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Ubiquity of arsenobetaine in marine animals and degradation of arsenobetaine by sedimentary micro-organisms

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Arsenic compounds were extracted with chloroform/methanol/water from tissues of marine animals (four carnivores, five herbivores, five plankton feeders). The extracts were purified by cation and anion exchange chromatography. Arsenobetaine [$(CH_3)_3As^+CH_2COO^-$], dimethylarsinic acid [$(CH_3)_2AsOOH$], trimethylarsine oxide [$(CH_3)_3AsO$] and arsenite, arsenate, and methylarsonic acid [$(CH_3AsO(OH)_2$] as a group with the same retention time were identified by high-pressure liquid chromatography. Arsenic was determined in the collected fractions by graphite furnace atomic absorption spectrometry. Arsenobetaine found in all the animals was almost always the most abundant arsenic compound in the extracts. These results show that arsenobetaine is present in marine animals independently of their feeding habits and trophic levels.

Arsenobetaine-containing growth media (ZoBell 2216E; solution of inorganic salts) were mixed with coastal marine sediments as the source of micro-organisms. Arsenobetaine was converted in both media to trimethylarsine oxide and trimethylarsine oxide was converted to arsenite, arsenate or methylarsonic acid but not to dimethylarsinic acid. The conversion rates in the inorganic medium were faster than in the ZoBell medium. Two dominant bacterial strains isolated from the inorganic medium and identified as members of the *Vibro–Aeromonas* group were incapable of degrading arsenobetaine.

Keywords: Arsenobetaine, trimethylarsine oxide, marine animals, micro-organisms, arsenic metabolism

INTRODUCTION

Arsenobetaine and other organic arsenic compounds were isolated from or identified in many marine organisms during the past decade. Arsenobetaine[1–17] is the arsenic compound that has most frequently been found in marine animals from higher trophic levels. These observations led to the hypothesis that arsenobetaine is the final arsenic metabolite in marine ecosystems, in which phytoplankton or microalgae take inorganic arsenic from seawater and form organic arsenic compounds. This conversion of inorganic arsenic to arsenobetaine has received much attention. In contrast, the fate of arsenobetaine after the deaths of animals containing this compound was entirely unknown before our investigations of the degradation of arsenobetaine by micro-organisms living in marine sediments.[18,19]

This paper presents the results of a survey relating to the presence of arsenobetaine in marine animals from various trophic levels and further data on the microbial degradation of arsenobetaine.

EXPERIMENTAL

Marine animals belonging to the group of carnivores, herbivores or plankton feeders, bottom sediments, and seawater samples were collected from the coastal waters of Yoshimi, Shimonoseki, Japan, during July 1987.

Extraction and partial purification of the arsenic compounds

Samples (10.0 g) of the animal tissues were

homogenized in the presence of chloroform/methanol (2:1, v/v, 200 cm^3). The homogenate was filtered and the filtrate was then thoroughly mixed with water (50 cm^3).[20] The aqueous phase was separated and concentrated to a small volume. The concentration and evaporation were performed under reduced pressure below 40°C throughout the experiment. The concentrate was placed on a cation-exchange column (Dowex 50W-X8, H^+ form, 2.0 cm × 8.0 cm) and eluted with 1.5 mol dm^{-3} aqueous ammonia. Fractions (10 cm^3) were monitored with the graphite furnace atomic absorption spectrometer (GF AA, Nippon Jarrel Ash, model AA 845) as the arsenic-specific detector under the following conditions: drying at 200°C for 20 s and ashing at 500°C for 60 s, both under an atmosphere of air, and atomization at 2400°C for 10 s under an argon atmosphere; deuterium background correction; monochromator at 193.7 nm. Portions (15 mm^3) of each fraction plus 5 mm^3 of a 1,000 mg kg^{-1} nickel solution in 1 mol dm^{-3} HNO_3 were used for each analysis. The arsenic-containing fractions (40–70 cm^3) were concentrated and passed through an anion-exchange column (Dowex 1-X8, OH^- form, 2.0 cm × 8.0 cm). The arsenic-containing fractions (10–30 cm^3) eluted with water through this column were combined and evaporated to 10 cm^3.

