Microbial Methylation of Metalloids: Arsenic, Antimony, and Bismuth

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INTRODUCTION

Biomethylation and Bioalkylation

The synthesis and transfer of methyl groups is an important and widely distributed metabolic process. The following natural products containing one or more methyl groups attached to nitrogen atoms were discovered early in the 19th century: creatine, creatinine, choline, and trimethylamine (113). Another compound, trimethylglycine ([trimethylammonio]acetate), originally named lycine (from *Lycium barbarum*) was later renamed betaine (from *Beta vulgaris*) (113). This discovery contributed the term "betaine" to chemical nomenclature to describe zwitterionic types, for instance $(CH_3)_n X^+–CH_2–COO^-$ (if X = N, $n = 3$; if X = S, $n = 2$).

The first observation of a biological methylation (16) came when His, with an interest in the detoxification of aromatic compounds, administered pyridine to a dog (122). *N*-Meth-

ylpyridine was excreted in the urine. Similar examples of the methylation of aromatic compounds were the conversion, xanthine → methylxanthine in rabbits, and nicotinic acid → trigonelline in dogs (2).

Much later, nutritional experiments showing, for example, that rats could substitute homocystine for methionine in the diet if choline was present led to the concept of transmethylation. This was defined initially as the transfer of a methyl group from one compound to form another *N*-methyl or *S*-methyl compound (80). A very important role for methionine, a methylated sulfur compound (111), was slowly recognized, and *S*-adenosylmethionine (SAM) was identified as the product of the enzymatic activation of methionine in transmethylation reactions (206). The role of SAM as the methyl donor in hundreds of methylation reactions is now well established (36, 37). The mechanism for the de novo biosynthesis of methyl groups has also been determined (90).

Methylation of metalloids. The C, O, N, and S atoms of organic compounds frequently function as methyl group acceptors in primary and secondary metabolic processes (37, 206). This article concerns another phenomenon, the use of metal-

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TABLE 1. Nomenclature of arsenic compounds[a]

Group and compound	Formula
Pentavalent	
Arsenic acid (arsenate)	$(HO)_2AsO(OH)$
Methylarsonic acid (methylarsonate)	$(CH_3)(HO)AsO(OH)$
Dimethylarsinic acid (dimethylarsinate)	$(CH_3)_2AsO(OH)$
Trivalent	
Arsenous acid (arsenite)	$(HO)_2As(OH)$
Methylarsonous acid (methylarsonite)	$(CH_3)(OH)As(OH)$
Dimethylarsinous acid (dimethylarsinite)	$(CH_3)_2As(OH)$

[a] The above names are those used in this review. Alternative names and abbreviations are as follows: for arsenic acid, orthoarsenic acid and arsenic(V) acid; for methylarsonic acid, methanearsonic acid, methylarsinic acid, and monomethylarsonic acid (abbreviations, MMA[V] and MMAA); for dimethylarsinic acid, dimethylarsonic acid, cacodylic acid, and hydroxydimethylarsine oxide (abbreviations, DMA and DMAA); for arsenous acid, arsenious acid and arsonic (III) acid; for methylarsonous acid, monomethylarsonous acid (abbreviation, MMA[III]).

loids as methyl group acceptors with the major emphasis on the production of volatile compounds by microorganisms. Because of its widespread distribution and many uses in agriculture, industry and medicine, most of the research has concerned arsenic. Like nitrogen, arsenic is a member of group 15 of the periodic table (International Union of Pure and Applied Chemistry recommendation). Work with other members of this group, antimony and bismuth, will also be included. It is of interest that antimony and arsenic share some chemical and toxicological properties (96). Microbial biosynthesis of methylated metalloids is now of considerable academic interest, and the physiological actions of the methylated products are of concern in disciplines such as medicine, toxicology and environmental studies.

The term "biomethylation" describes the formation of both volatile and nonvolatile methylated compounds of metals and metalloids. For arsenic, the major volatile compounds formed by biomethylation have the structure, $(CH_3)_nAsH_{3-n}$; for n = 1, 2, and 3, the products are mono-, di-, and trimethylarsine, respectively. The major nonvolatile compounds are methylarsonate and dimethylarsinate. The nomenclature of the arsenic oxyacids is somewhat confusing (Table 1). Potassium arsenite preparations have a variable composition; the commercial article corresponding approximately to $KAsO_2 \cdot HAsO_2$. The "meta-acid" $HAsO_2$ (i.e., H_3AsO_3 minus H_2O) and its salts are not known in solution. Acids will generally be referred to as ionized forms (e.g., arsenate for arsenic acid), since they are presumably present under physiological conditions.

The related term "bioalkylation" refers to "processes involving living cells that cause an alkyl group to become directly bonded through a carbon atom to some ′heavy element′ (defined as any element with atomic number larger than 10)" (60, 236). In view of the extensive occurrence of bioalkylation with a methyl group, i.e., biomethylation, the term "bioalkylation" specifically refers to "processes involving alkyl groups *other* than methyl." For the simplest alkyl groups such as ethyl, propyl, etc., this process is rare. However, $As(C_2H_5)(CH_3)_2$ has been recorded in landfill and sewage gas, and $As(C_2H_5)_3$ has been recorded in landfill gas; they are likely formed by microbial action, thus representing a true bioalkylation (86). Natural gas probably contains $As(C_2H_5)(CH_3)_2$ and $As(C_2H_5)_3$, and chemical mechanisms for their formation have been discussed (129). Relatively nonvolatile arsenic species, $As(C_2H_5)(CH_3)_2$, $As(C_2H_5)_2(CH_3)$, and $As(C_2H_5)_3$, are present in river and harbor sediments (144) as well as nonvolatile $Sb(C_2H_5)_3$ derivatives. Triethyllead is a toxic metabolic product of tetraethyllead (112) and volatile lead species with methyl and ethyl groups have been detected in sewage gas (86).

The term "bioalkylation" has sometimes been used for more-complex naturally occurring materials, for instance, trimethylarsonium betaine, $(CH_3)_3As^+CH_2COO^-$, discovered originally in Australian rock lobster (*Panulirus longipes cygnus*); lipoidal substances such as *O*-phosphatidyl trimethylarsoniumlactic acid (Fig. 1A), found in a marine diatom; and arsenoribofuranosides (Fig. 1B), found in marine algae (110), diatoms, the terrestrial alga *Nostoc commune* var. *flagelliforme* (148), and in oyster tissue (244). These structural types are not present in bacteria and fungi. However, methylarsonate and dimethylarsinate were found in several edible mushrooms (collected in Switzerland and Slovenia) and, as well, arsenobetaine (35). These materials occur in a wide range of terrestrial mushrooms and lichens; other compounds include arsenocholine and the tetramethylarsonium ion (140, 145, 146, 150, 220) and small amounts of some arsenoribofuranosides (140). The use of bioalkylation in this context appears incongruous; all of the materials contain methyl groups attached to the arsenic atom so presumably the biosynthetic process involved is biomethylation.

Although biomethylation of arsenic is very widespread, occurring not only in microorganisms but also in algae, plants, animals, and humans, it is not universal. In the animal kingdom, several monkeys (marmoset, squirrel, and tamarind), chimpanzees, and guinea pigs do not methylate arsenic to any
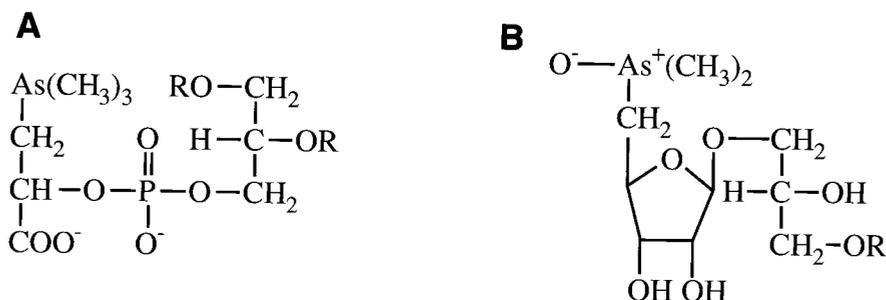

FIG. 1. Examples of arsenolipids. (A) *O*-Phosphatidyltrimethylarsonium lactic acid; (B) an arsenoribofuranoside. Several variations on this structure are observed. In each case, R is a fatty acyl residue.

significant extent (14). There are reviews of biomethylation in general (14, 43, 84, 94, 204, 236) and the methylation of arsenic in particular (15, 119, 173, 227, 232, 235).

The selected topics discussed here are a small component of a very wide area, the overall global role of metalloid elements. In particular, arsenic compounds are widely distributed and transformed on earth not only in the lithosphere but also in the atmosphere and hydrosphere (91, 168). Determination of arsenic species in the environment and the health effects of arsenic exposure have been reviewed (31, 49, 56, 75, 156, 164, 184, 185, 216, 235). The separation and identification of volatile arsines in gases (e.g., in headspaces above microbial cultures) is relatively straightforward, but the identification of nonvolatile components is more difficult. The nonvolatile metabolites may be converted to volatile arsines by hydride generation, for example, by reaction with sodium borohydride as a reducing agent. The advantages of hydride generation atomic absorption spectrometry (AAS) in analysis come from its much lower detection limits—parts per billion compared to parts per million for normal AAS—and from a lowered requirement upon matching the sample matrix which befalls normal AAS (59). At a pH of <4, arsenite is reduced to arsine itself, AsH_3; other conversions of oxyacids can be represented as follows: $(CH_3)_nAsO(OH)_{3-n} \rightarrow (CH_3)_nAsH_{3-n}$ where $n = 1$ to 3 (68, 69). Hence, methylarsonate and dimethylarsinate yield, respectively, monomethylarsine and dimethylarsine. Trimethylarsine oxide yields trimethylarsine. With such techniques, nonvolatile arsenic compounds can be partially identified as mono-, di-, or trimethyl species. Hydride generation can be coupled to separation instruments such as gas chromatography-mass spectrometry (MS) but most often is used with gas chromatography using atomic absorption spectrometric detection (9). With that said, high-pressure liquid chromatography–inductively coupled plasma–MS (HPLC-ICP-MS) is probably the most powerful tool.

Also becoming more widely used for nonvolatile organometalloid analysis are even more powerful so-called hyphenated instrumental techniques such as ion or HPLC-ICP-MS (110, 141, 147). And finally, the extraction of biological materials and subsequent determination of organoarsenicals has very recently been undertaken using microwave digestion followed by UV irradiation, hydride generation, and fluorescence spectrometry (244). This is similar to microwave-assisted extraction of arsenic in soils (114).

There is a wide difference in the arsenic level of marine and terrestrial organisms. In land creatures, the arsenic level is typically about 1 ppm (dry weight), but for marine organisms the values range from a few parts per million to as much as 100 ppm (5, 149, 164). There are practical consequences. While for the U.S. population the normal adult value for urinary arsenic excretion is <50 μg day^{-1}, the value for Japanese adults is three times as high (179). This level of urinary arsenic probably reflects a greater dietary use of marine organisms. A detailed evaluation of "arsenic eaters" has discussed the possibility of the chronic ingestion of arsenic trioxide by otherwise healthy people in hopes of improving a range of factors, from the beauty of women, prophylaxis against disease, to increased sexual potency (197).

Early history. Hofmeister observed that subcutaneous administration of 0.06 g of sodium tellurate to a 3-kg dog gave a strong garlic odor in the expired breath of the dog 30 min later (123). Similar odors were obtained when various organ pulps were treated with tellurium compounds. Influenced by the previously described work of His on pyridine methylation, Hofmeister obtained inadequate evidence that the odorous material in the expired dog breath was "tellurmethyl," i.e., dimethyl telluride, $(CH_3)_2Te$. Nevertheless, he has been credited as the first to observe the biomethylation of a metalloid. Moreover, he certainly recognized the overall significance of methyl group transfer and stated that when pyridine or tellurium are administered, methylation of these compounds may occur, while under normal conditions other methylated compounds such as choline and creatine may be formed. However, he did not propose a specific methyl group donor.

Hofmeister was not the first to have noted odor production during metalloid metabolism. The formation of odorous materials from both selenium and tellurium compounds was described in animal experiments before his work; thus, a garlic smell ("Knoblauchgeruch") was observed in 1824 on dissection of a rabbit poisoned with telluric acid (99). These odors, however, were attributed to hydrogen selenide or hydrogen telluride (for details, see reference 48). Interestingly, a 1951 organic chemistry text states that dimethyl telluride has "the most abominable odour of all organometallics" (50).

ARSENIC FUNGI

Wallpaper: Hazardous to Health

Just before Hofmeister's work, some fungi had been shown to produce a garlic odor when grown in the presence of arsenic. This work, not noted by Hofmeister, marks the beginning of the study of biomethylation of metalloids by microorganisms; surprisingly, the work involved problems with wallpaper.

