APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 8, 303–311 (1994)

Identification of Extracellular Arsenical Metabolites in the Growth Medium of the Microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis*

William R. Cullen,* Hao Li, Gary Hewitt, Kenneth J. Reimer and Nadia Zalunardo

Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia, Canada V6T 1Z1

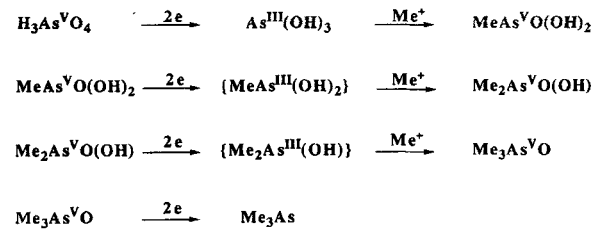
The separation and identification of some of the arsenic species produced in cells present in the growth medium when the microorganisms *Apiotrichum humicola* (previously known as *Candida humicola*) and *Scopulariopsis brevicaulis* were grown in the presence of arsenicals were achieved by using hydride generation–gas chromatography–atomic absorption spectrometry methodology (HG GC AA). Arsenite, monomethylarsonate, dimethylarsinate and trimethylarsine oxide were detected following incubation with arsenate. With arsenite as a substrate, the metabolites were monomethylarsonate, dimethylarsinate and trimethylarsine oxide; monomethylarsonate afforded dimethylarsinate and trimethylarsine oxide, and dimethylarsinate afforded trimethylarsine oxide. Trimethylarsine was not detected when the arsenic concentration was 1 ppm.

Keywords: Arsenic, extracellular, endocellular, methylation, growth medium, *Apiotrichum humicola*, *Scopulariopsis brevicaulis*, *Candida humicola*, hydride generation–gas chromatography–atomic absorption spectrometry, trimethylarsine oxide, methylarsonate, dimethylarsinate

INTRODUCTION

In 1933, Challenger *et al.*[1] successfully identified a volatile arsenic compound produced by molds growing in the presence of arsenic oxide (As_2O_3) as trimethylarsine. In these experiments the volatile metabolite produced by *Scopulariopsis brevicaulis* growing on sterile breadcrumbs treated with As_2O_3 was swept out of the flask with a stream of sterile air, and trapped in a solution of mercuric chloride in hydrochloric acid (Binginelli's solution[2]). The resulting precipitate was identified as a mercuric chloride adduct $[(CH_3)_3As.2HgCl_2]$ of the arsine. Following these studies with *S. brevicaulis*, a metabolic pathway was proposed for the biomethylation of arsenicals to trimethylarsine (Scheme 1).[1,3]

Support for Challenger's mechanism comes from studies that showed arsenate, arsenite, monomethylarsonate (MMAA), dimethylarsinate (DMAA), and trimethylarsine oxide (TMAO) to be substrates for the production of trimethylarsine $[(CH_3)_3As]$ by some microorganisms.[1,4–8] The source of carbonium ion (Me^+) is probably *S*-adenosylmethionine (SAM) and the reducing agent may be a thiol.[8] However, the presence of arsenite, MMAA, DMAA and TMAO as extracellular metabolites in the biomethylation of arsenicals has rarely been reported.[9] Challenger stated that arsenic intermediates from the proposed metabolic pathway (Scheme 1) were not found in the culture medium of *S. brevicaulis*, although no details were given regarding the methodology used to support this conclusion.[3] Cullen *et al.*[7] incubated labeled arse-



Scheme 1 Challenger's mechanism for the methylation of arsenic. The intermedites in {} are unknown as monomeric species. They are formulated as $(CH_3AsO)_n$ and $(CH_3As)_2O$, respectively, when prepared by conventional methods.

* Author to whom correspondence should be addressed.

CCC 0268–2605/94/040303–09

Received 14 February 1994
Accepted 29 March 1994

nicals with broken-cell homogenates of *Apiotrichum humicola*, previously known as *Candida humicola* but referred to exclusively as *A. humicola* in this paper, in order to look for metabolic intermediates in the growth medium. They employed a combination of molecular sieve chromatography, anion-exchange chromatography and electrophoresis to separate arsenicals from each other and from other biological material. Arsenite, MMAA and DMAA were found to be metabolites of [^{74}As]arsenate, and TMAO was a metabolite of [^{14}C]methylarsonate and [^{14}C]dimethylarsinate; in addition, a demethylation product, [^{14}C]methylarsonate, was observed from the latter. Replacement of the cell preparation by buffer failed to bring about any transformations. Probably these metabolites represent intermediates in the biosynthesis of trimethylarsine. The production of DMAA, together with MMAA and trimethylarsine, when *S. brevicaulis* and *A. humicola* were treated with the model arsenic(III) intermediate $(MeAsO)_n$ has been reported.[10] This was the first time that non-volatile methylated intermediates had been found in the growth medium of a pure culture.

