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Arsenic Compounds Accumulated in Sedimentary Microorganisms Cultivated in Media Containing Several Arsenicals

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Sediments, as sources of microorganisms, were added to two kinds of media, 1/5 ZoBell 2216E and a solution of inorganic salts, which contained inorganic arsenic(III), inorganic arsenic(V), methanearsonic acid, dimethylarsinic acid, trimethylarsine oxide, tetramethylarsonium salt or arsenocholine. After 17 days of incubation at 20 °C, the arsenicals that had accumulated in the microorganisms were analysed by high-performance liquid chromatography (HPLC). While the more toxic arsenicals [inorganic arsenic(III), inorganic arsenic(V), methanearsonic acid, dimethylarsinic acid] were not converted in the microorganisms, trimethylarsine oxide and tetramethylarsonium salt were considerably degraded to inorganic arsenic(V), and arsenocholine to arsenobetaine. Arsenobetaine that had accumulated in the microorganisms was extracted and confirmed by thin-layer chromatography (TLC) and fast atom bombardment (FAB) mass spectrometry.

Keywords: inorganic arsenic; methanearsonic acid; dimethylarsinic acid; tetramethylarsonium salt; arsenocholine; microorganisms; conversion; degradation

INTRODUCTION

Recently, we have investigated microbiological arsenic circulation in marine ecosystems. As a result, organic arsenic compounds were converted or degraded to other arsenicals aerobically or anaerobically by the microorganisms from all the sources investigated.[1–11] For example, arsenobetaine, an organic arsenic compound known to occur ubiquitously in marine animals,[3, 12, 13] was degraded to less methylated arsenicals *in vitro*.[1–9] Especially when the activity of the microorganisms was high, it was degraded to inorganic arsenic.[7–9] This degradation of arsenobetaine in organic arsenic was confirmed *in vivo* in shark[14, 15] and in mollusc.[16] Furthermore, this complete degradation may complete a cycle beginning with the methylation of inorganic arsenic in seawater: it terminates with degradation to inorganic arsenic via the synthesis of arsenobetaine.[7, 9, 14]

In the previous *in vitro* degradation experiments in which arsenicals were converted to other arsenicals, the metabolites were investigated only in the filtrates of the culture media, except when arsenobetaine was added as a starting material.[17] Thus, as for arsenicals other than arsenobetaine, the conversion behaviour in the microorganisms themselves was unknown. In the degradation experiments with arsenobetaine, arsenobetaine was the major arsenic compound in the microorganisms even when it completely disappeared from the filtrate.

In this study, sediments, as sources of microorganisms, were added to two kinds of media each containing several arsenicals: inorganic arsenic(III) [As(III)], inorganic arsenic(V) [As(V], methanearsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), tetramethylarsonium (TMAS) iodide and arsenocholine. The arsenicals occurring in both the microorganisms and the filtrates of the culture media were investigated after incubation at 20 °C.

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MATERIALS AND METHODS

Sediments

Sediments were collected from the coastal waters of Yoshimi, Japan, in August and October 1994 in front of the National Fisheries University, Shimonoseki, Japan, using an Eckman grab sampler.

Cultivation

Two culture media were used in this study as well as in our previous conversion experiments with arsenicals. These were 1/5 ZoBell 2216E [g dm^{-3} filtered seawater: peptone 1.0; yeast extracts 0.2, pH 7.5] and an aqueous solution of inorganic salts at pH 7.5 [g dm^{-3}: sodium chloride (NaCl) 30.0; calcium chloride ($CaCl_2 \cdot 2H_2O$) 0.2; potassium chloride (KCl) 0.03; iron(II) chloride ($FeCl_2 \cdot nH_2O$) 0.01; phosphates (KH_2PO_4) 0.5 and (K_2HPO_4) 1.0; magnesium sulphate ($MgSO_4 \cdot 7H_2O$) 0.5; ammonium chloride (NH_4Cl) 1.0]. In the latter medium, in which there was no carbon source, the microorganisms had to use the added arsenobetaine as the only carbon source except for trace amounts of organic matter introduced by addition of the sediments. Sediment (1 g) was added to each medium (20 cm^3) containing As(III), As(V), MMA, DMA, TMAO, TMAS iodine or arsenocholine (all with 0.084% as As). The flasks were aerobically incubated at 20 °C in the dark under an atmosphere of air. After 17 days of incubation, 0.1 cm^3 of the mixture was withdrawn, added to 2.0 cm^3 of water and filtered for analysis by high-performance liquid chromatography (HPLC). The remaining part of the mixture was centrifuged at 4000*g* for 14 min to obtain microbial pellets.