Microbial degradation of arsenobetaine

Two media [1/5 ZoBell 2216E (g dm^{-3} filtered seawater): peptone 1.0; yeast extract 0.2, 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 ($FeCl_2 \sim H_2O$) 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] were used for the degradation experiments. Synthetic arsenobetaine [$(CH_3)_3As^+CH_2COO^-$, 50 mg] and sediment (1.0 g) or seawater (2.0 cm^3) were added to each medium (25 cm^3) in a 50-cm^3 Erlenmeyer flask. The flasks kept at 25°C in the dark were shaken for 80 days under an atmosphere of air. Mixtures autoclaved at 120°C for 20 min served as controls. Filtered aliquots from the mixtures in the flasks were withdrawn in intervals of several days and diluted with distilled water to 20 times their volumes. Arsenic compounds in the diluted aliquots were identified and quantified by high-pressure liquid chromatography as described below.

Identification of arsenic compounds

The arsenic compounds in the partially purified extracts and in the diluted media samples were separated on a high-pressure liquid chromatograph (Toyo Soda Co., CCP 8000 series, TSK Gel ODS-120T column, 4.6 mm × 250 mm) with a 5.6 mmol dm^{-3} solution of sodium heptanesulfonate in water/acetonitrile/acetic acid (95:5:6 by vol.) as the mobile phase at a flow rate of 0.5 cm^3 min^{-1}.[21] Fractions (1.0 cm^3) were collected and an aliquot (50 mm^3) of each fraction injected into the graphite furnace atomic absorption spectrometer as described previously. Arsenite, arsenate and methylarsonic acid did not spearate under these conditions (Fig. 1). The total arsenic indicated in Fig. 1 was determined after digesting these samples (0.5 g) with nitric, sulfuric and perchloric acids. To the residue in the digestion beaker were added aliquots of 25% diammonium hydrogen citrate, hydrochloric acid, 1 mol dm^{-3} potassium iodide and 0.4 mol dm^{-3} stannous chloride and diluted with water to a concentration of 10–80 μg arsenic dm^{-3},[22] and determined by arsine (AsH_3) evolution–electrothermal atomic absorption spectrometry. The detection limit for arsenic was 1 ng. The calibration curve was linear from 1 to 100 ng arsenic.

Isolation and identification of trimethylarsine oxide as a microbial degradation product of arsenobetaine

The inorganic growth medium (25 cm^3) was mixed with arsenobetaine (50 mg) and sediment (1 g) in a 50 cm^3 flask. The flask was shaken in the dark for 120 h. The mixture was centrifuged and the supernatant was placed on a Dowex 50W-X2 column (1 cm × 58 cm) equilibrated with pyridine–formic acid buffer (pH 3.1) and eluted with the same buffer. The metabolite retained on the column was then eluted with 0.1 mol dm^{-3} pyridine. The effluent in which arsenic was detected was pooled and freeze-dried to yield a white crystalline powder (20 mg).[19] The purified metabolite was chromatographed on cellulose thin layers (Merck AG, 0.1 mm) together with synthetic trimethylarsine oxide (solvent system, R_f of metabolite, R_f of trimethylarsine oxide): ethyl acetate/acetic acid/water (3:2:1), 0.87, 0.88; chloroform/methanol/28% aqueous ammonia (3:2:1), 0.82, 0.83; 1-butanol/acetone/formic acid/water (10:10:2:5), 0.56, 0.57; 1-butanol/acetone/28% aqueous ammonia/water (10:10:2:5), 0.50, 0.50; 1-butanol/acetic acid/water (4:2:1), 0.72, 0.72. The ^1H and ^{13}C NMR (D_2O, Bruker-AAM-400, sodium 3-trimethylsilylpropionate-d_4) signals of the metabolite had the same chemical shifts as synthetic trimethylarsine oxide (^1H 1.79; ^{13}C 17.3). The FAB mass spectra (JEOL JMS DX-300, Xe at 6 keV) of trimethylarsine oxide and the