In this paper we report on the effect of adding low levels of four arsenic compounds (arsenate, arsenite, MMAA and DMAA) to cultures of the microorganisms *A. humicola* and *S. brevicaulis* growing aerobically in a liquid medium. Hydride generation–gas chromatogaphy–atomic absorption spectrometry (HG GC AA) was used to identify the extracellular arsenic metabolites present in the pure culture.

EXPERIMENTAL

All chemicals used were of reagent grade. Deionized water was used for all dilutions. Glass and plasticware were cleaned by soaking overnight in 2% Extran solution, followed by a water rinse, a soak in dilute hydrochloric acid, and finally a water rinse.

Arsenic standards were prepared freshly by serial dilutions from stock solutions (1000 ppm as elemental arsenic) of the following compounds; sodium arsenate, $Na_2HAsO_4.7H_2O$ (Baker); sodium arsenite, $NaAsO_2$ (Baker); disodium monomethylarsonate, $CH_3AsO_3Na_2.6H_2O$ (Alfa); dimethylarsinic acid, $(CH_3)_2AsO(OH)$ (Alfa); trimethylarsine oxide, $(CH_3)_3AsO$, which was synthesized according to the literature.[11] Solutions of 1 M hydrochloric acid (HCl), 4 M acetic acid (CH_3COOH) and 2% (w/v) $NaBH_4$ in 0.1% (w/v) caustic soda (NaOH) were freshly made daily.

A. humicola was obtained from the American Type Culture Collection (ATCC 26699) and *S. brevicaulis* was obtained from the Fungus Culture Collection of the Chemistry Department at the University of British Columbia. The cultures were grown aerobically in a synthetic inorganic liquid medium at pH 5 as described by Cox and Alexander.[4,12] Aqueous solutions of the appropriate arsenical were filter-sterilized (0.2 μm membrane) separately and added to the autoclaved culture medium in the flask. Typically, 10 ml of an actively growing culture of *A. humicola* or 2 ml of an actively growing culture of *S. brevicaulis* was added to 250 ml of the medium which contained 1 ppm of arsenic. During the growth period the cultures were maintained at 21–22 °C and were agitated by a rotary shaker at 130 rpm. Once each day for two weeks, a 6 ml culture aliquot was removed and stored frozen prior to HGGC AA analysis. After the first two weeks of incubation, a glass microfiber paper pre-soaked in 5% mercuric chloride was suspended in the headspace of cultures to trap the volatile arsines (chemofocusing).[13] The medium (6 ml) was then collected once each week for another two to three weeks. The experiment was terminated after four weeks incubation of *A. humicola* and five weeks incubation of *S. brevicaulis*. Terminal cultures were centrifuged, and the cells were washed and freze-dried for future analysis.

For the arsenic speciation analysis in the cells, the freeze-dried samples were weighed and transferred into an Erlenmeyer flask (250 ml) containing 30 ml of mixed solvent $CHCl_3$–MeOH–H_2O (1:1:1). The mixture was sonicated for 2 h and then agitated on a mechanical shaker for 24 h. The extracts were centrifuged to separate the aqueous fraction from the organic fraction. The aqueous layer was kept at −4 °C prior to analysis. The organic extract and the residue were air-dried, digested in 4 ml of 2 M NaOH in a water bath at 95 °C for 3 h, then neutralized with concentrated HCl prior to analysis.

A hydride generation system was used to identify hydride-forming arsenicals in growth media as well as in the cells.[14] A peristaltic pump was used to mix the sample solution (1–3 ml) with acid (1 M HCl or 4 M CH_3COOH) and 2% (w/v) $NaBH_4$ solutions. The volatile arsines so produced were trapped in a Teflon® U-tube (30 cm

long, 0.4 cm i.d.) immersed in liquid nitrogen (−196 °C). After immersing the Teflon® coil in a 70 °C water bath, the arsines were volatilized and separated on a Porapak-PS column (mesh 80–100; Chromatographic Specialties, Canada) by using a Hewlett–Packard Model 5830A gas chromatograph with a pre-set temperature program. The GC column outlet was connected directly to a hydrogen–air flame quartz cuvette. A 810 Jarrell-ash atomic absorption spectrometer was used as the arsenic detector at 193.7 nm and the signal was recorded by using a Hewlett–Packard 3390A integrator. To monitor the production of arsenite from arsenate by microorganisms, the hydrochloric acid solution was replaced by 4 M CH_3COOH (pH 2.1). At this pH, arsenate is not reduced to arsine by sodium borohydride solution.[14,15]