In the 17th century, a dissertation by Caroli de la Font concerning "The Nature and Causes of the Plague . . . deducing the Pestilential venom from the Air infected and corrupted chiefly by Arsenical Exhalations" had been reviewed in the *Philosophical Transactions of the Royal Society* (10). In the 1809 abridgment of this series, the dissertation was omitted and replaced with the statement that this work was "filled with absurd conjectures and reasoning" (127). However, some two centuries later, a type of toxic "arsenical exhalation" did come to prominence. Beginning about 1800, cases of poisoning in Germany were ascribed to wallpapers (and tapestries) printed with arsenical pigments.

Some of the decorative pigments used in printing were bright green arsenical materials such as Scheele's green (copper arsenite, $CuHAsO_3$) and Schweinfurt (or Schweinfurth) green, also termed Paris green, Vienna green, or emerald green (copper acetoarsenite, $3CuO \cdot As_2O_3 \cdot Cu[OOC \cdot CH_3]$). The use of these pigments was extensive (20). In 1871, arsenical papers were used "in the majority of dwelling houses, from the palace down to the navvy's hut. It is rare to meet with a house where arsenic is not visible on the walls of at least some of the rooms" (12). Moreover, arsenical pigments were used for coloring purposes in many other ways, even in foodstuffs. Many cases of poisoning were recorded (130), and a leading article in 1860 stated that "In the most emphatic manner, we feel it to be a duty to call the attention of medical practitioners to recent lamentable case of fatal poisoning, occasioned by the atrocious practice of coloring hanging-papers with arsenic pigments" (11).

The toxic effects observed in rooms coated with green pigments might have resulted from inhalation of arsenic-containing particles. However, poisoning was also observed where arsenical paper had been covered by fresh paper lacking arsenic, hence formation of dust particles was unlikely. The distinguished chemist Leopold Gmelin recorded in an 1839 newspaper article that an adverse, mouse-like odor was usually present in rooms where poisoning had occurred (100; a translation was kindly provided as a personal communication to T. G. Chasteen by M. Wiggli). He believed that the smell was caused by arsenic that had volatilized as alkarsine [cacodyl oxide, $(CH_3)_2AsOAs(CH_3)_2$]. Cacodyl itself is denigrated by the *Oxford English Dictionary* as "of most disgusting odour." Whether volatilization of arsenic was possible and caused poisoning was a much debated question (40, 157, 207, 208).

Work of Bartolomeo Gosio

Slowly, it was realized that damp and moldy conditions favored the production of a volatile arsenic compound. The Italian physician Bartolomeo Gosio initiated a controlled program to determine how arsenic preparations of use in industry and the domestic economy became a health hazard (101, 102, 104, 105, 107). Although a biography of Gosio (3) lists a "nota preventiva," dated 1890, on the role of fungi in the "gasification" of arsenic, this may be misdated. Gosio stated that he worked on this subject from May 1891 to April 1892; a preliminary account of his work was given in 1891 (103). He used a simple technique to obtain microorganisms producing a garlic odor when grown on a potato mash containing arsenic trioxide (arsenous oxide). Such a substrate, exposed to the air of a cellar, quickly developed colonies of fungi and bacteria and produced the garlic odor. Since pure cultures of the bacteria were odor-free, the gasification was ascribed to the action of fungi.

Initially, Gosio focused on arsenic volatilization by "*mucor mucedo* [*sic*]," presumably the common bread mold. Copper arsenite, the basis for the arsenical colors used in dyeing, was converted to the garlic-odored gas, but sulfide-containing pigments (e.g., orpiment and realgar) were not. Gosio aspirated gas from mixed microbial cultures into a silver nitrate solution; within 3 days there was a marked reduction of the silver salt. The presence of arsenic in the liquid after removal of silver was shown by the chemical Marsh test (102, 104). From 800 g of potato mash containing 0.12 g of arsenic trioxide, Gosio obtained 2.8 mg of metallic arsenic corresponding to the gasification of 4.3 mg of "As^2O^3 [*sic*]." This was a small yield to be sure, but it was a clear demonstration of arsenic volatilization by some saprophytes. Arsenical gases were also obtained from "hangings colored with Scheele's and Schweinfurth's greens, through the vegetation of the mucor . . . hence the danger to those who live in such an atmosphere." At this time, Gosio mistakenly believed that the gas contained arsine itself.

Another fungus showing a very active production of arsenical gas when grown with arsenic compounds was isolated from a piece of carrot exposed to air (102, 104). It was identical to "*penicillium brevicaule* [*sic*]" discovered earlier on rotted paper ("papier putride"). Gosio stated that this organism developed such an intensity of arsenical gas that it was dangerous to approach it ("il est dangereux de s'en approcher"). A rat exposed to the vapor was quickly killed ("Un rat qu'on expose à ces sortes d'émanations tombe rapidement dans des convulsions mortelles"), and a small mouse placed in a vessel with the fungus producing the gas died after a few seconds. When Emmerling reported contradictory results with *Mucor mucedo* and *Aspergillus glaucus*, Gosio vigorously defended his work and regretted that Emmerling had not attempted to obtain suitable cultures from him (82, 106). At the Harvard Laboratory, an American investigator, Charles Robert Sanger, had investigated cases "of chronic arsenical poisoning" from wallpapers but had been unable to demonstrate the volatilization of arsenic. Unlike Emmerling, he corresponded with Gosio, received cultures of *Penicillium brevicaule*, and confirmed Gosio's results (207, 208). Gosio's work was recognized by naming the garlic-odored volatile material as "Gosio gas." It is, perhaps, the only gas named eponymously.

P. brevicaule is now named formally as *Scopulariopsis brevicaulis* (Sacc.) Bainier (200). Historically, *Penicillium* and *Scopulariopsis* species have often been confused; there is, however, one important difference—*Scopulariopsis* species are never green. *Scopulariopsis* species, especially *S. brevicaulis*, are abundant in nature in material such as soil, stored grain and forage, and slowly decaying semidry vegetation (200). Quite typically, Gosio's organism was isolated from rotting paper and a slice of carrot.

Gosio had also shown volatilization of arsenic to a garlic-odored product with *A. glaucus* and *Aspergillus virens*, *Mucor ramosus*, *Cephalothecium roseum*, and *Sterigmatocystis ochracea*. Such organisms, termed "arsenic fungi," were later shown to be more common than previously supposed (237); thus, active strains were found among other aspergilli (*Aspergillus fischeri*, *Aspergillus sydowi*). From soil samples containing enough arsenic to prevent normal growth of certain crops, active gas producers were also obtained; they were two strains of *Fusarium*, a strain of *Paecilomyces*, and a sterile brown fungus. The volatile materials were not identified chemically. This work led to the definition of three microbial responses to arsenic: some microorganisms were inhibited by it, some tolerated arsenic but did not produce arsenical gas, and some tolerated it with production of gas. Further work with arsenic fungi is described later.

Microbiological Test for Arsenic

Gosio developed a sensitive microbiological test for arsenic based on the garlic odor (102, 105, 107, 108). A material possibly containing arsenic was extracted with water or dilute acid, and the extract was evaporated. Small quantities of the residue were added to a substrate and incubation with *S. brevicaulis* followed. After a few hours at 25 to 30°C, the presence of arsenic was indicated by a garlic odor. In an improved method, the suspected material was added to a pregrown culture on a potato slice; a garlic odor developed in about 10 min when arsenic was present. As little as 1 μg of arsenic trioxide in 1 g of material could be detected; the odor threshold for Gosio gas (trimethylarsine) in dilute aqueous solution is now said to be 2 ng kg^{-1} (75). Czapek's solution agar can also be used for growth of *S. brevicaulis* (222). Earlier research has been summarized (165). Although qualitatively more delicate than the Marsh test, the Gosio method is not adapted for

TABLE 2. Formation of mixed alkyl arsines by *S. brevicaulis*

Substrate[a]	Formula	Product
Ethylarsonic acid	$(C_2H_5)AsO(OH)_2$	$(CH_3)_2As(C_2H_5)$
Diethylarsinic acid	$(C_2H_5)_2AsO(OH)$	$(CH_3)As(C_2H_5)_2$
Propylarsonic acid	$(C_3H_7)AsO(OH)_2$	$(CH_3)_2As(C_3H_7)$
Methylpropylarsinic acid	$(C_3H_7)(CH_3)AsO(OH)$	$(CH_3)_2As(C_3H_7)$
Allylarsonic acid	$(C_3H_5)AsO(OH)_2$	$(CH_3)_2As(C_3H_5)$

[a] The substrate names have been modernized from those used by the Challenger group (40).

quantitative use. A modern use of it is described later in connection with the bacterial methylation of arsenic.

WORK OF CHALLENGER AND ASSOCIATES ("LEEDS SCHOOL")

When Gosio gas was passed into a solution of mercuric chloride in dilute HCl (Biginelli's solution) a crystalline precipitate was formed. A small vial of the Gosio/Biginelli mercurichloride is to this day preserved at The Museum of the History of Medicine (Museo di Storia della Medicina) in Rome, Italy. Under the printed name "Laboratorio Batteriologico della Sanità Pubblica" is handwritten, "arsina penicillare comp. mercurico" (C. Serarcangeli, personal communication). From analysis of this material (25, 26) and of the gas itself (107) it appeared that the gas was diethylarsine, $(C_2H_5)_2AsH$. More recently, the aspiration of culture headspace gases into Biginelli's solution has been termed "chemofocusing" (65).

Gosio Gas Is Trimethylarsine

When the reaction between Gosio gas and $HgCl_2$ was reexamined by Challenger and his associates, two crystalline mercuric chloride precipitates were obtained, depending on the time of aspiration (40, 46). These compounds were the di- and mono-mercurichloride of trimethylarsine, $(CH_3)_3As \cdot 2HgCl_2$ and $(CH_3)_3As \cdot HgCl_2$, respectively. Hence, Gosio gas was trimethylarsine. Ironically, trimethylarsine had been discovered as early as 1854, whereas diethylarsine was not known until after Gosio's work. Trimethylarsine is a colorless, oily liquid with a boiling point of 51 to 53°C (760 mm). It was obtained by reaction of magnesium methyl iodide, prepared in pentyl ether, with arsenic trichloride in xylene (81) among other more modern methods.

Trimethylarsine was obtained from the organic compounds sodium methylarsonate, $CH_3AsO(ONa)_2$, and sodium dimethylarsinate, $(CH_3)_2AsOONa$, by action of *S. brevicaulis*. Mixed methylarsines containing other alkyl groups were formed by growth of *S. brevicaulis* on suitable substrates (40) (Table 2).

