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Formation of arsenobetaine from arsenocholine by micro-organisms occurring in sediments

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As one of the experiments to pursue marine circulation of arsenic, we studied microbiological conversion of arsenocholine to arsenobetaine, because arsenocholine may be a precursor of arsenobetaine in these ecosystems. Two culture media, 1/5 ZoBell 2216E and an aqueous solution of inorganic salts, were used in this *in vitro* study. To each medium (25 cm^3) were added synthetic arsenocholine (0.2%) and about 1 g of the sediment, and they were aerobically incubated at 25 °C in the dark. These conversion experiments were performed in May and July 1990. In both seasons, two or three metabolites were derived in each mixture. These metabolites were purified using cation-exchange chromatography. Their structures were confirmed as arsenobetaine, trimethylarsine oxide and dimethylarsinic acid by high-performance liquid chromatography, thin-layer chromatography, FAB mass spectrometry and a combination of gas-chromatographic separation with hydride generation followed by a cold-trap technique and selected-ion monitoring mass spectrometric analysis. From this and other evidence it is concluded that, in the arsenic cycle in these marine ecosystems, as recently postulated by us, the pathway arsenocholine → arsenobetaine → trimethylarsine oxide → dimethylarsinic acid → methanearsonic acid → inorganic arsenic can be carried out by micro-organisms alone.

Keywords: Arsenocholine, arsenobetaine, trimethylarsine oxide, dimethylarsinic acid, methanearsonic acid, micro-organisms, sediments, arsenic metabolism, marine ecosystems

INTRODUCTION

Recently, we have studied the microbiological circulation of arsenic in marine ecosystems.[1–9] On the basis of the results from these studies, we have presented the following hypothesis: there is an arsenic cycle that begins with the methylation of inorganic arsenic from seawater on the way to arsenobetaine and terminates with the degradation of arsenobetaine to the original inorganic arsenic.[6,10] Arsenobetaine is the organoarsenic compound which was isolated and identified by Edmonds and Francesconi for the first time in 1977.[11] This compound has been proved to be ubiquitous in marine animals and has been accepted as the final metabolite of arsenic in marine food chains. On the other hand, various arsenic compounds besides arsenobetaine have so far been identified from many species of marine organisms.[12,13] Arsenocholine is one of these compounds and is thought to be a precursor of arsenobetaine. As a matter of fact, it was reported that arsenocholine is converted to arsenobetaine when administered to mammals[14,15] and fish.[16] Microbial conversion, however, of arsenocholine to arsenobetaine in marine environments is unknown so far.

In this study, we have tried to study the microbiological conversion of arsenocholine to arsenobetaine: viz. conversion of arsenocholine by micro-organisms occurring in the sediments was studied.

MATERIALS AND METHODS

Sediment

Bottom sediment was collected with an Ekman grab sampler from the coastal waters of Yoshimi, Shimonoseki, Japan, in May and July 1990.

Microbial conversion of arsenocholine

Two culture media, which have been used so far in microbial degradation experiments of arsenobetaine,[1–9] were also used in this *in vitro*

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study: 1/5 ZoBell 2216E ($g\,dm^{-3}$ filtered seawater: peptone, 1.0; yeast extract, 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), 0.3; 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; and ammonium chloride (NH_4Cl), 1.0]. The conversion experiment was performed under the same conditions as those used for the degradation experiments of arsenobetaine. Arsenocholine [$(CH_3)_3As^+CH_2CH_2OH$, 50 mg] and the sediment were added to each medium (25 cm^3) in a 50-cm^3 Erlenmeyer flask. The flasks were shaken at 25 °C in the dark under an atmosphere of air. Mixtures autoclaved at 120 °C for 20 min served as controls for these experiments. An aliquot of each mixture in the flask was withdrawn at intervals of several days of incubation and 0.1 cm^3 of it was added to 2.0 cm^3 of water. The arsenic compounds in the diluted aliquots were fractionated using high-performance liquid chromatography.

