced dimethylselenide from inorganic selenium compounds was isolated from raw sewage. Sulfate and methionine enhanced growth of the fungus and its production of dimethylselenide in media containing selenite. In solutions containing selenate, methionine inhibited dimethylselenide formation while stimulating proliferation of the fungus. Dimethylselenide was also generated from inorganic selenide. Alkylation did not appear to be a significant mechanism of selenium detoxication by this organism. Dimethyltelluride was also produced by the organism from several tellurium compounds, but this product was synthesized only in the presence of both tellurium and selenium. The yields of dimethylselenide and dimethyltelluride varied with the relative concentrations of selenium and tellurium in the medium.

Recent reports of the microbial methylation of mercury (5, 10, 17) have refocused attention on the possibility of the transformation of an inorganic form of a toxic element to a volatile and possibly hazardous organic product. Although the methylation of arsenic, selenium, and tellurium has been known for some time (2), factors affecting the reaction sequences are not adequately understood, and the possibility that such alkylations may take place in nature has largely been ignored.

Selenium is of particular interest as a potential environmental toxicant because of the small safety margin between the levels necessary in the diet and the concentrations which are hazardous to man (3). Moreover, Schroeder and co-workers (13) found that organic selenium compounds had longer retention times in rats than did inorganic selenium salts, thus providing a greater opportunity for the accumulation of toxic levels of this element. Thus, because of the narrow safety factor for selenium and the increased retention time of organic selenium compounds, it is of considerable importance to assess the possible genesis of organic selenium compounds by microorganisms.

The purpose of the present investigation was to establish some of the factors affecting the methylation of selenium. Inasmuch as the toxicity of selenium to microorganisms is overcome by additions of organic or inorganic sulfur compounds (6, 14, 16), the influence on the methylation reaction of some of these protective agents was characterized. During the study, it became evident that methylation of tellurium also was brought about by the test fungus, and this alkylation was therefore also investigated.

**MATERIALS AND METHODS**

Raw municipal sewage was amended with 1,000 μg of Na₂SeO₄/ml, and samples from which volatile selenium compounds were produced were plated on a solid medium containing 5.8 g of maleic acid, 1.0 g of glucose, 0.50 g of NH₄NO₃, 0.25 g of MgSO₄·7H₂O, 0.15 g of CaCl₂·2H₂O, 99 mg of K₃HPO₄, 2 mg of FeSO₄·7H₂O, 6.02 g of tris(hydroxymethyl)aminomethane (Tris), and 15 g of agar, per liter. A fungus which produced dimethylselenide from inorganic selenium compounds was isolated, and it was identified as a strain of *Penicillium*. The fungus was regularly grown in this medium but with agar being omitted. Tris-maleate buffer (8) was used in media containing HgCl₂ to avoid the formation of a precipitate in the presence of phosphate buffer. The *Penicillium* was unable to utilize the organic buffer as a carbon source, as indicated by its inability to grow in the medium in the absence of glucose. The medium was buffered at pH 7.6 when Na₂SeO₄ was the selenium source and at pH 6.6 when Na₂SeO₄ was employed. The formation of dimethylselenide from selenite was somewhat greater at pH 6.6 than at pH 7.6, hence the choice of acidities.

To measure the formation of volatile products, 50
ml of medium was placed in a 165-ml dilution bottle. Rubber serum stoppers, which were easily penetrated for withdrawal of gas samples, were used to cap the incubation vessels. A spore inoculum was prepared by growing the organism on Sabouraud dextrose agar (Difco). The spores were washed from the agar surface with sterile medium, and they were dispersed and suspended to a final optical density of 0.20 to 0.25 at 520 nm. A 1.0-ml fraction of this suspension was used as the inoculum for each 50 ml of medium. The cultures were incubated at room temperature without shaking, and they were routinely capped with serum stoppers about 72 hr after inoculation. Since little dimethylselenide was ever detected during the first 72 hr, the vessels were sealed only with foam plugs during this period to permit entry of air into the bottles. The yield of volatile product refers to the amount produced by 50 ml of culture.

The extent of growth was measured by filtration of each culture through a preweighed membrane filter (0.45-nm pore size; Millipore Corp.). The filter was dried to a constant weight at about 105°C and reweighed.