Challenger Mechanism for Arsenic Biomethylation

The Leeds School suggested a mechanism for trimethylarsine formation (40, 41, 42) that, in its broad details, has stood the test of time. It was proposed before SAM was identified as a nearly universal methyl donor in biological systems and when compounds such as formaldehyde, choline, and betaine were considered to be possible methyl donors in *S. brevicaulis*. It was assumed that a "positive methyl group," CH_3^+, would need to be generated and transferred to the metalloid. For the purpose
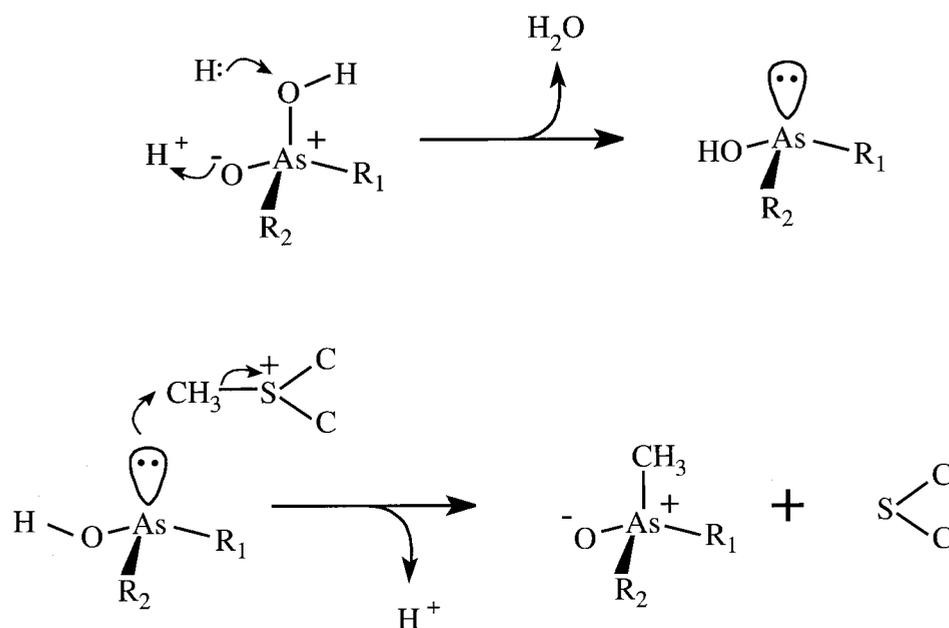
FIG. 2. Typical reactions of the Challenger mechanism. The top line indicates a mechanism for the reduction, As(V) → As(III), resulting in an unshared pair of electrons on As. Structures are as follows: $R_1 = R_2 = OH$, arsenate; $R_1 = CH_3$, $R_2 = OH$, methylarsonate; $R_1 = R_2 = CH_3$, dimethylarsinate. For reduction of trimethylarsine oxide to trimethylarsine, the process is a little different. Following proton addition, the structure $H-O-As^+(CH_3)_3$ reacts with hydride ion leading to elimination of H_2O. The bottom line indicates the methylation of an As(III) structure with SAM [shown in abbreviated form as $CH_3-S^+-(C)_2$]. A proton is released and SAM is converted to *S*-adenosylhomocysteine [abbreviated form, S-(C)$_2$].
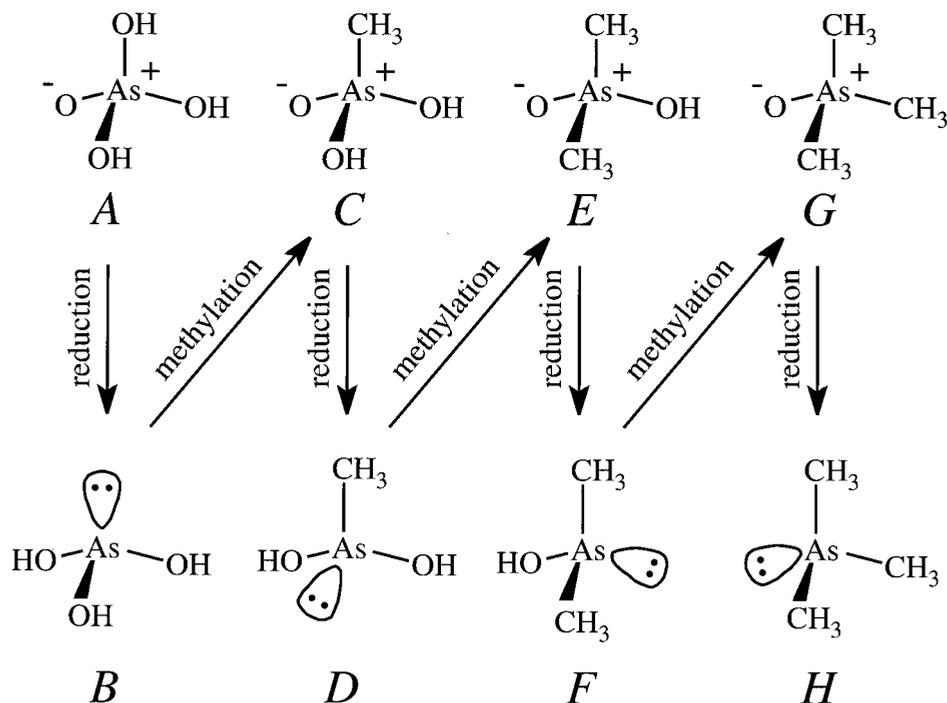
FIG. 3. Challenger mechanism for the conversion of arsenate to trimethylarsine. (A) Arsenate; (B) arsenite; (C) methylarsonate; (D) methylarsonite; (E) dimethylarsinate; (F) dimethylarsinite; (G) trimethylarsine oxide; (H) trimethylarsine. The top line of structures shows the As(V) intermediates. The vertical arrows indicate the reduction reactions to the As(III) intermediates (bottom line), and the diagonal arrows indicate the methylation steps by SAM (see Fig. 2 for details of the reduction and methylation processes).

of the present discussion it will be taken that SAM is, in fact, the methyl donor, and evidence for the assumption is presented later. To proceed from arsenate to trimethylarsine, four two-electron reductions are required. Each reduction produces a compound with an electron lone pair on the arsenic atom; with the exception of the final step, each reduction product is then methylated by SAM. Basic mechanisms for reduction using a hydride ion and methylation using SAM are shown in Fig. 2, as well as a complete and modern version of the individual reaction steps from arsenate to trimethylarsine in Fig. 3. A chemical model for the Challenger mechanism uses $(CH_3)_3S^+PF_6^-$ as the methyl donor and SO_2 as the reducing agent (13a).

Two of the proposed intermediates are poorly defined As(III) compounds—methylarsonous acid, $CH_3As(OH)_2$, and dimethylarsinous acid, $(CH_3)_2AsOH$. For experimental purposes, use is made of the fact that aqueous solutions of the cyclic methylarsine oxide, $(CH_3AsO)_x$, also termed methylarsonous acid anhydride, contain methylarsonous acid: $(CH_3AsO)_x + H_2O \rightarrow CH_3As(OH)_2$. This acid is also present when diiodomethylarsine, CH_3AsI_2, is dissolved in water. Similarly, dimethylarsinous acid may exist as its anhydride, tetramethylarsinous acid anhydride, $(CH_3)_2As-O-As(CH_3)_2$ (239): $[(CH_3)_2As]_2O + H_2O \rightarrow 2(CH_3)_2AsOH$.

It is of interest that methylarsonous acid has been detected in human urine by using ion-pair chromatographic separation of compounds with hydride generation atomic fluorescence spectrometry detection (153, 154). The individuals who were examined normally drank water with a high arsenic level, and urine samples were analyzed after treatment of some of the

subjects with the chelating agent sodium 2,3-dimercapto-1-propanesulfonate.

Understanding the chemical details of the overall scheme has sometimes caused difficulty, in part because of confusion between the term "oxidation number," which seeks to define electron distribution, and "valency" (or "valence"), which refers to the number of combining entities. The normal valency of arsenic is 3 or 5 (e.g., AsF_3 and AsF_5), and the most prominent oxidation states are −III, +III, and +V; the latter two states, As(III) and As(V), are of most concern here. The reductive steps are straightforward in concept but not in practice. Thus, arsenate with the oxidation number +V and a positive charge on the As atom is converted by a two-electron reduction to arsenite, with an oxidation number of +III [As(V) → As(III)]. The other reductions are conceptually similar (Fig. 3).

To convert arsenate to trimethylarsine, the reductions are likely to be enzyme catalyzed with reductants providing the necessary $2e^-$. The reductants are almost certainly thiols, and particular attention has been given to glutathione (GSH) and lipoic acid (6,8-dithiooctanoic acid). There is little experimental evidence from microbial systems, but it is assumed that the reactions resemble those for formation of methylarsonate and dimethylarsinate in mammals (239).

A disulfide redox couple, for instance involving GSH (equation 1), would drive the reduction of As(V) to As(III) in the general process of equation 2 (74).

$$2\,GSH \rightarrow GS\text{-}SG + 2H^+ + 2e^- \qquad (1)$$

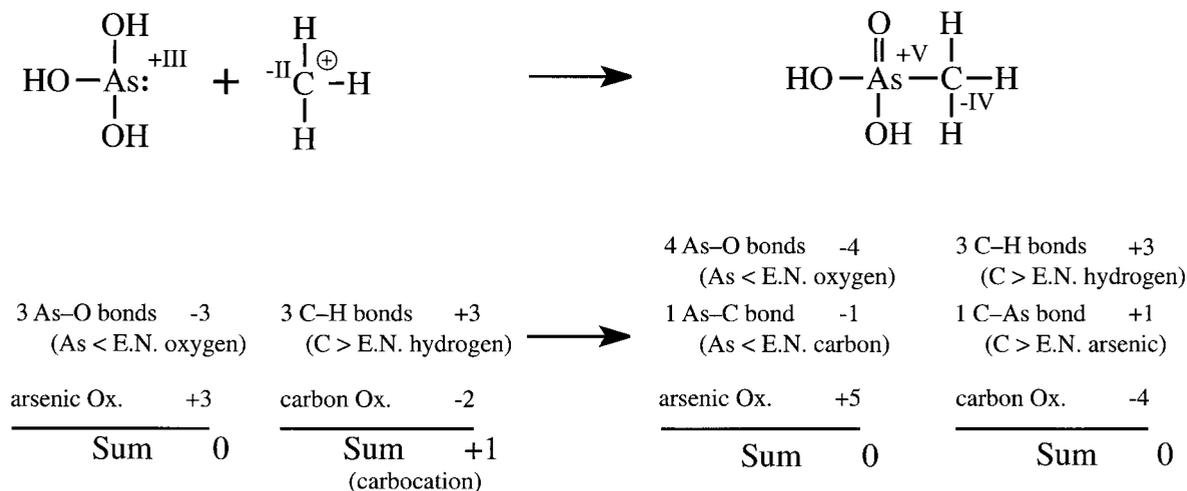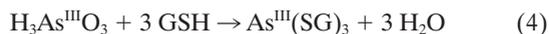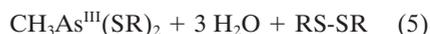$$Thiol + As^V \rightarrow Disulfide + As^{III} + 2H^+ \qquad (2)$$

FIG. 4. Changes in oxidation numbers on As and C during methylation according to Hanselmann's method (117). Abbreviations: Ox, oxidation number; E.N., electronegative (than).
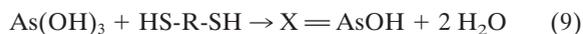
GSH does reduce arsenate to arsenite in vitro (equation 3), and further reaction eventually forms arsenotriglutathione [tri(γ-glutamylcysteinylglycinyl)-trithioarsenite] (equation 4):

$$H_3As^VO_4 + 2 GSH \rightarrow H_3As^{III}O_3 + H_2O + GS\text{-}SG \quad (3)$$

$$H_3As^{III}O_3 + 3 GSH \rightarrow As^{III}(SG)_3 + 3 H_2O \quad (4)$$

For the second reductive step, there is evidence that both in vitro and in vivo GSH does function as a reductant (239). Again, the mechanism is complicated by the ability of arsenic compounds to react with thiols. In model experiments, disodium methylarsonate was easily reduced by cysteine, GSH, or mercaptoethanol, to give derivatives of As(III) (74). For the overall reaction of equation 5, individual steps (equations 6, 7, and 8) were postulated:

$$CH_3As^VO(OH)_2 + 4 RSH \rightarrow$$

$$CH_3As^{III}(SR)_2 + 3 H_2O + RS\text{-}SR \quad (5)$$

$$CH_3As^VO(OH)_2 + 2 RSH \rightarrow$$

$$CH_3As^V(SR)_2(OH)_2 + H_2O \quad (6)$$

$$CH_3As^V(SR)_2(OH)_2 \rightarrow CH_3As^{III}(OH)_2 + RS\text{-}SR \quad (7)$$

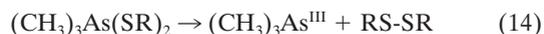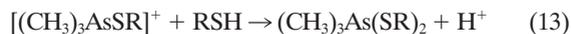$$CH_3As^{III}(OH)_2 + 2 RSH \rightarrow CH_3As^{III}(SR)_2 + 2 H_2O \quad (8)$$

Similar equations can be written for the subsequent reduction steps; it does seem likely that in vivo compounds with RS groups attached to the arsenic atom are involved. Dithiol derivatives such as lipoic acid could be involved, perhaps as a membrane-bound or enzyme-bound component. Thus, for arsenite itself, the reaction of equations 9 and 10 might be involved with the formation of cyclic disulfide derivatives.

$$As(OH)_3 + HS\text{-}R\text{-}SH \rightarrow X=AsOH + 2 H_2O \quad (9)$$

(X represents -S-R-S-)

$$X=AsOH + SAM \rightarrow X=AsOCH_3 \quad (10)$$

The final step of the Challenger mechanism, the reduction of trimethylarsine oxide to trimethylarsine, was possible with a variety of thiol reagents (cysteine, dimercaptopropanol, lipoic acid, mercaptoacetic acid, and mercaptoethanol) (73). On the basis of kinetic and other observations, the detailed mechanism of equations 11 to 14 was postulated. Enzyme- or membrane-bound lipoic acid was regarded as a likely in vivo reductant.

$$(CH_3)_3As^VO + RSH \rightarrow (CH_3)_3As(OH)(SR) \quad (11)$$

$$(CH_3)_3As(OH)(SR) + H_3O^+ \rightarrow$$

$$[(CH_3)_3AsSR]^+ + 2 H_2O \quad (12)$$

$$[(CH_3)_3AsSR]^+ + RSH \rightarrow (CH_3)_3As(SR)_2 + H^+ \quad (13)$$

$$(CH_3)_3As(SR)_2 \rightarrow (CH_3)_3As^{III} + RS\text{-}SR \quad (14)$$
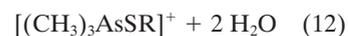
If reductive steps using GSH occur in vivo, the recycling of GS-SG to GSH by GSH reductase is necessary. It is of interest that this enzyme is inhibited by various arsenic compounds, including arsenotriglutathione (230, 231).