High-performance liquid chromatography

Arsenicals in the diluted mixtures were fractionated with a high-performance liquid chromatograph (HPLC Tosoh Co., CCP 8000 series) using a TSK Gel ODS-120T column (4.6 mm × 250 mm) with a 11.2 mmol dm^{-3} solution of sodium heptanesulfonate in water/acetonitrile/acetic acid (95:5:6 by volume) as mobile phase.[17] Portions of 10 or 20 (μl) each eluted fraction were analyzed with a graphite furnace atomic absorption spectrometer (GF AA, Nippon Jarrel Ash, model AA 845) serving as the arsenic-specific detector as described previously.[3]

Purification of metabolites

About 5 cm^3 of the medium containing arsenocholine and its metabolites was taken from the flask on day 14. After filtration, the mixture was applied to a cation-exchange column (Dowex 50W × 8, 100–200 mesh, H^+ form, 2.0 cm × 40 cm), and eluted with water (600 cm^3) and 1.5 mol dm^{-3} aqueous ammonia (600 cm^3) successively. Fractions (5 cm^3) were monitored with the graphite furnace atomic absorption spectrometer as previously described.[3] The arsenic-containing fractions were pooled, concentrated and placed on a Dowex 50W × 8 (100–200 mesh, pyridinium form, 1 cm × 50 cm) 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), successively. Arsenic-containing fractions were pooled and freeze-dried.

Identification of metabolites

The purified metabolites were chromatographed on a cellulose thin layer (Funakoshi Yakuhin Co. Ltd, Avicel SF, 0.1 mm). $SnCl_2$–KI reagent[18] and Dragendorff reagent were used for the detection of the spots.

FAB mass spectrometry (FAB MS, JEOL JMS DX-300 mass spectrometer equipped with fast atom bombardment, xenon atoms at 6 keV) and a combination of gas chromatographic separation with hydride generation followed by a cold-trap technique and selected ion monitor mass spectrometric analysis (hydride generation/cold trap/GC MS/SIM)[19] were used for the confirmation of the structure of the purified metabolites obtained as described in the preceding section.

RESULTS

Microbial conversion of arsenocholine

Figure 1 shows the time course pattern of arsenocholine and its microbial metabolites in the experiment performed with the sediment collected in May 1990. Two (inorganic salts medium) or three kinds (ZoBell medium) of metabolites were detected with HPLC. In this paper, they are labelled metabolite-1, metabolite-2 and metabolite-3; their retention times agreed with those of arsenobetaine, dimethylarsinic acid and trimethylarsine oxide, respectively. Higher activity was shown in the inorganic salts medium, in which arsenocholine disappeared after 14 days of incubation.

The time course pattern in the experiment performed with the sediment collected in July 1990 is shown in Fig. 2. On the whole, this pattern was similar to that with the sediments collected in May. The conversion activity, however, was lower than that in May: a considerable amount of

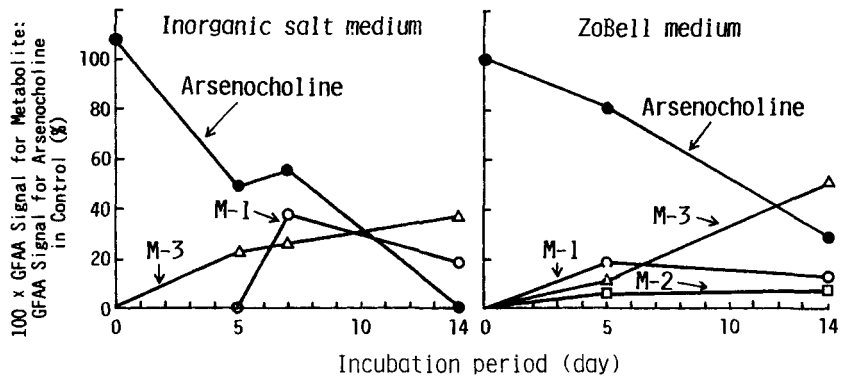

Figure 1 The conversion of arsenocholine to two or three metabolites (metabolite-1, metabolite-2 and metabolite-3) during aerobic incubation at 25 °C in an inorganic salts medium and a ZoBell medium added to the sediment collected in May 1990. M-1, metabolite-1; M-2, metabolite-2; M-3, metabolite-3.

arsenocholine still remained after 32 days of incubation. Metabolite-2 also appeared in the inorganic salts medium in this case.