For gas chromatography of volatile metabolites, a Varian Aerograph gas chromatograph, model 1700, with flame ionization detector was used. The column components, Chromosorb 101 (60/80 mesh) and 5% FFAP on Chromosorb G (100/120 mesh), were obtained from Applied Sciences Laboratories, State College, Pa. The column temperatures were 80°C for FFAP and 200°C for Chromosorb 101, and the flow rate of the carrier gas, N₂, was 106 ml/min. The finding of retention times on these columns identical to those of authentic dimethylselenide and dimethyltelluride, obtained from Alfa Chemicals, Beverly, Mass., was considered as presumptive evidence for the identification of the gases. The yield of various volatile products was determined from the peak areas in gas chromatograms as compared with the areas given by known amounts of the authentic compounds, or the yield was expressed as the peak areas per unit weight of mycelium.

The identification of the microbial metabolites was confirmed with a Perkin-Elmer gas chromatograph-mass spectrometer, model 270, fitted with a Chromosorb 101 column. The temperature of the chromatograph was programmed at 10°C/min from 100 to 250°C. Mass spectral analyses were performed at 70 eV. Dimethylselenide was characterized by major peaks at m/e (mass to charge ratio) of 80, 81, 93, 95, and 110, with an overall fragmentation pattern like that of authentic dimethylselenide.

RESULTS

The ability of the Penicillium isolate to produce dimethylselenide was tested initially in the defined medium, municipal sewage sterilized by autoclaving, and raw sewage, each supplemented with 1,000 μg of Na₂SeO₃/ml. Dimethylselenide was evolved from both the defined medium and sterile sewage (Fig. 1). After the initial phase of volatile selenium production, an anomalous rise was noted in the release of the gas from the amended sterile sewage. Only 1.7 μg of dimethylselenide was generated in raw sewage in 7 days, a result either of the inability of the fungus to establish itself in the heterogeneous community or of the activity of some native sewage resident. In these experiments, the culture vessels were not sealed and gas production was not measured until 7 days after inoculation of the raw sewage. In other studies, up to 2.7 μg of dimethylselenide was generated in the raw sewage.

In the previous study, a maximum of less than 2.0% of the added selenium was recovered as dimethylselenide in the 7-day period following sealing of the culture vessels. On the other hand, in tests performed under identical conditions in defined medium containing 10 μg selenite/ml, 13 to 24% of the added selenium was converted to the volatile metabolite.

The fungus grew well in defined medium containing 1,000 μg of Na₂SeO₃/ml, but it developed quite slowly in solutions with as little as 100 μg of Na₂SeO₃/ml. However, the inhibitory effect of selenate was reversed as the sulfate level in the medium was increased (Table 1). With 0.1 mM sulfate, the total fungal growth was similar in the presence of 1,000 μg of
either Na₂SeO₃ or Na₂SeO₄/ml. Sulfate also had a dramatic influence on dimethylselenide production (Fig. 2). The data are expressed as the peak area (m²) of dimethylselenide formed/mg of hyphal dry weight to correct for the effects of sulfate on mycelial development. It is evident that dimethylselenide formation in defined medium with selenium increased with increasing sulfate concentrations, although the quantity of the gaseous metabolite declined somewhat at the lower selenium level in the presence of 0.1 M MgSO₄.

The reversal of selenium toxicity by MgSO₄ and its enhancing effect on dimethylselenide evolution were apparently the result of the sulfate rather than of magnesium ions. Thus, adding concentrations of MgCl₂ ranging from 10⁻⁴ to 10⁻¹ M failed to cause a significant change in the amount of dimethylselenide produced per unit weight of mycelium.

The possible influence of DL-methionine, DL-homocysteine, and DL-homocystine on growth and gas release was also evaluated. The latter two compounds had no effect on fungal proliferation or dimethylselenide formation in defined media containing selenium or selenate when the sulfur compounds were added at concentrations up to 5 mg/ml. By contrast, methionine markedly influenced growth and the methylation of selenium. Thus, a stimulation of growth was found upon the addition to a selenate-containing medium of as little as 20 μg of DL-methionine/ml (Table 2). High concentrations of this amino acid did not fully overcome the selenate inhibition. By contrast, methionine greatly suppressed the formation of dimethylselenide from selenium, the extent of inhibition being correlated with the selenate concentration in the medium.

Methionine had a different effect on gas production from selenium (Table 3). The amino acid enhanced rather than inhibited dimethylselenide evolution in media containing sulfate at 10⁻⁵ and 10⁻³ M and had a slight stimulatory effect on growth in the presence of 1,000 μg of selenium/ml in the medium with the lower sulfate level. The greater stimulation at lower sulfate levels may result from the fact that methionine can replace sulfate as a protective agent against selenium toxicity, as reported by Weissman and Trelease (16).