During culture incubations, trimethylarsine presence was assessed by two methods, based on odor and chemofocusing. The intense and distinctive garlic-like odor of arsines has been used as qualitative evidence of arsine production.[16–20] The odor threshold for $(CH_3)_3As$ now appears to be 2 pg g^{-1} in dilute aqueous solution.[21] This allows qualitative evaluation of arsine production by cautious sniffing of culture headspace gas. The chemofocusing method has also proved to be an effective means to trap the volatile arsines.[13] If trimethylarsine is produced, crystals of the $HgCl_2$ adduct are formed on a glass fiber filter soaked in 5% mercuric chloride solution that is suspended in the headspace of cultures. Subsequent heating of the filter decomposes the mercuric chloride adduct and the volatile arsines are freed for mass-spectrometric analysis.[13]

RESULTS

A. humicola and *S. brevicaulis* grown in the presence of each of the four substrates arsenate, arsenite, MMAA and DMAA produced a number of compounds; therefore each substrate is discussed separately.

Experiment 1: Transformation of arsenate

The growth medium was analyzed by using HG GC AA in order to determine the biotransformation products of *A. humicola* and *S. brevicaulis* from arsenate.

A. humicola exposed to 1 ppm arsenate reduced more than 90% of the substrate to arsenite (detected as AsH_3) within two days of incubation (Fig. 1). Oxidation of the arsenite to arsenate was not observed during the rest of the growth period although the concentration of arsenite further decreased with time, and only background levels were detected after four weeks of incubation. In addition to arsenite, small amounts of DMAA and TMAO were detected as $(CH_3)_2AsH$ and $(CH_3)_3As$, respectively, in the culture medium collected on day 5. The concentration of DMAA increased to 0.02 ppm by day 7 but a further increase was not observed. The TMAO concentration increased rapidly with time, and reaching 0.75 ppm at the end of the fourth week. When 1 M HCl was used for HG GC AA analysis, trace amounts of MMAA were detected as CH_3AsH_2 in the culture medium after two weeks of incubation and the quantity remained constant

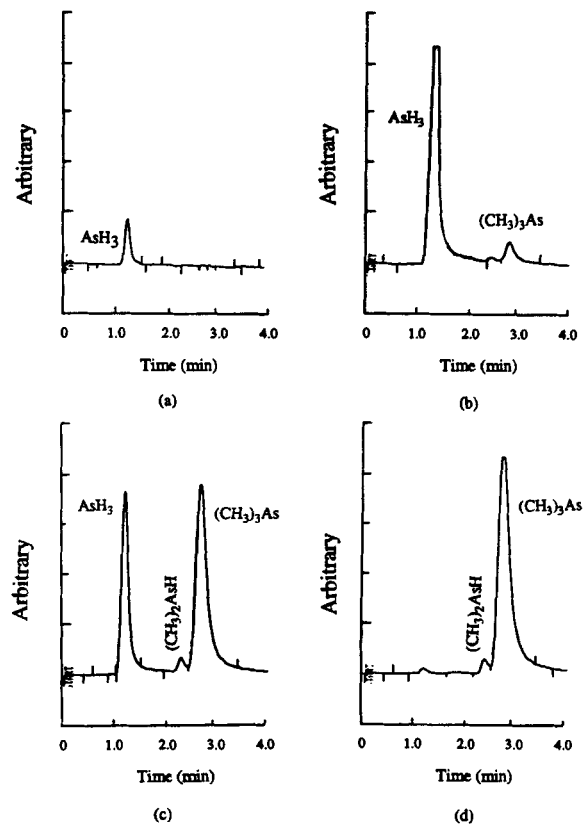


Figure 1 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm arsenate) obtained by using HG GC AA with 4 M CH_3COOH (pH 2.1) and 2% (w/v) $NaBH_4$. At this pH arsenate is not reduced to arsine by $NaBH_4$. The growth medium was collected on (a) day 0, (b) day 5, (c) day 15, and (d) day 28.

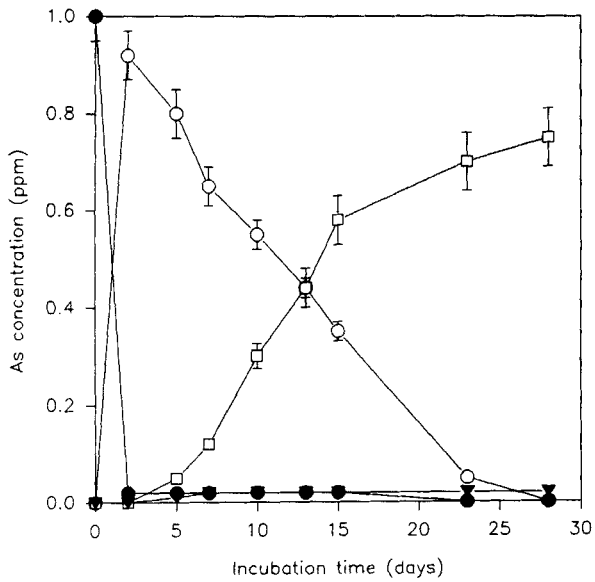


Figure 2 The change in arsenic concentrations in the growth medium of *A. humicola* enriched with 1 ppm arsenate: ○, arsenite; ●, arsenate; ▼, DMAA; □, TMAO.

for a further two weeks. The change in concentrations of arsenic species as a function of incubation time is shown in Fig. 2.