Purification of the metabolites

A 5 cm^3 portion of the incubated mixture (14 days) of inorganic salts medium and the sediment collected in July 1990 was taken up and applied to Dowex 50 $\times 8$ (H^+ form). The metabolites were eluted with 1.5 mol dm^{-3} aqueous ammonia. The eluates were then chromatographed with Dowex 50×8 (pyridinium form). Two and one arsenic fractions were eluted with 0.1 mol dm^{-3} pyrydine–formic acid buffer and 0.1 mol dm^{-3} pyridine, respectively. The two arsenic fractions eluted with the buffer had retention volumes slightly different from each other (42–74 cm^3 and 76–136 cm^3). The compound with lower retention volume corresponded to metabolite-2 and the other to metabolite-3 in HPLC. On the other hand, the arsenic compound eluted with

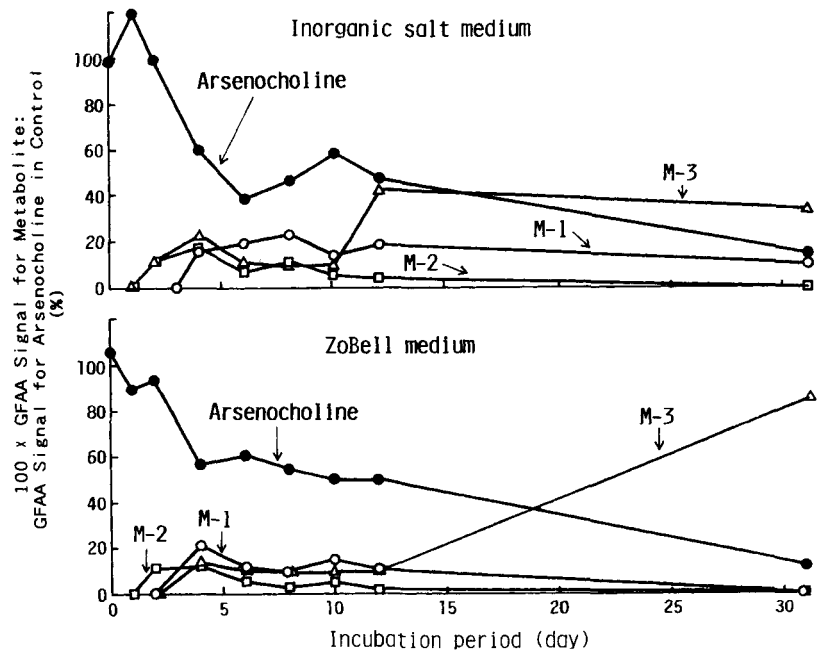

Figure 2 The conversion of arsenocholine to three metabolites (metabolite-1, metabolite-2 and metabolite-3) during aerobic incubation at 25 °C in an inorganic salts medium and a ZoBell medium added to the sediment collected in July 1990. M-1, metabolite-1; M-2, metabolite-2; M-3, metabolite-3.

Table 1 R_f values in thin-layer chromatography of metabolite-1 and metabolite-3

Sample	R_f value				
	Solvent system[a]				
	1	2	3	4	5
Arsenobetaine	0.71	0.66	0.61	0.32	0.59
Metabolite-1	0.70	0.67	0.59	0.32	0.59
Trimethylarsine oxide	0.80	0.80	0.76	0.43	0.71
Metabolite-3	0.80	0.80	0.76	0.44	0.69

[a] Solvent systems: 1, ethyl acetate/acetic acid/water (3:2:1); 2, chloroform/methanol/ammonia (28%) (3:2:1); 3, 1-butanol/acetone/formic acid (85%)/water (10:10:2:5); 4, 1-butanol/acetone/ammonia (28%)/water (10:10:2:5); 5, 1-butanol/acetic acid/water (4:2:1).