The methylation steps are at first sight somewhat confusing, since in all cases an oxidation has occurred, As(III) → As(V). To keep the process in balance there must have been a concomitant reduction. Since no redox cofactor is involved, the process appears at first glance to be a simple methyl transfer process. However, a detailed analysis of the reaction indicates that the necessary reduction is found in the corresponding decrease in oxidation number from −II to −IV for the transferred carbon. Using a formal method for assigning oxidation states to redox-labile elements in organic compounds helps in following the reduction and methylation steps with arsenic as shown in Fig. 4 (117).

Role of SAM

Since the methyl group of [$^{14}CH_3$]methionine was transferred to arsenic in the formation of trimethylarsine by *S. brevicaulis* (47), the postulated role for SAM was substantiated. Similar work has confirmed methyl transfer processes from [C^2H_3] methionine to materials such as As_2O_3, $CH_3AsO_3Na_2$, and $(CH_3)_2AsO_2H$ by the fungi *S. brevicaulis* and *Gliocladium roseum* and the yeast *Cryptococcus humiculus* (for taxonomy of this yeast, see below); the transfers resulted in the formation of ^2H-labeled trimethylarsine (66). The latter organism and *S. brevicaulis* carried out the previously unknown conversion of disodium butylarsonate, n-$C_4H_9AsO_3Na_2$, to butyldimethylarsine.

Nonvolatile di- and trimethylarsenic species were identified in cultures of *C. humiculus*, and a dimethylarsenic compound was identified in cultures of the marine alga *Polyphysa penicilus* (68, 69) by the hydride generation technique (see above). When [C^2H_3]methionine was used as a methyl donor, the ^2H-labeled nonvolatile metabolites produced ^2H-labeled arsines. These observations were consistent with the operation of the Challenger mechanism, with methyl groups being derived from methionine by way of SAM. Moreover, trimethylarsine formation from $(CH_3)_2AsO(OH)$ by these organisms was inhibited by ethionine. Ethionine is an antagonist for methionine; hence, these observations support the role of a methionine-based synthetic path (66, 75).

ARSENIC METHYLATION

Fungi and Yeasts

Challenger et al. observed that *Penicillium chrysogenum* and strains of *Penicillium notatum*, *Aspergillus niger*, and *A. fischeri* did not methylate arsenous acid in the usual bread crumb cultures (27). *A. glaucus* and *Aspergillus versicolor* formed trimethylarsine to a limited extent. However, all of these organisms converted methylarsonate to trimethylarsine. Trimethylarsine was also formed from dimethylarsinate by *P. notatum*, *P. chrysogenum*, and two strains of *A. niger*. The latter organism converted ethylarsonate to dimethylethylarsine and *P. chrysogenum* converted allylarsonate to dimethylallylarsine.

A wood-rotting basidiomycete, *Lenzites trabea*, produced a garlic odor when growing on wood treated with arsenical preservatives. When 65 species of wood-rotting fungi were examined with growth in presence of As_2O_3, only *L. trabea* and *Lenzites saepiaria* produced an unidentified, garlic-odored gas (176). Another wood decaying fungus, *Phaeolus schweinitzii*, produced volatile methylated products of arsenic when grown with "As_2O_5" (19). The strong garlic smell aided the recognition of plates containing this organism in isolation work with a selective medium. More recent work has confirmed the volatilization of arsenic by this fungus, but again the product was not specifically identified (189). The pathogenic fungus *Trichophyton rubrum* produced a "peculiar, nauseating, garlic-like odor" when it was grown in the presence of arsenate or arsenite (262). The volatile product contained an unidentified arsenic component(s).

A historical connection, where the wheel comes full circle, may be noted here. The widely used wood preservative, chromated copper arsenate, is converted to trimethylarsine by the yeast *C. humiculus* (72). Chromated copper arsenate is quite

similar to the arsenical wallpaper pigments that had triggered Gosio's work.

The work of the Leeds School was carried out by tedious chemical operations, usually the formation and analysis of mercuric chloride complexes. Moreover, the useful radioactive isotopes of arsenic were not available; however, as already noted ^{14}C was used in studies of methyl transfer reactions. With the advent of improved analytical techniques such as gas chromatography and gas chromatography-MS, and with the availability of arsenic isotopes, the detection of trimethylarsine and related compounds can be carried out with less labor and greater certainty. A simple gas chromatographic assay was used to monitor production of trimethylarsine by three organisms isolated from sewage and described as fungi (53). Two of these organisms, *G. roseum* and a *Penicillium* species, converted methylarsonate and dimethylarsinate to trimethylarsine under a range of culture conditions; however, neither arsenate nor arsenite was volatilized. The third organism, identified tentatively as *Candida humicola* was said later (69) to have been reclassified as *Apiotrichum humicola.* It had been deposited with the American Type Culture Collection as ATCC 26699 and in the American Type Culture Collection catalog it is now described as *Cryptococcus humiculus* (Daszewska) Golubev. The name *C. humiculus* is used throughout this article; the organism is clearly a yeast. When grown at pH 5.0, it produced small amounts of trimethylarsine from arsenate, arsenite, and methylarsonate, with dimethylarsinate being the best substrate for volatilization.

The variables affecting the methylation of arsenic by *C. humiculus* have been studied. Only a brief account will be given here since two authoritative reviews are available (52, 75). All of the organic As(V) compounds of the Challenger pathway (methylarsonate, dimethylarsinate, trimethylarsine oxide) have been observed in cell extracts metabolizing arsenate, and all of them are precursors for trimethylarsine biosynthesis (70, 195). Preconditioning of cells with dimethylarsinate improves trimethylarsine formation from arsenate and dimethylarsinate. In later experiments, the nonvolatile components of *C. humiculus* and *S. brevicaulis* were identified (67). These experiments used low levels (1 ppm) of the arsenic substrates, and under these conditions there was no detectable formation of trimethylarsine with either organism.

With methylarsonate as substrate, *C. humiculus* produced low levels of dimethylarsinate and trimethylarsine oxide by the second day, with slow increases of both metabolites over 4 weeks. For *S. brevicaulis*, the metabolism was much slower, with trace amounts of dimethylarsinate and trimethylarsine oxide not appearing until after 2 weeks of incubation.

This was the first report of trimethylarsine oxide as an end product metabolite for the two organisms; under the experimental conditions, it was the major product rather than trimethylarsine. Apparently the organisms tolerated low levels of this oxide, and further "detoxification" by conversion to trimethylarsine was not necessary. An extended model (Fig. 5) for arsenic methylation involving both cells and medium was proposed (67). The observed reaction rates suggested a possible sequential transfer of 2 methyl groups from SAM to methylarsonate as indicated. These observations emphasized that the methylation of arsenic by microorganisms is a very complex
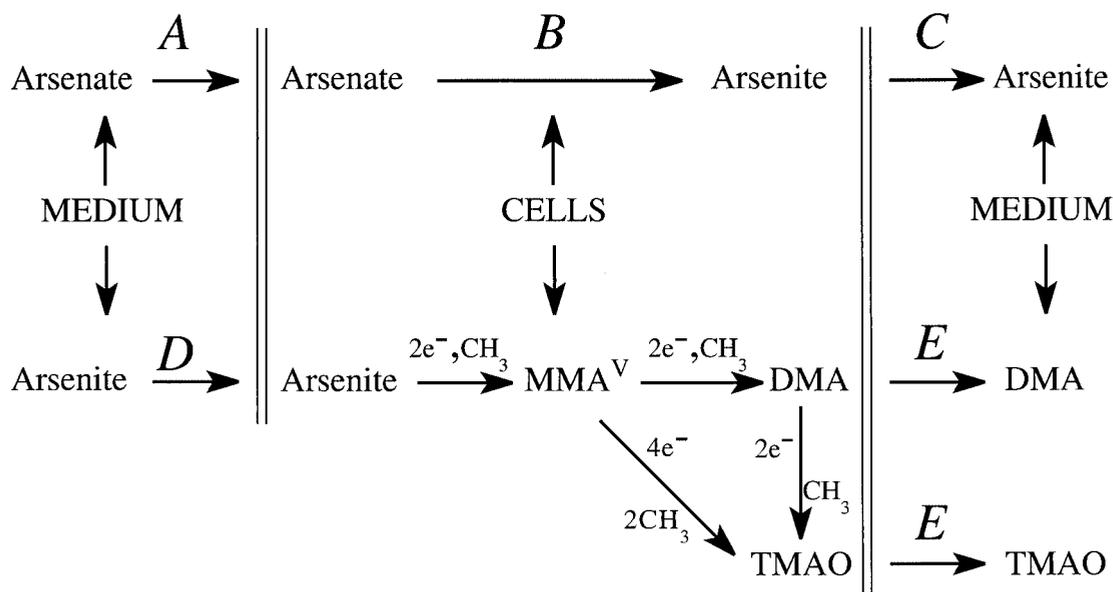
FIG. 5. Expanded version of the Challenger mechanism. This mechanism, proposed for *C. humiculus*, indicates roles for components both in the cells themselves and in the culture medium. The double vertical lines indicate cell walls. (A) Phosphate transport system; (B) thiols and/or dithiols; (C) active transport system; (D) active/passive transport; (E) passive diffusion. Abbreviations: MMA^V, methylarsonic acid; DMA, dimethylarsinic acid; TMAO, trimethylarsine oxide. This diagram is redrawn from scheme 2 of reference 67.

process despite the general simplicity of the Challenger mechanism.

Phosphate strongly inhibited formation of trimethylarsine in cultures growing with arsenate, arsenite, and monomethylarsonate but had no effect when dimethylarsinate was used (54). The reason for this behavior is not clear (75) but it is of interest that phosphorous and arsenic are both members of group 15 of the periodic table. Sodium salts of selenite and selenate were also strongly inhibitory, and sodium tellurate was a moderate inhibitor. *C. humiculus* is known to methylate both selenate and selenite to dimethylselenide (52).

C. humiculus produced a garlic odor when grown in the presence of benzenearsonic acid (phenylarsonic acid, $C_6H_5As[OH]_2$) (65). By use of the chemofocusing technique, the volatile product was shown to be $C_6H_5As(CH_3)_2$. Similarly, methylphenylarsinic acid, $(C_6H_5)(CH_3)AsO(OH)$, was converted to the same dimethylphenylarsine. Arsanilic acid, $H_2NC_6H_4AsO(OH)_2$, was not methylated, but 2-hydroxy-4-aminobenzene-arsonic acid yielded trimethylarsine.

The fungicide methylarsine sulfide, $(CH_3AsS)_x$ (Rhizoctol), produced a garlic odor when grown with *C. humiculus*; the volatile material was a mixture of trimethylarsine and methylarsine in the ratio 90:10. The conversion was rapid, with 50% of added arsenic being converted in 3 days; compare arsenate or dimethylarsinate → trimethylarsine, which resulted in 1% conversion in 10 days (71). Methylarsine sulfide was actually more toxic to *C. humiculus* than either arsenite or the corresponding oxide, $([CH_3]_2AsO)_x$, suggesting that at least in this case, methylation was not a detoxification process. Methylarsine oxide was also rapidly transformed by *C. humiculus* to dimethylarsinate (24 h); small amounts of unidentified volatile arsines were also formed.

The marine yeast *Rhodotorula rubra* an obligate aerobe, converted arsenate to arsenite, methylarsonate, and dimeth-

ylarsinate. Unidentified "volatile arsines" were also produced (243).

Modern analytical techniques for the determination of arsenic species continue to be developed and used (64, 110, 147, 149, 151–154, 190–192, 244). In one study a *Penicillium* species was isolated from an agricultural evaporation pond in an area with high concentrations of soil arsenic. The organism formed trimethylarsine from methylarsonate; addition of carbohydrate and sugar acids to the minimal medium suppressed volatilization, whereas glutamine, isoleucine, and phenylalanine had a stimulatory effect (128). Various elements known to be toxic to living systems generally inhibited the conversion of methylarsonate to trimethylarsine by this *Penicillium* sp. (92). The most inhibitory element was V, followed by Ni, Sn, B, Te, Mn, Mo, Cr, and Ag; at low concentrations the following elements were stimulatory: Hg, Fe, Cu, Al, Zn, and Se. In soil contaminated more than 30 years ago by arsenic trioxide from a cattle dipping vat, a low level of in situ volatilization of arsenic was demonstrated by subsurface probes. From this soil, a *Fusarium* sp. producing unidentified volatile arsenicals was isolated (238). A recent monograph provides more information in this area (92a).