The change of arsenic speciation in the growth medium of *S. brevicaulis* was less dramatic. The total reduction of arsenate to arsenite was achieved four days after inoculation of the stock culture. The quantity (~1 ppm) of arsenite in the medium did not decrease significantly over a five-week incubation period. TMAO (0.01 ppm) and a trace amount of DMAA was detected on day 5. The amount of TMAO had increased slowly to approximately 0.04 ppm at the end of the experiment. During this period, the quantity of DMAA did not change significantly. No MMAA was detected in these samples.

Neither *A. humicola* nor *S. brevicaulis* produced trimethylarsine as judged by the odor test or by the use of the chemofocusing trap. In the latter case no arsine was detected by mass spectroscopy when strips of the filter were analyzed.

No significant amounts of arsenic were found in cells of *A. humicola* or *S. brevicaulis* at the end of their incubations by using HG GC AA.

Experiment 2: Transformation of arsenite

Actively growing cultures of *A. humicola* (10 ml) and *S. brevicaulis* (2 ml) were inoculated with 250 ml of liquid media containing arsenite (1 ppm) and grown aerobically at 21–22 °C for four and five weeks, respectively.

The concentration of arsenite in the culture medium of *A. humicola* decreased rapidly to 0.29 ppm in two weeks, and reached a background level after four weeks of incubation (Fig. 3). The rate of arsenite disappearance is similar to that observed in Experiment 1. The oxidation of arsenite to arsenate in the growth medium was not observed. Both DMAA and TMAO were detected as $(CH_3)AsH$ and $(CH_3)_3As$, respectively, in the growth medium on day 5. The DMAA concentration increased to 0.02 ppm after two weeks and no further change was found thereafter. The TMAO concentration increased from 0.05 ppm on day 5 to 0.69 ppm at the end of the fourth week. Trace amounts of MMAA were detected after two weeks of incubation, but the quantity of MMAA did not increase significantly

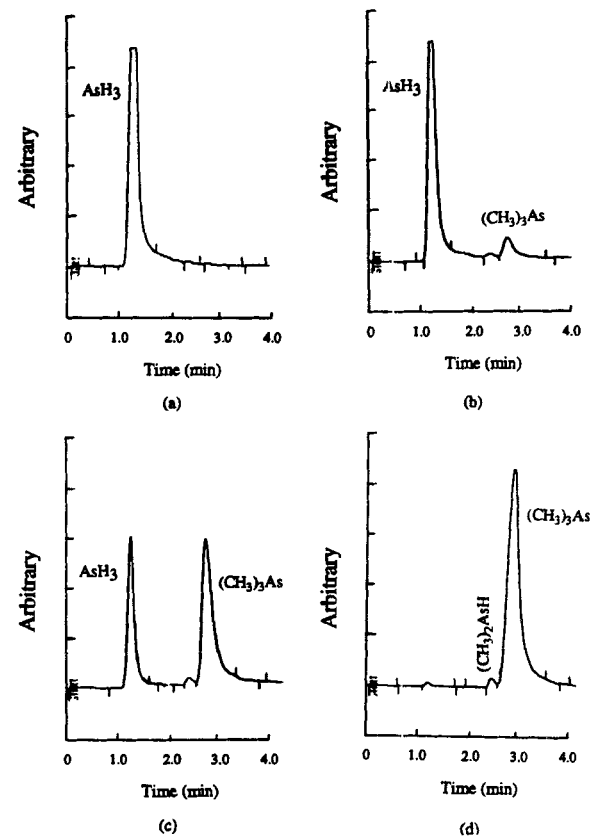


Figure 3 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm arsenite) obtained by using HG GC AA with 4 M CH_3COOH (pH 2.1) and 2% (w/v) $NaBH_4$. At this pH arsenate is not reduced to arsine by $NaBH_4$. The growth medium was collected on (a) day 0, (b) day 5, (c) day 15, and (d) day 28.