0.1 mol dm^{-3} pyridine (retention volume 52–74 cm^3) corresponded to metabolite-3. Each arsenic fraction was pooled and freeze-dried.

Identification of the metabolites

The purified metabolite-1 was subjected to thin-layer chromatography and FAB MS. The R_f value of metabolite-1 agreed with that of synthetic arsenobetaine in five solvent systems on a cellulose thin layer (Table 1). FAB mass spectra of metabolite-1 and synthetic arsenobetaine are shown in Fig. 3. Both are essentially the same, showing the most intense peak at m/z 179 $(M+1)^+$ along with peaks of the characteristic fragments (m/z 135 $(CH_3)_4As^+$; 120 $(CH_3)_3As^+$; 105 $(CH_3)_2As^+$) and adduct ions (m/z 201 $[M+Na]^+$; 313 $[M+(CH_3)_4As]^+$; 357 $[2M+1]^+$; 379 $[2M+Na]^+$). The 211 peak corresponds to M + As. It is not well known why this phenomenon occurs. We did not detect inorganic arsenic in the incubaton mixture by HPLC in this study. Trace amounts of inorganic arsenic, however, may be derived from arsenocholine and contaminate the purified trimethylarsine oxide, showing

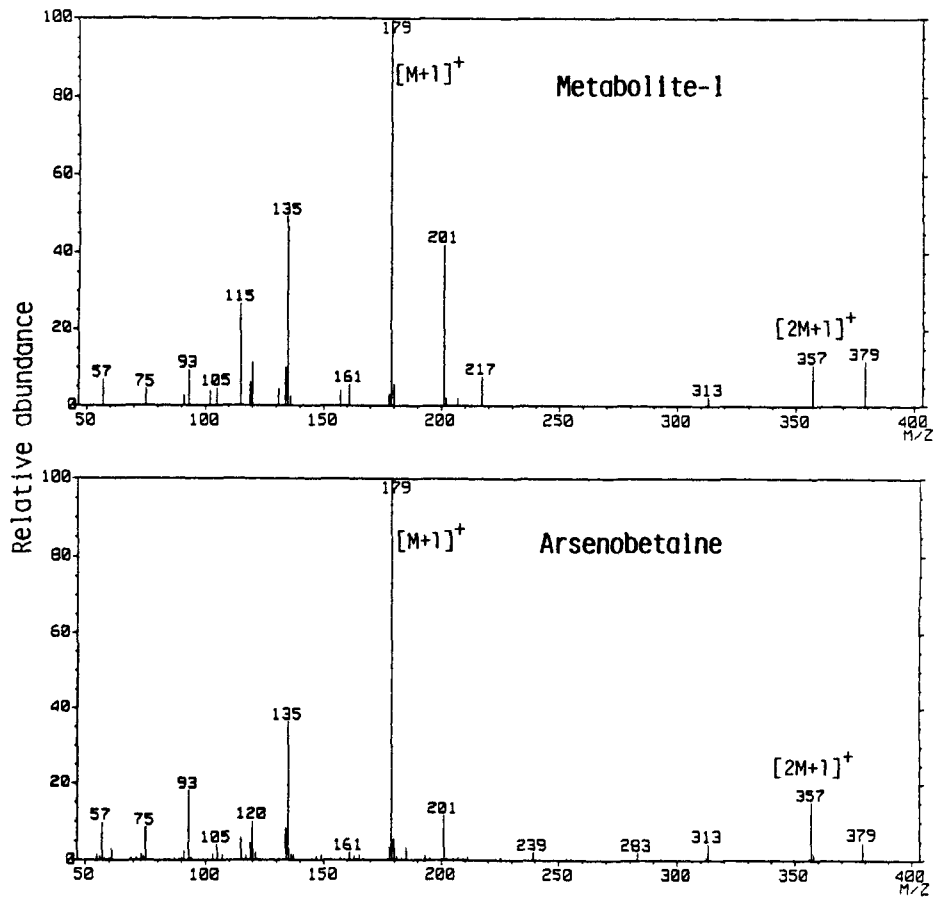

Figure 3 FAB mass spectra of metabolite-1 and synthetic arsenobetaine.