Extraction from microorganisms and purification of arsenic compounds

The microbial pellet was repeatedly suspended in 20 cm^3 of the medium and centrifuged to wash out the arsenicals present in the medium. Water-soluble arsenic compounds were extracted from the pellet with chloroform–methanol (2:1, 70 cm^3) as described previously.[3] One of the water-soluble extracts was applied to a cation-exchange resin, Dowex 50W-×8 (50–100 mesh, H^+ form; 2.2 cm × 18.5 cm column); and eluted with 400 cm^3 of water, 400 cm^3 of 2.0 mol dm^{-3} pyridine and 400 cm^3 of 1.0 mol dm^{-3} HCl, successively. The concentrated fraction eluted with the pyridine solution was further applied to a Dowex 50W-×2 (200–400 mesh, pyridinium form; 1 cm × 50 cm colunm) equilibrated with 0.1 mol dm^{-3} pyridine–formic acid buffer (pH 3.1) and eluted with the same buffer (200 cm^3) and 0.1 mol dm^{-3} pyridine (200 cm^3).

High-performance liquid chromatography

Each extract was analysed by HPLC (Tosoh Co. Ltd, CCP 8000 series) using an ODS 120T 4.6 mm × 250 mm column (Tosoh Co. Ltd) with a mobile phase of 11.2 mmol dm^{-3} sodium heptanesulphonate in water–acetonitrile–acetic acid (95:5:6, by vol.; flow rate 0.8 cm^3 min^{-1}; sample size 5 mm^3).[18] Portions of 20 or 50 mm^3 of each eluate collected every 25 s were injected into the graphite furnace atomic absorption spectrometer and analysed as described previously.[3] A mixture of the authentic arsenic compounds (all with 100 mg dm^{-3} as As, including those which had been detected in the previous *in vitro* experiments on the degradation of arsenobetaine,[9] was also fractionated [retention times, s: As(III) 225–300; As(V) 150–225; MMA 225–300; DMA 325–400: arsenobetaine 525–625; TMAO 725–850; TMAS ion 1125–1275].

In order to determine the presence (MMA) or absence [As(v)] of a methyl group in the arsenical whose retention time was 225–300 s, atomic absorption spectrometry with hydride generation derivitization followed by cold trapping was performed without the previous hydrolysis with NaOH.[19]

Confirmation of the metabolite

The purified arsenic metabolite which was derived within the microorganisms cultivated in the medium added with arsenocholine was subjected to thin-layer chromatography (TLC) performed on a cellulose thin layer (Avicel SF, thickness 0.1 mm; Funakoshi Yakuchin Co. Ltd). Dragendorff reagent was used to indicate the position of the metabolite. $SnCl_2$–KI reagent[20] and iodine vapour were also used to indicate the positions of the authentic arsenic compounds. FAB mass spectrometry was performed with a

JEOL JMS DX-300 mass spectrometer equipped with a fast atom bombardment ion source and xenon atoms at 6 keV.

RESULTS

The microorganisms from ZoBell (Z) medium had a tendency to accumulate larger amounts of arsenicals than the salt (S) medium except for the media with added AC and TMAO (Table 1).