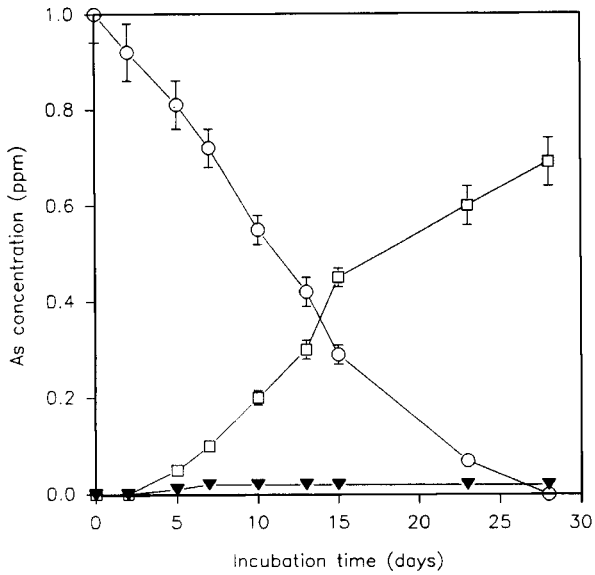


Figure 4 The change in arsenic concentrations in the growth medium of *A. humicola* enriched with 1 ppm arsenite: ○, arsenite; ▼, DMAA; □, TMAO.

over a further two to three weeks of incubation. The change in concentrations of arsenite, DMAA and TMAO in the growth medium is shown in Fig. 4.

In the growing culture of *S. brevicaulis*, the substrate arsenite was not oxidized to arsenate and the change in its concentration was insignificant. TMAO and trace amounts of DMAA were detected after eight days of incubation. TMAO in the medium increased slowly with time, and reached about 0.04 ppm in the medium at the end of five weeks of incubation. There was no further increase in the concentration of DMAA over the same period. No MMAA was detected in the culture medium.

Trimethylarsine was not detected in either of the experiments. No significant amounts of arsenic were found in cells of *A. humicola* and *S. brevicaulis* by using HG GC AA at the end of each experiment.

Experiment 3: Transformation of monomethylarsonate

No trimethylarsine was detected when MMAA (1 ppm) was incubated with *A. humicola* or *S. brevicaulis*. The possible demethylation products, arsenate and/or arsenite, were not observed.

In the *A. humicola* culture, DMAA (0.02 ppm in the medium) and TMAO (0.01 ppm in the medium) were detected on the second day of incubation (Fig. 5). The quantity of DMAA incresed to 0.15 ppm at the time when the experiment was terminated. The TMAO concentration increased to 0.33 ppm after four weeks of incubation. The concentration of MMAA in the medium had declined to 0.5 ppm when the experiment was terminated. The change in arsenic concentration is shown in Fig. 6.

For *S. brevicaulis*, TMAO and trace amounts of DMAA were detected in the growth medium after two weeks of incubation. TMAO had increased to 0.02 ppm when the experiment was ended. The concentration of MMAA in the medium did not change significantly in the time period of the experiment.

Accumulation of arsenic in the cells was not detected.

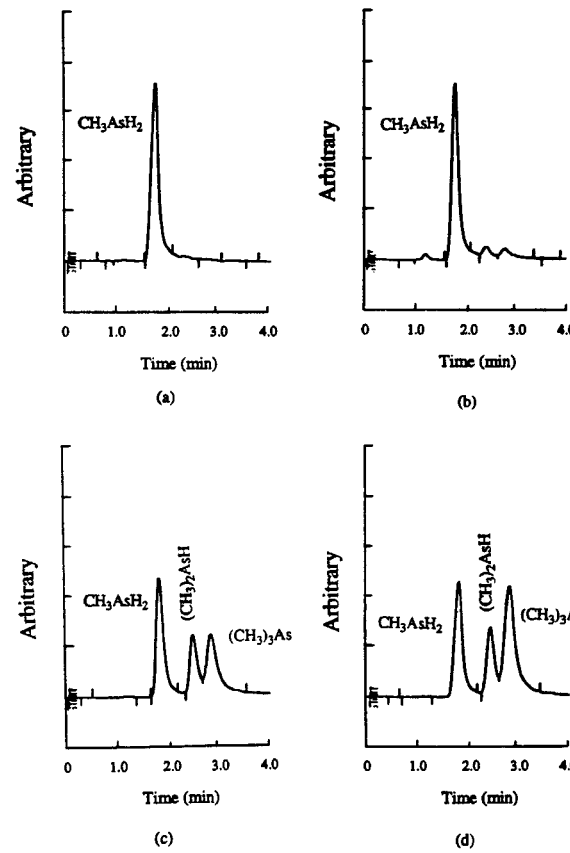


Figure 5 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm MMAA) obtained by using HG GC AA with 4 M CH_3COOH (pH 2.1) and 2% (w/v) $NaBH_4$. The growth medium was collected on (a) day 0, (b) day 2, (c) day 15, and (d) day 28.