this adduct ion (M+As). We believe that Metabolite-1 is arsenobetaine. From the informaton from HPLC, thin-layer chromatography and FAB MS, metabolite-1 was confirmed as arsenobetaine.

Hydride generation/cold trap/GC MS/SIM analysis of metabolite-2 gave only dimethylarsine without preceding hydrolysis with sodium hydroxide, indicating metabolite-2 to be dimethylarsinic acid (Fig. 4). Metabolite-2 was concluded to be dimethylarsinic acid from the HPLC results and the GC MS/SIM analysis.

The R_f value of metabolite-3 accorded with that of synthetic trimethylarsine oxide in the five solvent systems (Table 1). The FAB mass spectra of metabolite-3 and synthetic trimethylarsine oxide proved these two compounds to be identical with m/z 137 $(M+1)^+$, the most intense peak, and

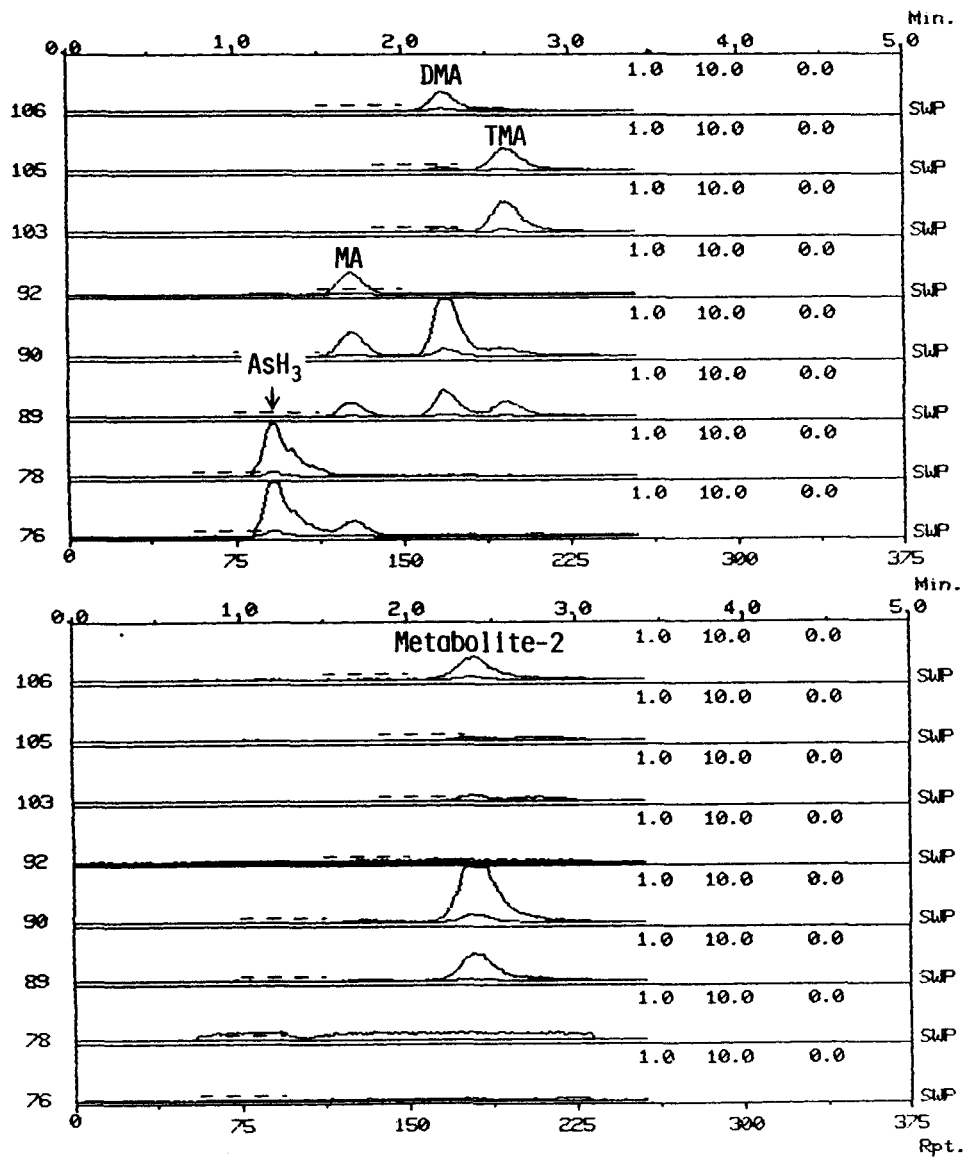

Figure 4 SIM chromatogram of metabolite-2 and those of arsine (AsH_3), methylarsine (MAA), dimethylarsine (DMA) and trimethylarsine (TMA) volatilized from each standard arsenical.

m/z 273 $(2M+1)^+$ (Fig. 5). From the information from HPLC, thin-layer chromatography and FAB MS, metabolite-3 was confirmed as trimethylarsine oxide.

DISCUSSION