Sodium selenide was also converted to dimethylselenide by Penicillium sp. An evaluation was not made of the percentage of the selenide which was volatilized because of the small amount of the selenide in solution.

The possible competitive influence of salts of other heavy metals on dimethylselenide formation was determined using 20 to 200 μg of TeCl₄/ml; 50 to 300 μg of H₂TeO₃/ml; 250 to 5,000 μg of H₄TeO₄/ml; 100 to 4,000 μg of AsCl₃/ml; 100 to 4,000 μg of Na₃H₂AsO₄·7H₂O or Na₂AsO₃·2H₂O; 10 to 100 μg of HgCl₂/ml; and 10 to 200 μg of CaCl₂/ml. Of these compounds, only TeCl₄, H₂TeO₃, and H₄TeO₄ affected dimethylselenide production. The influence of these compounds can be illustrated by the results of a study of the influence of TeCl₄ on

![Table 1. Yield of hyphae after 104 hr of growth in media with various levels of selenate, selenite, and sulfate](image)

![Figure 2. Effect of sulfate concentration on dimethylselenide production in media containing selenite. The cultures were incubated for 72 hr and were sealed, and dimethylselenide production was measured during the next 32 hr.](image)
TABLE 2. Effect of methionine on growth and dimethylselenide production in the presence of various concentrations of selenate

| Selenate concn (µg/ml) | Hyphal wt (mg) at: DL-Methionine concn (µg/ml) | Se(CH₃)₂ formed at* (µg/ml) |
|------------------------|---------------------------------------------|-----------------------------|
|                        | 0 20 100 200 500                           | 0 20 100 200 500             |
| 0                      | 9.8 9.2 12.0 10.1 13.1                     | 16.6 2.6 1.6 0.8            |
| 25                     | 1.2 6.8 6.6 7.6 9.7                       | 46.5 16.9 9.1 2.5 4.2       |
| 100                    | 0.0 3.7 4.2 5.6 5.4                       | 109 93.9 56.0 62.9          |
| 1,000                  |                                            |                             |

* Peak area (mm²)/mg dry weight.

TABLE 3. Effect of methionine on growth and dimethylselenide formation in the presence of various concentrations of selenite and sulfate

| Sulfate concn (m) | Selenite concn (µg/ml) | Hyphal wt (mg) at: DL-Methionine concn (µg/ml) | Se(CH₃)₂ formed at* (µg/ml) |
|-------------------|------------------------|---------------------------------------------|-----------------------------|
|                   |                        | 0 20 40 100 200                              | 0 20 40 100 200             |
| 10⁻²              | 0                      | 15.3 13.7 15.0 12.4                         | 24.6 123 81.4 116          |
| 1,000             | 1,000                  | 5.1 10.6 8.2 11.2                           |                             |
|                   |                        | 25.2 21.3 22.3 22.3                         |                             |
| 10⁻³              | 1,000                  | 14.2 15.6 15.4 18.4                         |                             |
|                   |                        | 14.2 15.6 15.4 18.4                         |                             |

* Peak area (mm²)/mg dry weight.

gas production in a medium supplemented with 100 µg of selenite/ml. The cultures were sealed 72 hr after inoculation, and the yield of volatile products was determined after an additional 48-hr incubation period. This longer trapping period was necessary to evaluate accurately the yield of a second volatile metabolite generated in the presence of TeCl₄.

The generation of dimethylselenide was inhibited by about half by as little as 20 µg of TeCl₄/ml, with the maximal inhibition occurring at a TeCl₄ concentration of 30 µg/ml (Fig. 3). Higher tellurium levels did not further suppress release of this metabolite. The increase in dimethylselenide yield at low TeCl₄ levels is unexplained. As the tellurium level in the medium increased, a second product was generated in increasing amounts. This product was identified as dimethyltelluride on the basis of the identity of its retention time with that of authentic dimethyltelluride in gas chromatographic analysis and the finding of major peaks at m/e of 128, 130, 143, 145, 158, and 160 in mass spectrometric analysis.