Bacteria

Gosio's pioneering work on arsenic volatilization by fungi was merely the tip of a very large iceberg. However, the bacteria that he isolated from his mixed cultures did not produce a garlic odor in the presence of arsenic. In 1945, Challenger asserted that "the weight of the evidence would indicate that bacteria are unable to produce volatile methyl derivatives of arsenic, selenium and tellurium, the statements to the contrary being based on observations of odor alone" (40); another reviewer stated that there was not a single case recorded in which a methylation was performed by bacteria (16). However, the

possibility of bacterial methylation had not been overlooked. People taking dimethylarsinate (cacodylate) by ingestion were known to exhale a garlic odor, and this was believed to be due to bacterial decomposition of the arsenic compound (198). Several strains of fecal bacteria (named as follows [with the genus not specified] *mesentericus vulgatus*, *B. mesentericus ruber*, and *B. subtilis*) produced a garlic odor when grown in the presence of either dimethylarsinate or arsenate. Although all strains examined produced the odor from dimethylarsinate, one strain each of *B. mesentericus vulgatus* and *B. mesentericus ruber* and three strains of *B. subtilis* did not produce Gosio gas from arsenate. Later workers using cultures of these organisms obtained from a culture collection, rather than being isolated from feces, could not repeat these results (45). Similarly, organisms named *B. coli communis* and *B. lactis aerogenes* did not yield trimethylarsine in the presence of arsenous oxide (again, the genus was not specified). Despite these apparently negative results, it does seem quite possible that bacterial methylation of arsenic using organisms isolated from feces had been observed. More recent evidence suggests that mammalian, intestinal bacteria are responsible for a portion of arsenic metabolism by a pathway different from that of animals. *Escherichia coli* strains from rat cecal contents converted dimethylarsinate into two unidentified, nonvolatile compounds and converted trimethylarsine oxide into a further, nonvolatile, unidentified material (147). Kuroda et al. (147) have detected trimethylarsine oxide in cultures of *E. coli* amended with dimethylarsinate. The fecal culture of rats, from which the bacteria were isolated, reduced arsenate to arsenite. Along with the isolated strains themselves, the urine and feces of those rats produced unidentified arsenic compounds. These unknowns were different from arsenate, arsenite, methylarsonic acid, dimethylarsinic acid, trimethylarsine oxide, tetramethylarsonium ion, arsenocholine, and arsenobetaine.

The metabolism of arsenic compounds widely used in agriculture has been studied in mixed microbial populations from soil. While evidence was obtained for arsenic volatilization, it was usually not clear whether bacteria or fungi were involved. For example, when ^{14}C-labeled dimethylarsinate was added to three soil samples under aerobic conditions, 35% of the added arsenic was converted to a volatile material or materials over a 24-week period, and 41% was converted to [14]CO$_2$ and AsO$_4^{3-}$ (253). Under conditions considered to be anaerobic, the amount of volatilized arsenic increased to 61% and a garlic-like odor was observed. If the conditions (flooding with water) were indeed anaerobic, fungal growth would have been unlikely, and the observed volatilization might have resulted from a mixed bacterial culture. The volatilization of arsenic from a soil treated with [[74]As]H$_3$AsO$_4$, sodium [^{14}C]methylarsonate, and [^{14}C]dimethylarsinate was most rapid under aerobic conditions, but significant yields of di- and trimethylarsine were obtained when the experimental flasks were swept with nitrogen (252). The formation of methylarsine was not detected.

Air samples collected over sodium arsenite-treated lawn grass for 2-day periods, with sample collections usually at 2- or 3-h intervals, reliably gave small amounts of trimethylarsine after about 20 h (29). Dimethylarsine was observed in small amounts only in the last two 2-h intervals. From methylarsonate, trimethylarsine formation began after the first 2-h interval and continued throughout the experiment with substantial

amounts after about 14 h. Specific organisms were not isolated from the mixed cultures, but the formation of dimethylarsine, not usually a fungal product, suggests bacterial action.

When three soil samples were incubated with arsenate, arsenite, methylarsonate, and dimethylarsinate, methyl- and dimethylarsine were only produced from methylarsonate and dimethylarsinate; some arsine production was also observed. Surprisingly, two soil bacteria (a *Pseudomonas* sp. and an *Alcaligenes* sp.) produced only arsine when incubated anaerobically with arsenate or arsenite (51). Arsine (AsH$_3$) formation is relatively uncommon but is discussed later in connection with anaerobic organisms.

Five bacterial species, (*Corynebacterium* sp., *E. coli*, *Flavobacterium* sp., *Proteus* sp., and *Pseudomonas* sp.) isolated from the environment and acclimatized to growth with sodium arsenate for 6 months, transformed arsenate to arsenite, and all of them produced dimethylarsine. The *Pseudomonas* sp. formed all three of the methylated arsines (214). Arsenic accumulated by the cells was in a protein fraction (212). Six bacterial species (*Achromobacter* sp., *Aeromonas* sp., *Alcaligenes* sp., *Flavobacterium* sp., *Nocardia* sp., and *Pseudomonas* sp.) produced both mono- and dimethylarsine from methylarsonate; only two of them produced trimethylarsine. A *Nocardia* sp. was the only organism that produced all of the methylarsines from this substrate (213).

C. humiculus was known to reduce trimethylarsine oxide to trimethylarsine (195), and this oxide was also reduced under both aerobic and anaerobic conditions by several bacteria (194). The most active (142 nmol min^{-1} g of cells^{-1} [wet weight] at 37°C) of four anaerobes (*Fusobacterium nucleatum*, *Veillonella alcalescens*, and two unidentified skin organisms) was *V. alcalescens*. However, anaerobically grown *Staphylococcus aureus* gave the highest rate for any of the organisms tested anaerobically (208 nmol min^{-1} g of cells^{-1} [wet weight]). Similar results were obtained using aerobic organisms; a marine pseudomonad showed the highest activity (585 nmol min^{-1} g of cells^{-1} [wet weight]) with substantial activity (range, 80 to 170 nmol min^{-1} g of cells^{-1} [wet weight]) for *B. subtilis*, *E. coli*, *S. aureus* (grown aerobically), and two unidentified skin aerobes. A further skin aerobe and *Streptococcus sanguis* were much less active. The same conversion was shown by river water, some sea sediments and sewage sludge (low levels in both cases analyzed by odor alone), and rumen fluid (under both aerobic and anaerobic conditions). The authors noted that "the odor of trimethylarsine becomes immediately apparent if some of the oxide is placed on the skin," presumably by the action of the mixed microbial flora (194).

Arsenic-resistant *Pseudomonas putida*, isolated from a contaminated *Chlorella* culture, contained a nonvolatile trimethylarsenic species and excreted into the medium all three methylated species (170). Similar results were obtained with arsenic-resistant *Klebsiella oxytoca* and a *Xanthomonas* sp. (169). None of these organisms formed volatile arsenic compounds.

A sample of soil with a low level of arsenic contamination (As, 1.5 ppm) yielded two arsenic-resistant, nonmethylating bacteria (*Bacillus* sp. and *Pseudomonas fluorescens*) and a new, arsenic-resistant organism, assigned to the *Flavobacterium-Cytophaga* groups, with methylating capacity. It produced only trimethylarsine as the volatile material; arsenite was trans-

formed more rapidly than arsenate. Dimethylarsinate did not yield trimethylarsine (124). Contaminated soil (contaminated with As, Cr, and Cu) from a wood-impregnating plant had methylating activity (production of methylarsonate and dimethylarsinate). There was no mention of arsenic volatilization in this work (241).

A brief report has described the bioleaching of columns of arsenic-contaminated soil percolated with a nutrient medium (240). Arsenic was converted to unidentified volatiles, most efficiently under anaerobic conditions, thus suggesting possible bacterial action. Using garden soil spiked with As_2S_3, the undefined microbial population converted the insoluble material to "nearly 50% water-soluble form within two months" (240). At the same time, small amounts of unidentified volatile arsenicals were produced. Bacterial leaching of arsenic-contaminated materials was suggested as a possible means for bioremediation. Solubilization of arsenic by oxidation from the mineral arsenopyrite (FeAsS) by mesophilic and moderately thermophilic acidophiles has been described without mention of any formation of volatile products (79, 240).

Role of Anaerobic Microorganisms

Following the landmark discovery of biomethylation of mercury (32, 136, 251), it was shown that *Methanobacterium bryantii* produced dimethylarsine from several arsenic compounds (175). The initial chance observation of alkylarsine synthesis was made when a strong, nauseating, garlic-like odor was detected in reaction mixtures containing cell extract, hydrogen, ATP, arsenate, and methylcobalamin. In fact, the intense garlic odor was used cautiously as a rapid qualitative assay for the formation of alkylarsines–-an interesting revival of Gosio's biological method!

Further work on the anaerobic volatilization of arsenic has focused on anaerobic ecosystems such as sewage sludge and the use of pure cultures of anaerobes. As noted earlier, sewage sludge had yielded arsenic volatilizing organisms in 1973. Soon thereafter, sludge from an anaerobic digester was treated with $[^{74}As]Na_2HAsO_4$ and then incubated anaerobically (172, 173). Volatile, radioactive arsenic compounds were produced, as evidenced by the use of rubber traps, but when the sludge was preheated to 90°C for 15 min, volatilization did not occur. Unfortunately, experimental difficulties with the system precluded extensive observations. Some evidence for the assumption that methanogens were producing dimethylarsine was obtained by taking advantage of the fact that the main precursor of methane formation in sewage sludge was carbon atom 2 (the methyl carbon) of acetate. In experiments with $[2-^{14}C]$acetate, radioactivity was incorporated into the traps only when arsenate was present. Other preliminary experiments indicated that rumen fluid and compost were particularly effective in volatilizing arsenic from $[^{74}As]Na_2HAsO_4$.

A more-sophisticated analytical technique (on-line coupling of gas chromatography with ICP-MS) later detected volatile metal and metalloid species in landfill and sewage gases (85, 86, 121). For arsenic, the gases from both sources contained $(CH_3)_2AsH$, $(CH_3)_3As$, and, interestingly, $(CH_3)_2C_2H_5As$. Only sewage gas formed CH_3AsH_2, and only landfill gas formed $(C_2H_5)_3As$. Particularly noteworthy is the formation of the ethylated species. Assuming that the ethyl group transfers

are a result of microbial action and thus an instance of bioalkylation, it would be of considerable interest to identify the organisms responsible. Similar work has shown that gases released from anaerobic wastewater treatment plants contained arsine as well as the three trimethylarsines (178).

An interesting feature of the work with the anaerobic ecosystems just described was the formation of arsine itself (86, 178). Although the formation of arsine from arsenate and arsenite by soil bacteria (*Pseudomonas* sp. and *Alcaligenes* sp.) was known (51), the microbial formation of this nonmethylated metabolite is uncommon. However, as will be seen, arsine formation by pure cultures of anaerobes has been described.

Cell extracts of *Desulfovibrio vulgaris* strain 803 produced an unidentified, garlic-odored gas when incubated with sodium arsenate (175), and unpublished results (75) have indicated the production of both di- and trimethylarsine from arsenate by cell extracts of *Methanobacterium thermoautotrophicum*. When this organism was grown under stable chemostat conditions with H_2 and CO_2 as growth substrates, arsenate was transformed to volatile products, mainly arsine itself with small amounts of dimethylarsine (17). Lowered phosphate concentrations increased the efficiency of the volatilization by 25%.

Enrichment cultures of anaerobes isolated from an arsenic-contaminated lake in the Canadian sub-Arctic region with five selective media showed extensive formation of methylarsenicals (30). Anaerobic oligotrophs were most active, producing a high proportion of dimethylarsinate. Sulfate-reducing consortia with acetate produced mainly trimethylarsine oxide, with relative concentrations of methylarsonate and dimethylarsinate increasing with time. Sulfate-reducing organisms with lactate produced only trimethylarsine oxide by day 4, with a range of products being produced on extended incubation. Iron- and manganese-reducing organisms grew poorly, producing only low levels of methylated arsenicals. Some of the compounds were probably methylarsenic(III) thiols, and volatile arsines were also produced in some cases. In sulfate-reducing (acetate) cultures, AsH_3 was formed with monomethylarsine as the major volatile compound. Sulfate-reducing (lactate) cultures gave AsH_3 and trimethylarsine as the major products.

Several representatives of sewage sludge microflora (methanogenic archaea) and sulfate-reducing anaerobes have been examined in pure culture in the presence of various arsenic concentrations (178). The most efficient organism for arsenic volatilization was *Methanobacterium formicicum*; arsine and all three methylarsines, as well as an unidentified volatile arsenic compound, were produced over the As concentration range of 0.05 to 0.3 mM KH_2AsO_4. *Methanosarcina barkeri* produced only arsine over the same range, and *Methanobacterium thermoautotrophicum* formed arsine at 0.1, 0.3, and 0.5 mM concentrations, especially so at the higher arsenic levels. Three organisms—*Clostridium collagenovorans*, *D. vulgaris*, and *Desulfovibrio gigas*—produced small amounts of trimethylarsine. The facultative marine anaerobe *Serratia marinorubra* converted arsenate to arsenite and methylarsonate when grown aerobically; volatile arsines were not produced under either aerobic or anaerobic conditions (243).