The formation of arsenobetaine from arsenocholine was clearly found in this study. This conversion is without doubt the result of the action of micro-organisms occurring in the sediment because no conversion of arsenocholine was observed in the mixtures incubated after being autoclaved. Although this conversion or oxidation of arsenocholine has been reported in mammals[14,15] and fish,[16] participation of marine micro-organisms occurring in the sediments in the conversion was shown for the first time. We have already reported that arsenobetaine is degraded by marine micro-organisms to inorganic arsenic via trimethylarsine oxide, dimethylarsinic acid and methanearsonic acid.[1-10] This degradation and the microbial conversion of arsenocholine to arsenobetaine in this study led to the following conclusion: in the arsenic cycle previously proposed by us,[6,10] the pathway arsenocholine → arsenobetaine → trimethylarsine oxide → dimethylarsinic acid → methanearsonic acid → inorganic arsenic can be carried out by marine micro-organisms alone. On the other hand, a trimethylarsonioriboside in a marine alga is reported to be anaerobically converted to arsenocholine.[20] Taking account of this fact, it is suggested that micro-organisms play a very important role in arsenic circulation in marine ecosystems.

In this conversion study, trimethylarsine oxide and dimethylarsinic acid were derived as well as arsenobetaine. Trimethylarsine oxide has always been detected in experiments on degradation of arsenobetaine so far; dimethylarsinic acid has also