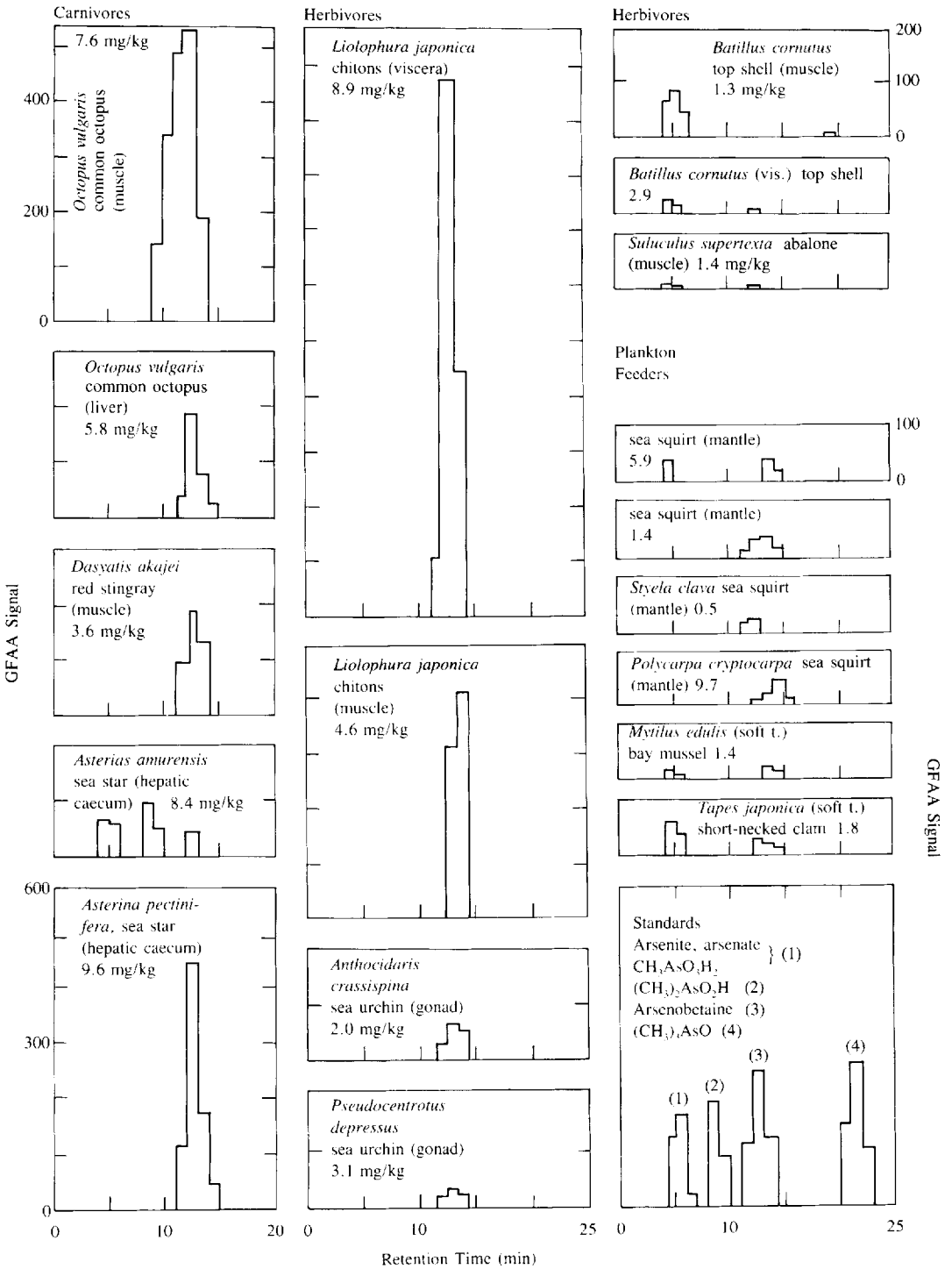

Figure 1 HPLC–GF AA chromatograms of partially purified extracts from carnivorous, herbivorous and plankton-feeding marine animals with total arsenic concentrations given as μg arsenic per gram wet weight. For the standards, only their retention times are shown and the GF AA signal is on an arbitrary scale.