Methyl Donor in Anaerobes

While SAM is clearly the methyl group donor for many arsenic methylation processes, the situation with respect to bacteria and anaerobic microorganisms is less clear-cut. Dimethylarsine formation by *M. bryantii* required H_2, ATP, and a methyl donor in addition to the substrate [^{74}As]Na_2HAsO_4 (175). Except for arsenate, the same materials are required for methane biosynthesis. The most efficient methyl donor was methylcobalamin, with carbon dioxide as a poor second. Neither serine nor 5-methyltetrahydrofolate was a methyl donor. The addition of vitamin B_{12} did not lead to dimethylarsine formation, and ethylcobalamin did not transfer the ethyl group. These results suggested that the reaction with methylcobalamin was enzyme catalyzed; there was, in fact, a linear relationship between the amount of methylcobalamin used and the yield of dimethylarsine. It is clear that the transfer of methyl groups from methylcobalamin to arsenic over and above that of nonenzymatic controls was demonstrated (175). Methylcobalamin has been increasingly implicated in a range of methylation reactions in addition to its well-known role in methionine biosynthesis (94, 143, 226).

Since arsenite inhibited methane biosynthesis, it seemed possible that methyl transfer to arsenite led to the formation of a nonvolatile compound. By the use of [$^{14}CH_3$]methylcobalamin, this compound was identified as methylarsonate (175). An abbreviated, Challenger-like mechanism was postulated for dimethylarsine synthesis, with dimethylarsinate undergoing a $4e^-$ reduction as the final step: arsenate → arsenite → methylarsonate → dimethylarsinate → dimethylarsine. Unfortunately, in the schematic mechanism presented by these authors, there appears to be confusion between oxidation numbers and valency. The methyl groups were represented as derived from methylcobalamin with formation of vitamin B_{12r} (notation representing the demethylated form of B_{12}).

One difficulty is the existence of nonenzymatic methyl transfers from this methylcobalamin, e.g., to mercury (251). The methylation of arsenite by methylcobalamin occurred at a low rate in the presence or absence of rat liver extract (33). In 1973, the nonenzymatic transfer of the methyl group of methylcobalamin to arsenite was shown to require a reducing agent such as dithioerythritol (209, 210). Typically, incubation of methylcobalamin, As_2O_3, and dithioerythritol in water gave arsine, methylarsine, and methane. The reductant, Zn/NH$_4$Cl, behaved similarly. Using [C^2H_3]cobalamin, the arsenical product was $C^2H_3AsH_2$, demonstrating transfer of an intact methyl group. There was no alkyl transfer from ethylcobalamin. Selenite inhibited arsenite methylation and selenite underwent methylation to dimethylselenide in the absence of arsenite.

Arsenite methylation by methylcobalamin in the presence of GSH has also been observed (259). The reaction was studied in capped tubes, one reason for this being "to prevent any as yet unknown volatile intermediates from escaping" (259). After treatment of the reaction mixtures with H_2O_2 to oxidize arsenical intermediates to the As(V) state, methylarsonate and smaller amounts of dimethylarsinate were identified. In view of this experimental procedure, the initial products of methyl transfer were not identified. In addition to containing arsenite, methylcobalamin, and GSH, the complex incubation mixtures contained vitamin B_{12} and SAM. The unexplained presence of
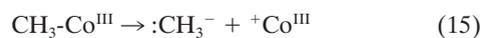
the latter slightly increased the yield of methylarsonate; in the absence of GSH, methylation was not observed. Sodium 2,3-dimercapto-1-propane-sulfonate and sodium selenite also functioned as reducing agents; in combination these two agents behaved synergistically.

Work with *E. coli* cultures isolated from rats fed dimethylarsinate for 6 months has shown that cysteine but not GSH was required for dimethylarsinate metabolism. Thus, although GSH contributes to arsenic metabolism in animals, there may be a different metabolic pathway in bacteria (147).

Some workers add vitamin B_{12} (34) to assays for methyl transfer in animal extracts, but there is little evidence for the requirement. Methylcobalamin, added to incubations of rat liver cytosol, produced no effect (228). However, for arsenic methylation by the microbial contents of mouse intestinal cecum, methylcobalamin addition increased production of methylarsonate from inorganic forms of arsenic but had little effect on the formation of dimethylarsinate. These increases may have resulted from increased production of SAM by the cecal microflora or from the use of methylcobalamin in undefined enzymatically catalyzed methylation reactions (116).

The possible chemistry for nonenzymatic and enzyme-catalyzed methyl transfers from methylcobalamin to arsenic has been discussed in detail (83, 196, 203, 209, 210, 250). There are strongly held opinions, particularly with respect to the methyl donor situation in anaerobic organisms. Wood et al. stated that, taking into account all of the available experimental data, "we believe that the biomethylation of arsenic by methyl-B_{12} occurs in anaerobic ecosystems" (203). However, Cullen and Reimer claimed that "until strong evidence is advanced, there seems little need to invoke a different mechanism for arsenic biomethylation by bacteria from that discussed above for fungi" (75). Moreover, in a discussion at a 1978 symposium dedicated to Challenger, Cullen expressed himself more forcefully: "We are brain-washed by the idea that methyl B_{12} is involved in all of these reactions. We firmly believe that there is no B_{12} involved in arsenic methylation. Professor Wood pointed out that the nature of the methylating species had to be established in nature. Much of our discussion now involves base-on, base-off details, yet the compound may not in fact be the methylating agent" (62). It should be noted that the literature contains confusing statements with respect to oxidation numbers and electron flow details (66, 261).

Clearly, there is no general consensus as to a mechanism for anaerobic dimethylarsine formation. There are two general explanations for the observed results with *M. bryantii*: either there is a direct transfer from methylcobalamin or, if not, then methylcobalamin transfers methyl to a further carrier molecule. Three mechanisms are available for the direct transfer of methyl from methylcobalamin, represented as CH_3-Co(III). The methyl group may be transferred as an anion (equation 15), as a radical (equation 16), or as a cation (equation 17):

$$CH_3\text{-}Co^{III} \rightarrow :CH_3^- + {}^+Co^{III} \qquad (15)$$

$$CH_3\text{-}Co^{II} \rightarrow \cdot CH_3 + \cdot Co^{II} \ (B_{12r}) \qquad (16)$$

$$CH_3\text{-}Co^{III} \rightarrow {}^+CH_3 + {}^-Co^{I} \ (B_{12s}) \qquad (17)$$

In terms of the enzymatic situation, the cation transfer (equation 17) would appear to be advantageous; it is analogous to
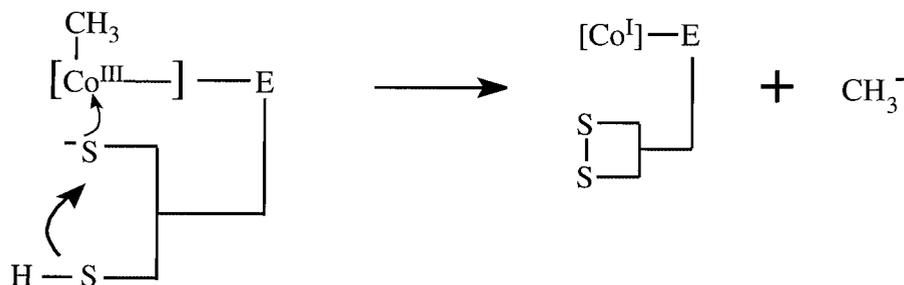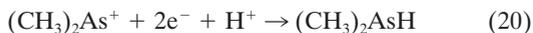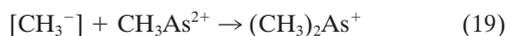
FIG. 6. Possible mechanism for formation of a "cryptocarbanion" from a methyl cobalamin. E indicates an enzyme containing two SH groups (209, 210).

the situation with SAM. This mechanism requires formation of $^-$Co(I), also represented as B_{12s}. However, the compound formed after methyl transfer was B_{12r} (equation 16); it was described as the normal cobamide product formed by demethylation of methylcobalamin (175). If correct, this identification suggests the intervention of a methyl radical, and in fact, consideration of the standard reduction potential (-0.67 V) for As(V) or As(III) is suggestive of a reductive homolytic cleavage (83, 249).

An alternative explanation (209, 210) invoked a role for a thiol, likely GSH, in formation of methane, acetate, and methylarsines by anaerobes. For the first two metabolites, the process is more complex than originally postulated, but the overall concept may be valid for methyl transfer to arsenic. The role of the thiol is that of a reductant; in fact, methylcobalamin can be reductively cleaved nonenzymatically in neutral or weakly acid solutions to form methane. The addition of the thiol to the cobalt atom promoted the heterolytic cleavage of the Co-C bond, leading to formation of a methyl carbanion (or similar species) which may be enzyme bound. Such a species was described as a "kryptocarbanion"; however, "cryptocarbanion" would be more appropriate.

For the enzyme reaction, two thiol units were involved as well as linkage of methylcobalamin as a corrinoid protein (Fig. 6). The methyl carbanion was represented as attacking arsenic in the ionized state and carrying three positive charges (equations 18 to 20). For arsenite the ionization would be represented as $As(OH)_3 \leftrightarrow As^{3+}(OH^-)_3$. Other species would presumably be possible, for instance, those with GSH replacing the OH groups: $As(SG)_3 \leftrightarrow As^{3+}(SG^-)_3$.

$$[CH_3^-] + As^{3+} \rightarrow CH_3As^{2+} \qquad (18)$$

$$[CH_3^-] + CH_3As^{2+} \rightarrow (CH_3)_2As^+ \qquad (19)$$

$$(CH_3)_2As^+ + 2e^- + H^+ \rightarrow (CH_3)_2AsH \qquad (20)$$

The ionized species, CH_3As^{2+}, would correspond to methylarsonate or a similar structure $[CH_3As(OH)_2 \rightarrow CH_3As^{2+}(OH^-)_2]$, and $(CH_3)_2As^+$ would correspond to dimethylarsinate $[(CH_3)_2AsOH \rightarrow (CH_3)_2As^+(OH^-)]$ in the usual Challenger mechanism. This proposed mechanism requires production of Co(I), i.e., B_{12s} rather than the observed B_{12r}. Perhaps an interconversion occurred during workup of the enzymatic incubation mixtures. For the nonenzymatic transfer, this mechanism was dismissed (259) as "less likely in the highly reductive conditions of our experiments," and instead a "nucleophilic

attack on the Co-C bond by an arsenite-GSH complex" was proposed. It is not clear why the reaction conditions for this work were characterized as "highly reducing." The tubes, although capped, contained air, and the incubation components in addition to arsenite and methylcobalamin were the required GSH in Tris-HCl buffer with Mg^{2+}, SAM and vitamin B_{12}. "Highly reducing" seems to be an exaggeration.

If the transfer is not direct, a possible intermediate would be required; the case of methane biosynthesis may provide a model. It is known that the methyl transfer processes for methane biosynthesis are very complex, with a multitude of cofactors and enzymes being involved; in addition, certain proteins contain bound cobalamin (89, 131, 155, 245). One material that plays an important role in methane biosynthesis is coenzyme M (CoM), $HS-CH_2-CH_2-SO_3^-$; it accepts a methyl from a corrinoid protein forming methyl-CoM, $CH_3-S-CH_2-CH_2-SO_3^-$. In fact, amino acid sequences are known for two CoM methyl transferases from *M. barkeri* and *Methanobacterium thermoautotrophicum* (89, 155, 163). Methyl-CoM is the last intermediate before methane, being reduced with the involvement of 7-mercaptoheptanoylthreonine phosphate (93).

It has been suggested that for metal alkylation, CoM would be a molecule of interest (173), and it has also been suggested that in methanogens arsenic methylation was coupled to the methane biosynthetic pathway (235), apparently implying a role for CoM. Moreover, it has been asserted that "*Methanobacterium* methylates arsenate in a side reaction of methane biosynthesis using CoM (2,2'-dithiodiethanesulphonic acid)" (126). The McBride and Wolfe paper (175) cited in reference 126 in support of this statement does not refer to CoM, and another paper by the same authors in the same year (174) describing CoM does not mention arsenic methylation. In 1971, the CoM structure was not known, and in any case, the disulfide form of CoM, CoM-S-S-CoM, noted by Hughes and Poole (126) is not likely to be an acceptor for methyl groups. Another assertion without experimental support is that for *M. bryantii* the pathway "involves methylation by a low molecular weight cofactor Coenzyme M (CoM)" (234).