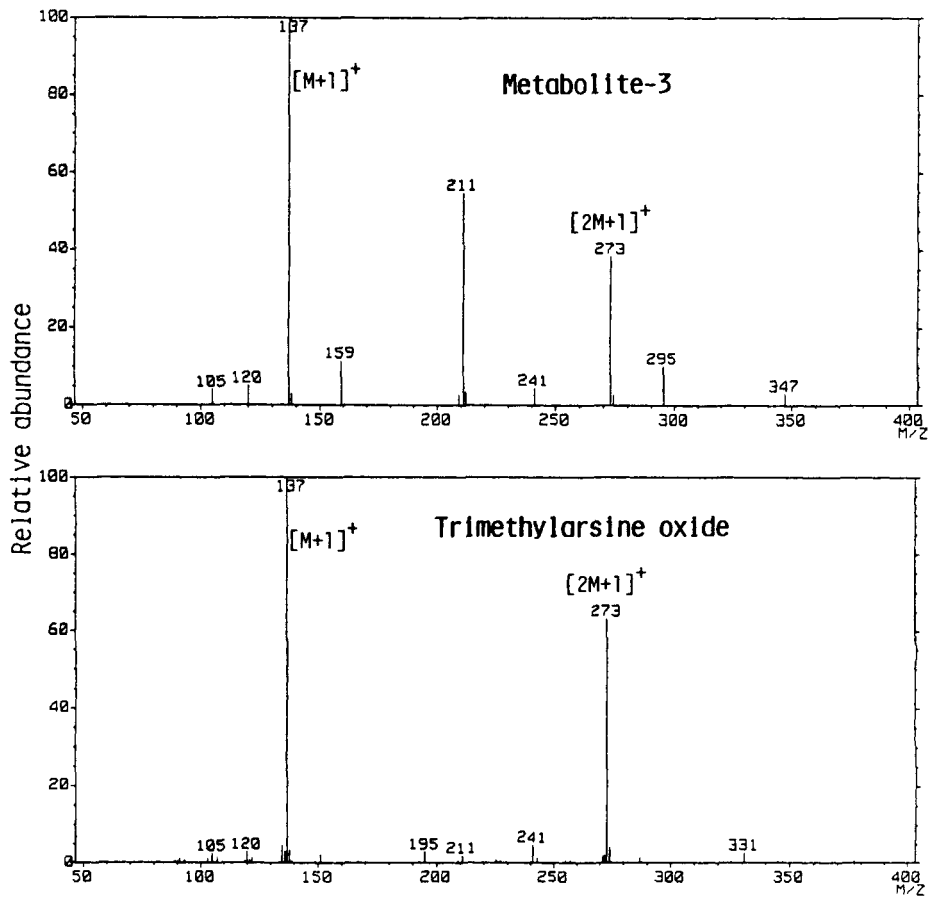

Figure 5 FAB mass spectra of metabolite-3 and synthetic trimethylarsenine oxide.

been found in most cases. Therefore, these metabolites in this study, at least in part, were considered to be metabolites of arsenobetaine which was derived from arsenocholine. Trimethylarsine oxide and dimethylarsinic acid, however, appeared prior to the appearance of arsenobetaine in the inorganic salts medium (May) and the ZoBell medium (July), respectively, suggesting that arsenocholine can be directly degraded to trimethylarsine oxide or dimethylarsinic acid.

The activity of micro-organisms in the sediments to convert arsenocholine to arsenobetaine or other metabolites was higher in May than in July. This difference suggests that there may also be a seasonal difference in the rate of the conversion of various arsenic compounds other than arsenocholine. Actually, the conversion rate of arsenobetaine to its metabolies has been observed seasonaly different.[1,3,5,6] These differences are important regarding the rate of arsenic circulation, although neither the number of micro-organisms nor their species occurring in the sediment was known in this study. In order to clarify the circulation of arsenic, we would like to investigate it also from a more detailed microbiological point of view.

On the other hand, activity in the inorganic salts medium was the same as or higher than that in the ZoBell medium. It was therefore proved that there are micro-organisms which can use arsenocholine as the only carbon source, because the inorganic salts medium contained no carbon sources other than arsenocholine except for trace amounts of organic matter contained in the sediment added to the medium. Probably arsenocholine-oxidizing micro-organisms occur ubiquitously in marine environments in the same way as arsenobetaine-decomposing micro-organisms, whose existence has already been reported in sediments,[1-3,5,6] on the surface of macro-algae,[4] in the intestine of a mollusk[7] and in suspended substances.[9] In order to prove their ubiquitous existence, various micro-organisms may have to be examined in order to determine their ability to convert arsenocholine.

CONCLUSION

The formation of arsenobetaine from arsenocholine by sedimentary micro-organisms is clearly demonstrated in this study. Trimethylarsine oxide and dimethylarsinic acid were also derived from arsenocholine as microbial degradation products. From results in this and other studies, it was concluded that, in the arsenic cycle previously proposed by us, the pathway arsenocholine → arsenobetaine → trimethylarsine oxide → dimethylarsinic acid → methanearsonic acid → inorganic arsenic can be carried out by marine micro-organisms alone.

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