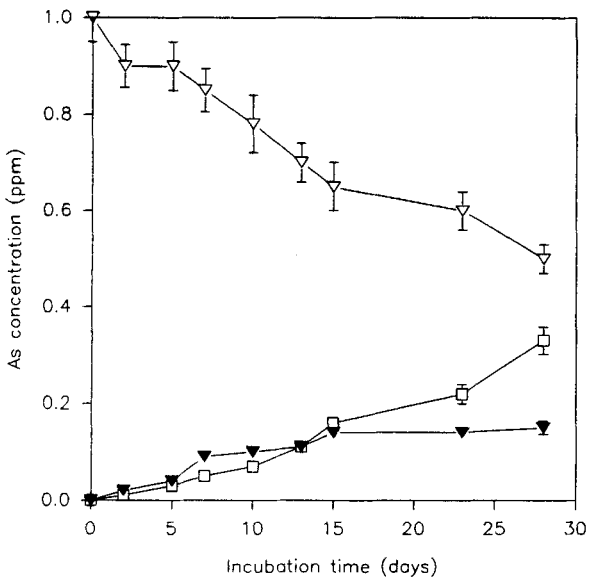


Figure 6 The change in arsenic concentrations in the growth medium of *A. humicola* enriched with 1 ppm MMAA; ▽, MMAA; ▼, DMAA; □, TMAO.

Experiment 4: Transformation of dimethylarsinate

In the growing culture of *A. humicola* spiked with 1 ppm DMAA, only trace amounts of TMAO were detected after 15 days of incubation, and the change in the DMAA concentration was insignificant.

TMAO was detected in the culture medium of *S. brevicaulis* but not until day 10. The concentration of TMAO increased with time and was about 0.07 ppm in the growth medium after five weeks of incubation.

Incubations of *A. humicola* and *S. brevicaulis* with DMAA (1 ppm) produced no detectable amounts of trimethylarsine or demethylation products, such as MMAA.

Accumulation of arsenic in cells of *A. humicola* and *S. brevicaulis* was not detected by using HG GC AA.

DISCUSSION

A combination of molecular sieve chromatography, anion-exchange chromatography and electrophoresis has proved to be a reliable method to isolate and identify the arsenicals present in biological material.[7,10] These procedures, however, are lengthy and time-consuming, especially when dealing with large numbers of samples; there may also be problems with detection when the concentration of analytes is low. The HG GC AA system used in this study can minimize the time for analysis (less than 10 min per sample) and can be used to detect low levels of arsenate, arsenite, MMAA, DMAA and TMAO in samples. It is assumed here that these are the precursors to the arsines that are ultimately formed and quantified. On this basis, the technique is capable of determining the concentration of these extracellular arsenic metabolites at concentrations which were too low to be detected by other convenient techniques. However, the separation and identification of the arsenic species are based on the properties of derivatives of arsenicals in the medium rather than on the properties of the arsenicals themselves.

Exposure of *A. humicola* and *S. brevicaulis* to arsenate yields arsenite, DMAA and TMAO in the growth medium, and MMAA is found in the growth medium of *A. humicola* but not in that of *S. brevicaulis*. The substrate arsenite is metabolized by *A. humicola* to produce MMAA, DMAA and TMAO and by *S. brevicaulis* to produce DMAA and TMAO. Both microorganisms transform MMAA to DMAA and TMAO, and DMAA to TMAO. The identification of these arsenicals is noteworthy, as it is the first time that non-volatile arsenic intermediates (shown in Scheme 1) have been identified in the growth medium of a pure culture spiked with either arsenate, arsenite, MMAA or DMAA. As mentioned in the Introduction, methylated intermediates have been isolated from broken-cell extracts of *A. humicola*[7] and from the growth medium of cultures of *A. humicola* and *S. brevicaulis* and also of *V. alcalescens* and *L. brevis*) when methylarsine oxide $[(CH_3AsO)_n]$ was used as a substrate.[10] Baker *et al.*[22] incubated arsenate and arsenite, separately, in a nutrient medium containing sediment collected from a small acidic, oligotrophic lake. By using the HG GC AA technique, they reported that 0–0.7% of the total arsenic had been transformed to MMAA and DMAA. It is believed that a variety of microorganisms including bacteria can contribute to the biological methylation.