Dimethyltelluride was not formed in media devoid of selenium even though TeCl₄, H₂TeO₃, or H₂TeO₅ was added up to concentrations of 200, 300, or 5,000 µg/ml, respectively. This suggests that some step in the reaction sequence leading to the biosynthesis of the methylated compounds is induced by selenium but not by tellurium. The yields of dimethylselenide and dimethyltelluride depended on the relative concentrations of selenium and tellurium in the medium; thus, if the Na₂SeO₃ concentration was increased to 500 µg/ml, dimethyltelluride was not detected unless 50 µg of TeCl₄/ml was present.

The formation of dimethyltelluride also occurred in a medium containing H₂TeO₃ or H₂TeO₅. Of the three compounds tested in the sequence leading to the biosynthesis of the methylated compounds is induced by selenium but not by tellurium. The yields of dimethylselenide and dimethyltelluride depended on the relative concentrations of selenium and tellurium in the medium; thus, if the Na₂SeO₃ concentration was increased to 500 µg/ml, dimethyltelluride was not detected unless 50 µg of TeCl₄/ml was present.

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defined medium with 100 µg of selenite/ml, the best tellurium source for dimethyltelluride formation was TeCl$_4$; for example, detectable quantities of the methylated tellurium compound were noted in media with 30 µg of TeCl$_4$/ml, 200 µg of H$_2$TeO$_4$/ml, or 1,000 µg of H$_2$TeO$_4$/ml. In media with 100 and 200 µg of selenide/ml, 50 and 100 µg of TeCl$_4$/ml were required, respectively, for detectable levels of dimethyltelluride. With selenite at 50 µg/ml, 10 µg of TeCl$_4$/ml was necessary for the appearance of dimethyltelluride, whereas 100 µg of TeCl$_4$/ml was needed if the selenite level was raised to 100 µg/ml. These experiments with selenate were conducted by using the defined medium containing $10^{-3}$ M sulfate, a condition in which selenate is quite toxic to the fungus.

**DISCUSSION**

The finding that dimethylselenide was generated from raw sewage, albeit at a slow rate, is of special significance. The maximal yield in a 7-day test period was 2.7 µg from a 50-ml sample containing 1,000 µg of selenite/ml, but no attempt was made to establish whether larger quantities could be obtained by varying environmental conditions or by using different sources of sewage. Inasmuch as enzymatic methylation is known to be a means by which microorganisms generate new toxicants from mercurials and arsenicals in nature, the potential for dimethylselenide evolution in natural ecosystems requires careful assessment. It is of importance, moreover, to assess whether dimethyltelluride is generated in nature also.

It has been proposed that sulfate partially overcomes selenium toxicity by competitively inhibiting selenium passage into the cell (14, 15). Such a competition might explain the observation that high sulfate levels depressed somewhat the production of dimethylselenide from selenite at 100 but not at 1,000 µg/ml. In plants, however, some selenium enters the cells regardless of the ratio of sulfate to selenium in the medium (9), and the data in this report suggest that selenium still enters the cells of the *Penicillium* isolate at sulfate levels as high as 0.1 M.

Although methylation may be a mechanism of detoxification when the microorganism is grown in the presence of high levels of selenium, an enhancement of methylation does not appear to be responsible for the stimulation of growth by increasing sulfate concentrations. If this stimulation of fungal development by sulfate were indeed the result of selenium detoxication as a consequence of increased methyla-

tion, then probably there should have been a dramatic difference in the amount of the methylated compound formed in the presence of 100 and 1,000 µg of selenite/ml to account for the similar extents of growth. Fels and Cheddlin (7) similarly concluded that the protection against selenium toxicity afforded by methionine was not related to the methylation of the toxicant, although it is known that both D- and L-methionine can donate methyl groups to selenium (4).

Dimethylselenide has been found to be evolved from the lungs of rats (11), and trimethylselenide and trimethylselenonium ions have been reported to be present in the urine of rats fed a selenium-rich diet (1, 12). It is generally believed that the formation of these methylated compounds constitutes a major mechanism of selenium detoxication in the rat, but the findings of the present study do not lend support to the view that methylation prevents selenium toxicity to microorganisms. In addition to the role that the methylation reaction plays in the physiology of the microorganism, however, it is of considerable importance to establish whether such a microbial transformation has ecological significance.

**ACKNOWLEDGMENTS**

We thank J. M. Duxbury and A. J. Francis for performing the mass spectral analyses.

This investigation was supported by Public Health Service training grant ES-00068 from Division of Environmental Health Sciences and Office of Water Resources Research, U.S. Dept. of the Interior.

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