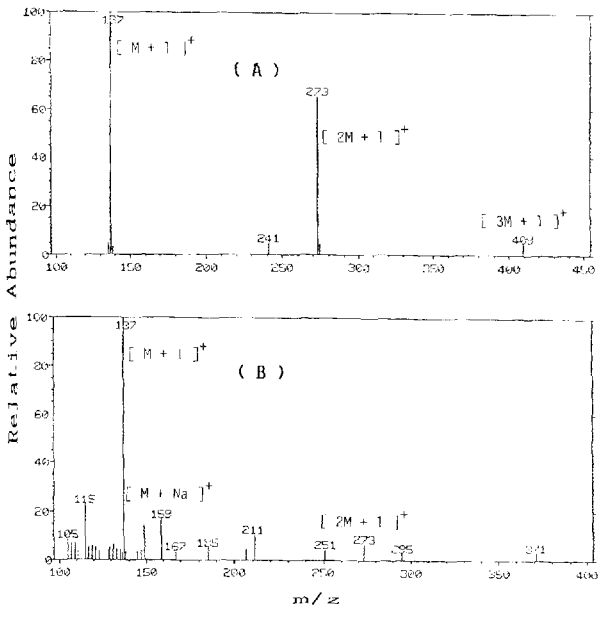

Figure 2 FAB MS (Xe, 6 keV) of trimethylarsine oxide (A) and metabolite 1 (B).

metabolite (Fig. 2) proved these two compounds to be identical with m/z 137 $[(CH_3)_3AsOH^+]$ the most intense peak. Several low-intensity peaks in the mass spectrum of the metabolite indicated that impurities were still present.

RESULTS AND DISCUSSION

Arsenobetaine in marine organisms

The tissues of carnivores contained arsenic in the concentration range 3.6–9.6 mg kg^{-1} wet weight. The herbivores (1.3–8.9 mg kg^{-1}) and the plankton feeders (0.5–9.7 mg kg^{-1}) had on the average lower arsenic concentrations than the carnivores (Fig. 1). HPLC separation of the arsenic compounds in the aqueous extracts obtained from specific tissues of these organisms including the viscera of *Batillus cornutus* showed that the major arsenic compound in these tissues is arsenobetaine. The muscle of the top shell *B. cornutus* is the only tissue in which arsenobetaine was not detected. Arsenite, arsenate and methylarsonic acid, compounds that were not separated under the chromatographic conditions used, or any combination of these three compounds were found in the hepatic caecum of the sea star *Asterias amurensis*, the muscle and viscera of *B. cornutus*, the muscle of the abolone *Suluculus supertexta*, the mantle of a sea squirt, and the soft tissues of the bay mussel *Mytilus edulis* and the short-necked clam *Tapes Japonica*. Trimethylarsine oxide was detected only in the muscle of *B. cornutus* (Fig. 1). These results indicate that arsenobetaine is not only present in marine animals belonging to higher trophic levels but also in animals at lower trophic levels.[16] Feeding habits do not seem to determine whether an animal contains arsenobetaine or not. A larger variety of animals and tissues need to be checked for arsenobetaine, other water-soluble arsenic compounds, and lipophilic arsenic compounds. It is possible that the ratios of arsenobetaine concentrations to the concentrations of the other groups of arsenic compounds will provide information about the sources and transformation of arsenic derivatives at various trophic levels.

Microbial transformation of arsenobetaine

When the arsenobetaine-containing (50 mg/25 cm^3) media (ZoBell, solutions of inorganic salts) were exposed to the micro-organisms introduced by addition of 1 g sediment collected in July 1987, arsenobetaine was converted to two types of arsenic compounds. The

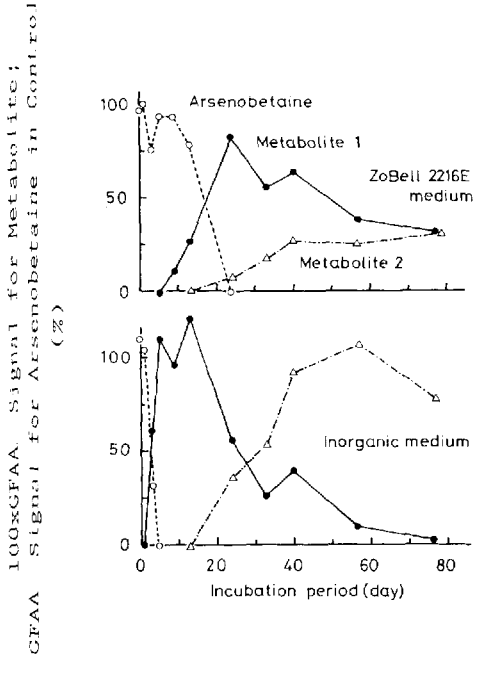

Figure 3 The microbial conversion of arsenobetaine to trimethylarsine oxide (metabolite 1) and metabolite 2 (arsenite, arsenate and/or methylarsonic acid) in a ZoBell medium and an inorganic salt medium.