The added arsenicals were converted to their metabolites in the filtrates of the medium and/or in the microorganisms themselves, except for inorganic As(III). There was no essential difference in conversion behaviour between the ZoBell medium and the inorganic salt medium, suggesting that the microorganisms can degrade or convert arsenicals regardless of the presence of organic matter. The conversion behaviour of each arsenical was as follows:

Inorganic arsenic(V)

DMA was derived in the filtrates of both media with added inorganic arsenic (V): 24.3% (Z medium) or 9.0% (S medium) of arsenic in the filtrate was detected as this compound. On the other hand, only inorganic arsenic(V) was detected in the harvested microorganisms.

Methanearsonic acid

A minor conversion or methylation occurred in both the media: 2.2% (Z medium) or 2.4% (S medium) of arsenic was detected as TMAS ion in the filtrates. This was the first time that this compound was detected as a metabolite in our *in vitro* conversion experiments. All the detected arsenical in the microorganisms, however, was MMA.

Table 1. Accumulation of arsenicals in Zobell and salt media

Arsenical added to medium	Total wt of metabolites (μm)	
	Zobell medium	Salt medium
AS(III)	3.06	1.88
AS(V)	29.1	5.57
MMA	1.54	0.57
DMA	1.44	0.56
TMAO	0.27	0.57
AC	10.2	10.8
TMAS ion	5.63	2.47

Dimethylarsinic acid

The same results were obtained as those with MMA: 0.7% (Z medium) or 3.0% (S medium) of TMAS ion was detected in the filtrates, the arsenical detected in the microorganisms being starting material (DMA) only.

Trimethylarsine oxide

In terms of the extent of conversion in the filtrates, TMAO was comparable with the compounds above; 9.3% (Z medium) or 11.1% (S medium) of TMAO was degraded to DMA. However, part of the TMAO was completely degraded in the microorganisms; 54.3% (Z medium) or 65.2% (S medium) of arsenic accumulated in them was detected as arsenic (V).

Tetramethylarsonium salt

While no metabolite was detected in either of the filtrates, 7.5% (Z medium) or 43.9% (S medium) of TMAS ion was degraded to inorganic arsenic(V) in the microorganisms, showing the same type of degradation behaviour as that of TMAO.

Arsenocholine

Conversion of this compound occurred both in filtrates and within microorganisms. In the microorganisms, 73.9% (Z medium) or 80.9% (S medium) of arsenocholine was converted to arsenobetaine. On the other hand, arsenobetaine was also detected in the filtrates (Z medium 3.2%; S medium 3.1%). Besides arsenobetaine, 6.8% (Z medium) or 3.6% (S medium) of DMA was detected in the filtrates.

Isolation and identification of arsenobetaine accumulated in the microorganisms

In order to confirm the formation of arsenobetaine within the microorganisms, which were

cultured in the medium with added arsenocholine, 1600 cm^3 of arsenocholine-containing (0.011% as As) Z medium was incubated again for 17 days.

The arsenic metabolite extracted from the harvested microorganisms was eluted from Dowex 50W-×8 (H^+ form) with 2 mol dm^{-3} pyridine and from Dowex 50W-×2 (pyridinium form) with 0.1 mol dm^{-3} pyridine–formic acid buffer. The arsenic-containing fractions were freeze-dried and analysed by TLC and FAB mass spectrometry.

As shown in Fig. 1, FAB mass spectra of the purified arsenic compound and synthetic arsenobetaine are essentially the same, showing the most intense peak at *m/z* 179 $(M+1)^+$ along with peaks of the characterized fragments {*m/z* 135, $(CH_3)_4As^+$ and adduct ions {*m/z* 313, $[M + (CH_3)_4As]^+$; 357, $[2M + 1]^+$; 535, $[3M + 1]^+$}. Some additional adduct ions {*m/z* 201, $[M + Na]^+$; 217, $[M + K]^+$; 379, $[2M+Na]^+$; 395, $[2M+K]^+$} were shown in the spectrum of the purified compound, having resulted from the presence of sodium and