Cullen has cited unpublished observations showing that methyl transfer does take place in model systems from methyl CoM to arsenic(III) (61). Apart from this, there is apparently no experimental evidence for a role for CoM in arsenic methylation by microorganisms, and as with so many other parts of the arsenic story, the picture is incomplete. A chemically attractive possibility for a methyl donor to arsenic would be a sulfonium derivative of CoM, $(CH_3)_2-S^+-CH_2-CH_2-SO_3^-$, but

methyl transfer to arsenite from this sulfonium compound could not be demonstrated (75). Moreover, S-adenosyl-CoM and methylsulfonium adenosyl-CoM, did not stimulate methane formation in *Methanobacterium thermoautotrophicum* extracts (63).

A possible intermediate for methyl transfer from methylcobalamin is, of course, SAM. The latter material is clearly synthesized by methanogens and has significant metabolic activity. For instance, methyl-CoM reductase from *Methanobacterium thermoautotrophicum* contains five modified amino acids, of which four involve methylation: 1-*N*-methylhistidine, 5-(*S*)-methylarginine, 2-(*S*)-methylglutamine, and *S*-methylcysteine (note that "*S*" indicates substitution at sulfur and "[*S*]" indicates a specific configuration). Methionine is taken up from culture medium by this organism and used for protein synthesis and in SAM-dependent methylation reactions (but not in methyl-CoM-dependent methane biosynthesis). The methyl groups of the four modified amino acids derive from methionine, almost certainly by way of SAM (211). Moreover, SAM is involved in methyl transfer to uroporphyrinogen III in *Methanobacterium ivanovii* (28) and in the biosynthesis of the methylated pterin in methanopterins in *M. formicicum* and *Methanococcus volta* (247).

However, apart from the role of methylcobalamin in methionine synthase, there is apparently no simple mechanism by which a methyl group from this corrinoid would become the methyl group of SAM; if methionine synthase were to be involved, methionine adenosyltransferase would also be required. The latter enzyme has, in fact, been purified to homogeneity from *Methanococcus jannaschii* and has been cloned and expressed in *E. coli* (109). In other words, for this hypothesis to be viable, the cell extracts of *M. bryantii* must also have contained both methionine synthase and methionine adenosyltransferase as well as the necessary substrates and cofactors for these enzymes and in addition must have contained methyl transferases for arsenic methylation and their associated redox cofactors. Perhaps this is not impossible, but it seems a tall order.

ENZYMOLOGY OF BIOMETHYLATION

The enzymology of arsenic biomethylation is complicated by the several oxidation states for this element, the previously discussed propensity of arsenic to react with sulfur compounds, and the fact that only very small amounts of the arsenic compounds are present in biological specimens. The chemical intermediates and reactions in the metabolism of arsenate are assumed to be generally the same in microorganisms and animals. However, in microorganisms, the reactions tend to proceed to methylarsines, whereas in mammalian species the major urinary metabolite is generally dimethylarsinate and only a very small amount of it is further reduced. There is considerable variation in the arsenic metabolic pathways among and between various animal species. With the exception of arsenate reductase (see below) most of the enzymatic experiments involve mammalian systems.
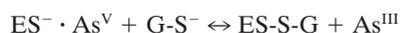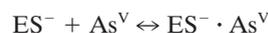
Enzymes Catalyzing Reductions

Arsenate reductase. Although GSH can reduce arsenate to arsenite nonenzymatically, several thiol-requiring arsenate reductases, are involved in connection with bacterial resistance to arsenic. While these enzymes are not known to have a connection with biomethylation they may be usefully described here as possible models for enzymes reducing As(V) compounds in methylation. This is especially so since no microbial reductases involved in biomethylation have been described. Moreover, the arsenate reductases utilize reductants such as thioredoxin and glutaredoxin rather than GSH which is usually postulated to play a role in biomethylation. Two of the sources for arsenate reductases are *E. coli* and *S. aureus.* Strains of both of these bacteria are known to convert trimethylarsine oxide to trimethylarsine, and in *E. coli*, dimethylarsinate is converted to trimethylarsine oxide.

For some bacterial strains that are resistant to heavy metals (including here, arsenic), the resistance determinants are located on plasmids (205, 217–219, 233). In *E. coli* and *S. aureus* there are operons induced by arsenate, arsenite, and antimony(III); each of these ions induces all three resistances. The arsenic resistance plasmid, pI258, of *S. aureus* contains three genes: *arsA* (which encodes a repressor regulator protein), *arsB* (which encodes a membrane efflux protein), and *arsC* (137, 138, 177). The last of these genes, required only for arsenate resistance, codes for the reductase ArsC, or more specifically, $ArsC_{sa}$ to distinguish it from the subsequently discussed $ArsC_{ec}$ from *E. coli*. This reductase is required since arsenite is the species transported from cells.

The $ArsC_{sa}$ enzyme is a monomer of about 14.5 kDa, with 131 amino acid residues (137). This reductase coupled to thioredoxin, thioredoxin reductase, and NADPH. Two enzyme-bound cysteine residues, Cys^{82} and Cys^{89}, were identified as a redox couple (177). The disulfide form of $ArsC_{sa}$ was only regenerated by thioredoxin (not by GSH), confirming the thioredoxin specificity.

In *E. coli* the arsenic resistance plasmid is termed R773. The $ArsC_{ec}$ reductase, a 16-kDa protein, contained 141 amino acid residues and had an essential thiol component—Cys^{12} (98, 162). In addition, a histidine residue, His^8, was required for catalytic activity by lowering the pK_a of the cysteine thiolate (97). Unlike $ArsC_{sa}$ it requires GSH (186) and glutaredoxin 2 (Grx2-SH); thioredoxin is not involved (162, 215). The suggested reaction sequence is as follows where ES^- is Cys^{12} of ArsC:

$$ES^- + As^V \leftrightarrow ES^- \cdot As^V$$

$$ES^- \cdot As^V + G\text{-}S^- \leftrightarrow ES\text{-}S\text{-}G + As^{III}$$

(probably involves several steps)

$$ES\text{-}S\text{-}G + Grx2\text{-}S^- \leftrightarrow ES^- + G\text{-}S\text{-}S\text{-}Grx2$$

$$G\text{-}S\text{-}S\text{-}Grx2 + G\text{-}S^- \leftrightarrow Grx2\text{-}S^- + G\text{-}S\text{-}S\text{-}G$$

$$G\text{-}S\text{-}S\text{-}G + NADPH + H^+ \leftrightarrow 2\ G\text{-}SH + NADP^+$$

The secondary structure and overall protein fold for $ArsC_{ec}$ were determined by use of multidimensional, multinuclear magnetic resonance (224). A model fold was structurally homologous to *E. coli* glutaredoxin, thiol transferases, and GSH

S-transferases that are α/β proteins. The $ArsC_{ec}$ protein contained large regions of extensive mobility, especially at the active site.

A gene cluster on chromosome XVI of *Saccharomyces cerevisiae*, confers arsenate resistance, and the arsenate reductase is labeled Acr2p (182). There is a requirement for GSH and glutaredoxin and a mixed disulfide between GSH and Acr2p is probably formed. There is no role for thioredoxin. A homolog of this reductase, Ygr203p, a 148 amino acid residue protein, was overexpressed in *E. coli*, and the protein was crystallized (180). X-ray diffraction data were obtained, but the crystal structure has not yet been solved.

A few anaerobes use arsenate, selenate, or in one case selenite as terminal electron acceptors, typically with reduction to arsenite or selenite. In view of a recent review (225) only a brief discussion is given here. The respiratory arsenate reductase from *Chrysiogenes arsenatis* gen. nov., sp. nov., in which acetate is the electron donor consists of two subunits, ArrA (87 kDa) and ArrB (29 kDa) (142). Molybdenum, iron, zinc, and acid-labile sulfur are required cofactors; this enzyme is very different from other arsenate reductases. More recently two further arsenate- and sulfate-reducing bacteria, *Desulfomicrobium* strains Ben-RA and Ben-RB, were isolated (166). In the Ben-RA strain, which cannot grow with arsenate as an electron acceptor, arsenate is reduced by an enzyme encoded by a chromosomally borne gene that is homologous with the *arsC* gene of *E. coli* plasmid R773. The nature of the electron donor for the reductase was not determined. For strain Ben-RB, arsenate was reduced by a membrane-bound enzyme, probably associated with a *c*-type cytochrome; the major cytochrome in this organism is c_{551}. It thus differs from the arsenate reductase of *C. arsenatis*.

Human liver arsenate reductase uses (nonnatural) dithiothreitol as the electron donor with GSH, thioredoxin or reduced lipoic acid having much less activity (199). Partially purified enzyme, with an apparent molecular mass of about 72 kDa, required an unidentified, heat-stable cofactor (not a divalent cation).

Methylarsonate reductase. The only other reduction studied at the enzyme level is that of methylarsonate. Rabbit liver enzyme, purified 118-fold, required GSH. Dithiothreitol alone did not substitute for GSH; however, in the presence of GSH, dithiothreitol almost doubled the formation of methylarsonite. This enzyme was rate-limiting in arsenic biotransformation. The same reductase was also identified in human liver (258).

Enzymes Catalyzing Methyl Group Transfers

The methyltransferases involved in biomethylation of arsenic have interesting chemical properties since they have a "hidden" redox component (Fig. 4). Such activities have been mainly investigated for the mammalian arsenate $\rightarrow$ dimethylarsinate transformation. Arsenite methyltransferase activity has been studied and partially purified in various animal tissues (14, 118, 258). Methylarsonite transferases have been purified from rabbit liver and Chang human hepatocytes (248, 260). Reviews describing them are available (14, 15, 227). There are apparently no accounts of the characterization of specific arsenic methyltransferases from microorganisms.

ARSENIC BIOMETHYLATION AS DETOXIFICATION?

Whether biomethylation of arsenic is a detoxification mechanism, particularly in animals, has been discussed at length (1, 14, 24, 125, 171, 227, 229). The most poisonous form of arsenic for humans (as measured by experiments that determine a lethal dose for some *other* test organism) is arsenous acid, $As(OH)_3$, or its anion arsenite, materials commonly present in the environment. In general As(III) derivatives, e.g., arsenite, are more toxic than As(V) compounds, e.g., arsenate (229). Toxicity here, in general, refers to measurements of concentrations that comprise a lethal dose for 50% of the test subjects at some predetermined time limit after exposure. Analogously, selenite (more than selenate) and tellurite (more than tellurate) appear to be the most toxic oxyanionic forms of those metalloids to bacteria (21, 257). In fact, one detoxification mechanism is simply the oxidation of arsenite to arsenate (75). While methylation is often assigned a role in detoxification (242) this assumption may not be universally true. For instance, the As(III) compound, methylarsine oxide $[(CH_3AsO)_n]$ is more toxic to *C. humiculus* than arsenite (71). The corresponding sulfide $[(CH_3AsS)_n]$ is even more toxic than the oxide or arsenite. The order of toxicity for various compounds in Chang human hepatocyte cultures was as follows (from most to least toxic except as indicated: methylarsonous acid, arsenite, arsenate, methylarsonic acid = dimethylarsinic acid (193). There is evidence that organoarsenicals may produce toxic and carcinogenic effects that differ from those of inorganic arsenic (228).

Although arsine and the methylarsines are undoubtedly very toxic in the gas form, a surprising result was obtained when trimethylarsine dissolved in olive oil was administered to mice by mouth (256). Under these conditions the acute toxicity of trimethylarsine "was found to be incomparably low"; the 50% lethal dose was calculated to be 7,870 mg kg of body weight^{-1}. The trimethylarsine was rapidly excreted in urine after oxidation to trimethylarsine oxide. The detoxification effect may in some cases be merely a function of increased volatility of more highly methylated forms. In other words, outgassing may be a significant means of removing metalloids from an organism's environment.

BIOMETHYLATION OF ANTIMONY

Introduction

Like arsenic, antimony is widely distributed on earth, usually at low concentrations, e.g., 4.3 to 7.9 ppm in soils (167). In addition to terrestrial sources, this element can be detected in oceans and freshwater environments in the parts per billion range. The metalloid and its inorganic compounds find a variety of industrial uses; the element is a frequent component of lead alloys. It is used in the ceramic and plastic industries and is widely present in many materials as a fire retardant. A few organic compounds of antimony are used in medicine. Tartar emetic, antimony potassium tartrate, $C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$, is used as an anthelmintic in treatment of parasitic infections and as an animal expectorant. Some other organic antimony derivatives have also been used as anthelmintics, and at one time antimonial materials were used in the treatment of syphilis.

Early information on the determination of antimony species in nature has been summarized (167). Within the last few

years, improved methods for the detection of small amounts of antimony and determination of antimony species have been published (58, 59, 78, 139, 144, 159–161).