first metabolite was isolated from the inorganic salt medium and identified by HPLC, TLC, ^{1}H and ^{13}C NMR spectroscopy, and FAB MS as trimethylarsine oxide. The second metabolite had an HPLC retention time similar to the retention time of arsenite, arsenate and methylarsonic acid that were not separated under the chromatographic conditions used.

The disappearance of arsenobetaine and the appearance of trimethylarsine oxide and metabolite 2 in the two media was followed by chromatography of aliquots of the diluted media. In the inorganic salt medium mixed with sediment, arsenobetaine was completely converted to trimethylarsine oxide within five days. Metabolite 2 began to appear on day 13 of the incubation period and on day 60 was practically the only arsenic compound(s) in the medium. At incubation times longer than 60 days, the concentration of metabolite 2 decreased (Fig. 3). In the ZoBell medium mixed with sediment, arsenobetaine was also converted to trimethylarsine oxide, and trimethylarsine oxide to metabolite 2, but at rates much slower than in the inorganic salt medium. The cause for the differences in the rates of the conversions of the arsenic compounds may be with the carbon sources available to the sedimentary micro-organism in the ZoBell and the inorganic salt media. In the inorganic salt medium arsenobetaine was the only carbon source, if one neglects the small amount of organic material in the 1 g of sediment added to the medium. The micro-organisms probably used the carboxymethyl moiety of arsenobetaine to satisfy their requirement for organic carbon and converted arsenobetaine to trimethylarsine oxide. After this source of carbon had become rapidly exhausted, the methyl groups in trimethylarsine oxide became attractive, they might have been cleaved from the arsenic compound, and were utilized by the micro-organisms with concomitant conversion of trimethylarsine oxide to methylarsonic acid, arsenate, or arsenite. The analytical methods employed did not distinguish these three arsenic compounds. Dimethylarsinic acid was not observed in these media.

Similar experiments with sediments collected in January 1987[18] gave qualitatively the same results as obtained with sediments collected in July 1987. The transformation of arsenobetaine to trimethylarsine oxide and of trimethylarsine oxide to metabolite 2 occurred in both the media. However, the conversion rates were faster in the ZoBell/January sediment mixture than in the ZoBell/July sediment mixture.

When the two arsenobetaine-containing media were mixed with 2 cm^3 of seawater as the source of micro-organisms, arsenobetaine was not converted to trimethylarsine oxide. The likely cause for the failure of these experiments is the absence or very low number of micro-organisms in seawater. These experiments should be repeated with concentrated seawater.

Two dominant bacterial strains were isolated from the inorganic medium/July sediment mixture and several from the ZoBell/July sediment mixture using the enrichment culture method. The two strains from the inorganic medium were identified, using biological reactions and morphological characteristics,[23] as members of the *Vibrio–Aeromonas* group. When the arsenobetaine-containing media were inoculated with the bacterial strains, arsenobetaine was the only arsenic compound detected by liquid chromatography in the media throughout the incubation period. Why these strains did not degrade arsenobetaine is not known. It is possible that other bacteria that could not be obtained by the isolation procedure used are responsible for the conversion of arsenobetaine to trimethylarsine oxide.

CONCLUSIONS

Arsenobetaine is widely distributed in marine animals independently of their feeding habits and the trophic level to which they belong. Micro-organisms living in sediments convert arsenobetaine to trimethylarsine oxide and trimethylarsine oxide to a less methylated metabolite(s) that could include monomethylated arsenic compound(s). This degradation of arsenobetaine to arsenite or arsenate closes the marine arsenic cycle that begins with the methylation of arsenite on the way to arsenobetaine.

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