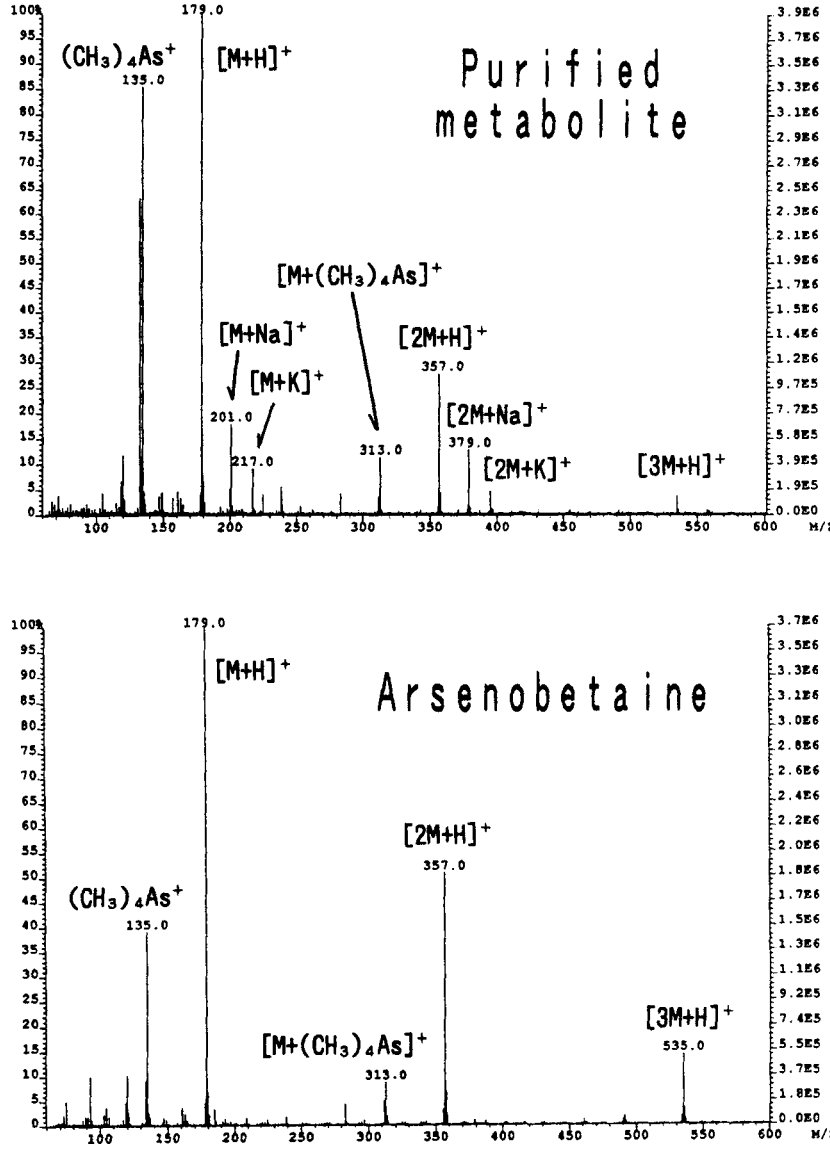

Figure 1 FAB mass spectra of the purified arsenic compound (above) and synthetic arsenobetaine (below).

Table 2. TLC identification of arsenocholine metabolite from microorganisms

Solvent system	R_f						
	Metabolite	Arsenobetaine	AS(III)	As(V)	MMA	DMA	Arsenocholine
Ethylacetate–acetic acid–water (3:2:1)	0.78	0.77	0.22	0.31	0.55	0.80	0.53
Chloroform–metahnol–28% aq. amonia (3:2:1)	0.75	0.74	0.30	0.00	0.22	0.78	0.87
1-Butanol–acetone–formic acid–water (10:10:2:5)	0.70	0.72	0.40	0.22	0.50	0.61	0.54
1-Butanol–acetone–28% aq. ammonia–water (10:10:2:5)	0.46	0.45	0.07	0.01	0.02	0.23	0.56
1-Butanol–acetic acid–water (4:2:1)	0.75	0.75	0.35	0.21	0.50	0.71	0.62

potassium in the medium. The purified metabolite was also chromatographed on cellulose thin layers together with authentic arsenic compounds. The R_f value of the metabolite agreed with that of synthetic arsenobetaine in five solvent systems (Table 2).