Antimony and SIDS

Prior to the middle nineteen nineties there was little evidence for microbial methylation of antimony (55). However, this possibility became of interest when a possible cause for sudden infant death syndrome (SIDS) (also known as cotdeath) was suggested (202). The hypothesis was that flame retardants in mattresses and covers might undergo methylation to toxic gases by the action of *S. brevicaulis* or other microorganisms. Since one flame retardant was antimony, trimethylstibine might have been produced. It was claimed that *S. brevicaulis* had actually been isolated from damp crib mattresses (202). The hypothesis was vociferously supported by some groups, and there were accusations of a cover-up of important facts by "authorities" (223). Later work indicated that the predominant organism isolated from crib mattresses was not *S. brevicaulis* but a mix of common environmental *Bacillus* sp. (246). A very detailed reexamination of old evidence and consideration of new information, carried out by an independent advisory committee for the British Department of Health, found no justification for the toxic-gas hypothesis (Lady Limerick, final report to British Government Department of Health, 1998 [www.doh.gov.uk/limerch.htm]).

The nonmicrobial leaching of antimony (as Sb_2O_3) from polyvinylchloride crib mattresses could account for the high variability associated with antimony levels in livers for both SIDS victims and other infants and for the elevated Sb levels in the hair of some healthy infants (132). The polyurethane innerfoam of crib mattresses might be a site for toxic gas formation of group 15 elements, but determination of the level of Sb, As, P, and Bi in crib mattress foam showed no correlation with the occurrence of SIDS. No volatile forms were detected in the headspace of mixed or monoseptic cultures of anaerobes containing polyurethane foams. There was no evidence for a causal relationship between levels of trimethylantimony or total trimethylantimony forms and SIDS (135).

Microbial Methylation of Antimony

Challenger has described a curious case of chronic antimony poisoning in Vienna, Austria, at the beginning of the 20th century. The problem was in a house decorated with silk curtains mordanted with an antimony compound. Although a volatile antimony compound was a prime suspect, none could be identified (43). Challenger and Ellis investigated growth of *S. brevicaulis* on media containing tartar emetic (44). The cultures were without odor, and aspiration of the air over them through Biginelli's solution gave no precipitate. Other attempts to volatilize antimony using *P. notatum* in addition to *S. brevicaulis* were made by one of Challenger's students (18). It appeared that stibonic acids, $RSbO(OH)_2$, would be useful substrates, and early experiments utilized the more readily available phenylstibonic acid (R = C_6H_5). Sterile air was slowly aspirated over cultures of both organisms and then into concentrated nitric acid. The latter solution was then carefully analyzed for antimony. Only *P. notatum* gave a positive result,

the amount of antimony being just detectable. Since the expected product, phenyldimethylstibine, might have been oxidized in the air stream, the experiments were repeated with an air-nitrogen mixture containing 8% oxygen. Under these conditions, neither organism produced a volatile antimony compound; however, *P. notatum* produced small amounts of a volatile antimony compound from growth in presence of potassium antimonate ($KSbO_3$).

A possible mechanism for biomethylation of phenylstibonic acid to phenyldimethylstibine would involve alternate reductions and methylations as in the Challenger mechanism for arsenic biomethylation. The final step would be the reduction of phenyldimethylstibine oxide, $C_6H_5(CH_3)_2SbO \rightarrow C_6H_5(CH_3)_2Sb$. To provide evidence for reductive capabilities, both organisms were grown with trimethylstibine dihydroxide, $(CH_3)_3Sb(OH)_2$ [i.e., $(CH_3)_3SbO \cdot H_2O$]. Trimethylstibine, detected both chemically and by odor, was produced in both cases. Thus, it was clear that the reduction Sb(V) $\rightarrow$ Sb(III) was biogenically possible and that trimethylstibine was sufficiently stable to be detected when formed in fungal cultures. Another potential intermediate in formation of trimethylstibine was dimethylstibonic acid, $(CH_3)_2SbO(OH)$. This compound was also converted to a volatile material; the best results were obtained with *S. brevicaulis* rather than with *P. notatum*. This careful work did establish a very low level of volatilization of antimony by fungi, but the amounts of volatile material were too small for identification. Fungi grown repeatedly in the presence of sodium dimethylstibonate or potassium antimonate showed no increase in antimony volatilization (18).

Despite other inconclusive results on the microbial volatilization of antimony (95, 189) the process is now well established. The reduction of trimethyldibromoantimony to trimethylstibine by *P. fluorescens* K27, an organism isolated from the selenium-contaminated Kesterson reservoir in central California, was observed but this organism did not biomethylate tartar emetic or potassium hexahydroxyantimonate. However, the headspace of certain soil samples (from another site) amended with either of these compounds and augmented with nitrate-containing growth media contained trimethylstibine. These experiments clearly established the anaerobic microbial biomethylation of antimony; however, the responsible microorganisms were not identified (115).

In similar experiments, mixed anaerobic soil enrichment cultures containing tartar emetic were also shown to produce trimethylantimony (134). Six soil types and three culture media were used; the formation of trimethylantimony was dependent on both the soil sample and the medium and was variable even for replicate analyses.

Other work with complex technology established a low level of antimony methylation under aerobic conditions with *S. brevicaulis* (7, 133). In typical experiments, the antimony volatilized was about one-millionth of that added. Small amounts of nonvolatile di- and trimethylantimony compounds were detected in some experiments with Sb(III) compounds but not Sb(V) compounds.

S. brevicaulis cultures incorporated labeled methyl groups ($^{13}C^2H_3$) from [$^{13}C^2H_3$]-L-methionine into nonvolatile tri- and dimethylantimony compounds. Hence, SAM is likely the microbial methylating agent for antimony (6, 8). Dimethylantimony species were shown to be true intermediates in the tri-

methylstibine biosynthetic pathway. Isotope incorporation into trimethylstibine from $[^2H_3]$-D-methionine was much less than that from the L enantiomer. This degree of stereospecificity indicated that the pathway was enzymatic. Since similar differences for L- versus D-methionine occur for arsenic biomethylation it is probable that the mechanism for the methylation of these metalloids is the same. Antimony biomethylation has not been studied at the enzymatic level.

When sewage sludge containing a substantial amount of Sb (1.7 ± 0.3 mg/kg [dry weight]) was incubated anaerobically, trimethylstibine was detected in the headspace gas as a dominant volatile metalloid. With pure cultures of various anaerobes, trimethylstibine was produced by *C. collagenovorans* and *D. vulgaris* (but not *D. gigas*). *Methanobacterium thermoautotrophicum*, and *M. barkeri* formed only very small amounts. One organism, *M. formicicum*, was a particularly active methylator, producing mono- and dimethylstibine as well as the trimethyl derivative and, in addition, stibine, SbH_3, itself (178). Trimethylstibine was also present in landfill as well as sewage gases. Concentrations of trimethylstibine in thermophilic sewage digestion gases in the range of 6.7 to 14.7 μg m^{-3} were proposed to originate from biological sources after this metalloid was supplied from anthropogenic sources (86). Volatile antimony species were detected in gases over hot springs in British Columbia, Canada (120). Analytical methods for antimony determination at trace levels in environmental samples have been reviewed (183, 221).

Small amounts (1 to 12 ng liter^{-1}) of nonvolatile methylstibonic acid, $CH_3SbO(OH)_2$, and dimethylstibinic acid, $(CH_3)_2SbO(OH)$, were detected in rivers and seawaters (4, 5). The compounds were present at levels ranging from 1 to 12 ng liter^{-1}. While these observations were probably the first to identify naturally occurring methylantimony compounds, there was no proof for a biological origin. Moreover, doubt has been cast upon these results, with further evaluation of the hydride generation process suggesting that some chemical species were, in fact, method artifacts (77). Methylated antimony species were detected in geothermal waters in New Zealand (120), but it was not clear whether these compounds were derived by biological (e.g., action of algae) or purely chemical means. Pondweed samples (*Potamogetan pectinatus*) from Canadian lakes influenced by mine effluents contained substantial levels of antimony (48 to 68 ppm [dry mass]). Antimony species in extracts made with acetic acid were obtained as stibines following reduction; these hydrides were SbH_3 produced from the Sb(III) species and the mono-, di-, and trimethylstibines (78). Cultures of the marine diatom *Thalassiosira nana* converted $^{125}SbCl_3$ to a stibnolipid. The structure was probably the same as that of 3'-O-(5''-dimethyl arsenoso-5''-deoxyribosyl)-phosphatidylglycerol (Fig. 1B), with Sb replacing As (22). There is little evidence for antimony methylation in mammals (96).

The wood rot fungus *P. schweinitzii* has produced trimethylarsine and trimethylantimony in solution. While trimethylarsine production from samples with added sodium arsenite was slight, separate samples amended with potassium antimony tartrate yielded conversion of up to 0.7% antimony to the methylated forms. Fungal cultures with added potassium hexahydroxyantimonate produced substantially less methylated antimony (9).

The fate of trimethylstibine in the atmosphere is important because knowledge of its lifetime contributes to an understanding of the involvement of the volatile forms in biogeochemical cycling. Craig et al. (57) have investigated the oxidation products of trimethylstibine in atmospheric oxygen and found the products to be trimethylstibine oxide as "a range of cyclic and linear oligomers" containing stibine oxide units. If these oxidized products can be metabolized by microorganisms, then the remobilization of antimony in biological systems can occur. The kinetics of this oxidation appear to be slower than those proposed by the only other antimony oxidation investigations in the literature (187, 188).

METHYLATION OF BISMUTH

Bismuth, the heaviest of the group 15 periodic table elements, occurs on earth in concentrations somewhat lower than those of antimony, i.e., at a crustal abundance of 0.17 to 0.2 ppm (167). Like antimony and arsenic, it is used in many ways. It is more metallic in character than these elements and forms low-melting-point alloys such as Wood's metal (Bi, 50%; Pb, 25%; Sn, 12.5%; and Cd, 12.5% [melting point, 154°F or 67.8°C]). Bismuth compounds have important applications in cosmetics, and bismuth is a semiconductor dopant.

Bismuth preparations have long been used by physicians, and most of the world's production of bismuth (about 50%) is used in the pharmaceutical industry (76, 201, 254). The 1899 edition of *Merck's Manual* lists 15 bismuth preparations, compared with only five for antimony (13). These preparations range from "Bismal," bismuth methylene digallate (to be used as "an intestinal astringent especially in diarrheas not benefited by opiates") to bismuth valerianate (basic bismuth valerate, to be used as a "sedative and antispasmodic for nervous headaches, cardialgia, chorea, etc."). Bismuth compounds continue to be used in the treatment of intestinal problems as antacids (bismuth carbonate), as hemostatic agents (bismuth gallate), and in treatment of diarrhea (bismuth salicylate). The well-known, over-the-counter preparation Pepto-Bismol (Procter and Gamble) contains bismuth subsalicylate, HO-C_6H_4-COOBiO, and is said to provide "soothing relief for indigestion, upset stomach, diarrhea, nausea, and heartburn." With the recognition of the role of *Helicobacter pylori* in the ulceration of the gut, much attention has been refocused on the use of bismuth preparations. Eradication of *H. pylori* infections is generally achieved with a combination therapy of three agents—a bismuth compound, an antibiotic (e.g., amoxicillin or metronidazole), and an anti (H^+) secretory agent (255).

Soluble, inorganic bismuth, as bismuth nitrate, was more easily absorbed and then eliminated by rats fed diets augmented with thiols such as L-cysteine, homocysteine, or mercaptopropionic acid (38, 39). Nonthiols such as methionine or alanine did not produce this effect. It is feasible that enteric microbial reduction and methylation were enhanced by these thiol-containing additions and that the metabolic products enhanced bismuth elimination; however, bismuth analogs of arsenical sugars were not investigated.

For detection of small amounts of bismuth a merging-zones flow injection-AAS has been used; volatile alkylbismuth species were generated with sodium tetraethylborate (181). The detection limit for urinary bismuth was 1.6 μg liter^{-1}.

Trimethylbismuth has been identified in landfill and sewage

gas (86, 87), and experiments with sediments also showed production of trimethylbismuth under anaerobic conditions. The annual worldwide generation of trimethybismuth was estimated at 30 to 4,980 kg. There was little evidence for nonvolatile methylbismuth compounds in sediments and freshwaters (88). Anaerobic wastewater treatment gases contained trimethylbismuth, and pure cultures of *M. formicicum* produced significant amounts of trimethylbismuth from Bi(NO₃)₃. Very small amounts of trimethylbismuth were produced by *C. collagenovorans* (178).

EPILOGUE

The Italian physician Bartolomeo Gosio, who gave his name to a toxic gas, also discovered the fungal metabolite now known as mycophenolic acid and demonstrated its antibiotic action against anthrax bacillus (23). Most of his career was spent in the Italian Public Health Service, especially dealing with malaria, tuberculosis, and influenza. He died in Rome in 1944 at a time when that city was still occupied by the Nazis. Although brief obituaries were published in Italian, the English-speaking world was preoccupied with World War II, and his death was not noted. Gosio must have known that his eponymous gas had been identified as trimethylarsine, but he could never have imagined the volume of work on biomethylation that was yet to come.

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