The reduction of arsenate to arsenite is the first step towards methylation in Challenger's mechanism.[1,3] Our results support this assertion, as most of the arsenate is reduced to arsenite prior to the detection of any methylated arseni-

cals in the medium. The production of the anticipated methylated intermediates from the substrates, the absence of oxidation of arsenite to arsenate, and the lack of demethylation products, strongly support the metabolic sequence of Scheme 1 proposed by Challenger.[1,3]

Cells of *A. humicola* take up and metabolize arsenate to arsenite, and then excrete the arsenite into the medium. The arsenate is believed to be reduced inside the cells by thiols or dithiols as a detoxification process.[8] The uptake of arsenate by the cells probably involves an active transport system: the mechanism of arsenate uptake in *A. humicola* is metabolism-linked because, when the phosphate concentration is equimolar with arsenate, the rate of arsenate uptake by *A. humicola* is reduced to 18% of that observed in the absence of phosphate.[23] The uptake of MMAA and DMAA probably occurs by means of a slow passive diffusion.[23]

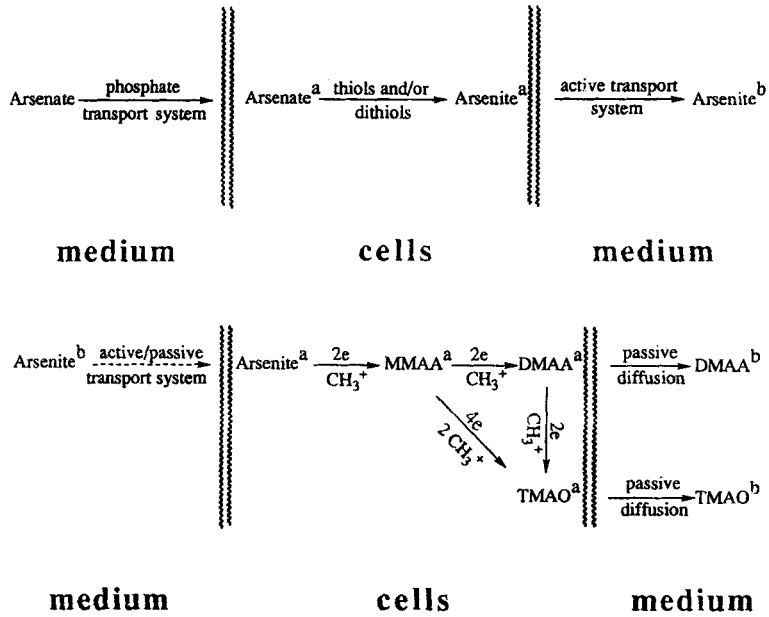
Cullen *et al.*[7] reported that only traces of MMAA were produced from arsenate by broken-cell homogenates of *A. humicola* and that MMAA was the least transformed arsenical by the broken-cell homogenates. They speculated that MMAA would not be found as a free intermediate in Challenger's arsenate-to-trimethylarsine pathway. The present results show that MMAA is a metabolite of arsenate and arsenite in whole-cell cultures of *A. humicola* but it is produced in a much lower concentration than either DMAA or TMAO. We also find that the production of DMAA in the growth medium of *A. humicola* is a rapid process when MMAA is used as a substrate; DMAA and TMAO are detected within two days of incubation. This result indicates that MMAA, if it exists as an intermediate in the arsenate/arsenite–trimethylarsine methylation process, could be metabolized rapidly inside the cells. Therefore, little MMAA would be excreted and detected as an extracellular metabolite of the cells. Cullen and Nelson[24] have studied the biomobility of MMAA and DMAA by measuring the rate at which these arsenicals diffuse through liposomes as models for biological membranes. It was found that DMAA was much more permeable to the membranes than MMAA. The permeability coefficient of MMAA can be 10 times lower than that of DMAA. Because of the low diffusion coefficient, it is possible that the cells metabolize the endocellular MMAA to DMAA and TMAO faster than the MMAA can diffuse into the growth medium. The DMAA thus produced has a higher permeability coefficient, and can diffuse into the growth medium. This surmise seems to be consistent with what we have observed.

In our work, the transformation of DMAA to other arsenic metabolites is a very slow process in cultures of *A. humicola*. This is a surprise as DMAA has been found to be a better precursor to trimethylarsine than arsenate or MMAA.[4] We suggest that these latter experiments were carried out with high concentrations (>100 ppm) of arsenicals and that this may be necessary to trigger the methylation process from DMAA to $(CH_3)_3As$, as is discussed below. Other factors may also affect the low production of TMAO; they are discussed in connection with the proposed methylation model (Scheme 2).

Apart from the substrate DMAA, the concentrations of all other arsenic substrates decrease with time in the growing culture of *A. humicola*, corresponding to an increase in the concentrations of arsenic metabolites. Only trace amounts of inorganic arsenic are detected in the growth medium at the end of incubations when arsenate or arsenite is used as a substrate: the transformation product is TMAO. The total concentration of arsenicals in the two growing cultures is relatively constant during the growth period (approximately 0.8–0.9 ppm), indicating that the uptake, the methylation, and the excretion of arsenic proceed simultaneously. Since neither the production of the volatile trimethylarsine is observed during the growth period nor significant amounts of arsenic are found in the cells harvested at the end of the incubations, the small decrease in the total arsenic concentration may be due to the adsorption of TMAO on cell surface of *A. humicola*. When MMAA is used as a substrate, the arsenic speciation in the growth medium is changed dramatically while the total concentration of arsenic is kept relatively constant (0.9–1.0 ppm). This indicates that the accumulation of arsenic and the production of volatile trimethylarsine by the cells are limited.