From the results of HPLC, FAB mass spectrometry and TLC the purified compound was confirmed to be arsenobetaine.

DISCUSSION

The microbial conversion behaviour of inorganic arsenic and some methylarsenicals has been investigated. Their conversion behaviour in the filtrates of the culture medium or in the bodies of the microorganisms was different from that of arsenobetaine. As we reported previously, arsenobetaine is subject to aerobic degradation with eventual formation of inorganic arsenic(V) in the filtrate. On the other hand, little or no conversion of the methylarsenicals which were used also in this study as starting materials (MMA, DMA, TMAO and TMAS ion) was observed in the filtrates in aerobic conditions during the incubation period, although they were considerably degraded under anaerobic conditions.[5,11] However, even when arsenobetaine was degraded in the filtrates, arsenobetaine was the only or major arsenical in the bodies of the microorganisms. Thus, the degradation behaviour of arsenobetaine is characterized by rapid degeneration in the filtrates and stability in the bodies of microorganisms.

On the other hand, the arsenicals investigated in this study may be classified into two groups from the standpoint of conversion behaviour. That is, one comprised arsenicals whose metabolites were not detected in the microorganisms [As(III), As(V), MMA and DMA] and the other those that were converted in them {TMAO, TMA ion and arsenocholine}. This phenomenon suggests that more toxic arsenicals, i.e. As(III), As(V), MMA and DMA, are hard to convert in the microorganisms, even though the microorganisms are able to bear the accumulation of these arsenicals in their bodies. The possibility, however, that the microorganisms excrete metabolites from these toxic arsenicals, cannot be denied.

Recently, Gailer *et al.*[21] investigated the metabolism of arsenicals by the blue mussel after accumulation from seawater spiked with dimethyl-(2-hydroxyethyl)arsine oxide and other five species of arsenicals which were used in this study also. In their results with arsenobetaine and arsenocholine, a similar conversion behaviour to those observed in microorganisms was obtained; while both arsenicals in the seawater were converted to TMAO, they accumulated in the mussel tissue without conversion. The fact that there was no great difference in the behaviours between microorganisms and mussel is a very interesting factor in the study of arsenic circulation in marine ecosystems. As for the TMAS ion, which was another arsenical that accumulated in mussel, the conversion behaviour of mussel differed from that of the microorganisms in this study. It accumulated in the mussel without conversion. The degradation of TMAS ion or TMAO to arsenic(V) and accumulation of arsenic(V) may characterize the conversion behaviour of microorganisms.

When metabolites were observed in both the filtrates and the bodies of microorganisms, they were not always the same. This may result from the combination of the conversion actions of exoenzymes and endoenzymes of the microorganisms. Whether the metabolites are toxic or of use to the microorganisms may also be an important factor in the distribution of the metabolites to the filtrates or within the microbial bodies. Further experiments will make this clearer.

Arsenocholine is considered as the precursor of arsenobetaine. The conversion of administered arsenocholine to arsenobetaine has been reported in mammals[22,23] and fish.[24] We also reported its conversion by sedimentary microorganisms: arsenobetaine, DMA and TMAO were detected as metabolites in the filtrates.[10] Arsenobetaine and DMA were also detected in this study. It is found however, that even when the formation of arsenobetaine is small (S and Z medium), arsenobetaine is accumulated in the bodies of microorganisms as a major arsenical. This fact is interesting in a consideration of arsenic circulation in marine ecosystems because arsenobetaine accumulated in microorganisms was difficult to convert. Thus, microorganisms taking up not only arsenobetaine but also arsenocholine retain arsenobetaine in their bodies for longer periods. This implies the possibility that detritivores live on detritus or suspended substances in which arsenobetaine-containing microorganisms are growing.

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