The detection of TMAO in the growing culture medium of microorganisms is very significant: not only is this the first time that TMAO has been found as an extracellular metabolite of fungi, but it is also the major arsenic metabolite of the microorganism. Previously, trimethylarsine was reported to be the major arsenic methylation product.

Trimethylarsine (b.p. 52 °C) is surprisingly stable in air at low partial pressures. A rough estimate of 10^{-6} M^{-1} s^{-1} for the rate constant of



Scheme 2 Proposed biotransformation model of arsenate in *A. humicola*. [a] Endocellular arsenicals. [b] Extracellular arsenicals.

the reaction of $(CH_3)_3As$ with oxygen in the gas phase has been made.[25] It is well known that both *A. humicola* and *S. brevicaulis* methylate arsenate, arsenite, MMAA and DMAA to trimethylarsine.[1,4-7] However, this volatile arsine metabolite was not detected during the present experiments, and it is unlikely that it was lost during sampling as the chemofocusing trap was an effective arsine collection device.[13] Pickett *et al.*[8] have demonstrated that TMAO can be reduced to trimethylarsine rapidly by whole cells of *A. humicola*. The rate of arsine production from TMAO increases linearly with the TMAO concentration, and is considerably faster than from arsenate or DMAA. Because of this rapid reduction of TMAO it was suggested that TMAO would not be detectable as an intermediate in cultures of *A. humicola* and it would be unlikely to be found in the environment.[8] However, in the present experiments TMAO is found to be the major methylation product and seems to be the end product of the methylation process. It now seems likely that low concentrations of TMAO (<1 ppm) do not greatly affect the fungus system. Therefore, further detoxification by reducing TMAO to trimethylarsine is not necessary. Previously, much higher concentrations (>100 ppm) of the arsenicals, including arsenate, arsenite, MMAA, DMAA and TMAO, were added to the growing culture of *A. humicola* to produce trimethylarsine.[4-7] These high concentrations were used because of analytical expedience only. The finding of TMAO in clams[26] seems to support this argument. Kaise[27] incubated arsenobetaine in inorganic medium containing bottom sediment collected from coastal waters. After 100 h of incubation, arsenobetaine was completely degraded into TMAO. This degradation is believed to be caused by indigenous microorganisms[27]. Apparently trimethylarsine was not detected in this experiment.

The limited metabolism of the arsenic substrates by *S. brevicaulis* in the growth medium is not surprising. This liquid medium was optimized by Cox and Alexander for the production of trimethylarsine by *A. humicola*: it has since been shown to support the growth of *S. brevicaulis* but no comparative growth studies were made,[4,12] Bread cultures of *S. brevicaulis* may be more productive than cultures in liquid medium, even though the reported rates of arsine production are not great.[8]

Although our findings lend general support to Challenger's proposed pathway, they indicate that it is an oversimplification of the many processes involved. Therefore, we propose an extended model for the methylation of arsenate by growing cells of *A. humicola* as outlined in Scheme 2. At first, the cells take up arsenate from the medium through a phosphate transport system, reduce the arsenate to arsenite inside the cells by thiols and/or dithiols, and excrete most of

the arsenite into the growth medium, probably by an active transport system.[28] This process can be achieved within two days. Any arsenite in the cells can be methylated to MMAA by *S*-adenosylmethionine (SAM), but because of the low passive diffusion coefficient of MMAA, most of the endocellular MMAA does not diffuse into the growth medium. Rather, the MMAA is more likely to be reduced and methylated to DMAA and then to TMAO. It is also possible that two methyl groups from SAM can be transferred to the intermediate MMAA sequentially to form TMAO, but without the formation of DMAA as a free intermediate. This is indicated by the low and constant level of extracellular DMAA in the growth medium and by the slow methylation process observed when DMAA is used as a substrate. Both DMAA and TMAO are able to diffuse into the growth medium. The excreted or added arsenite enters the cells of *A. humicola* by means of active or passive diffusion, and is metabolized in the same sequence to form MMAA, DMAA and TMAO. When MMAA and DMAA are used as substrates, their uptake is achieved by means of passive diffusion and the methylation process is similar to those presented above. Although this model can explain some of the results obained in our work, the biotransformation process is undoubtedly more complex in reality.

Acknowledgement We thank NSERC Canada for financial support.

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