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Microbial Degradation of Arsenobetaine Accumulated in Marine Animals

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The microbial degradation of arsenobetaine, which is ubiquitous in marine animals, was investigated *in vitro* and also in a natural environment. This compound was aerobically degraded in culture media with every microbial origin used in this study such as sediments, suspended substances, macro algae, etc. The highest degradation activity was shown by the sediments where all the arsenobetaine were completely degraded to inorganic arsenic (V) via trimethylarsine oxide (TMAO) and dimethylarsinic acid. Arsenobetaine, however, was consistently the major arsenical in the bodies of microorganisms themselves even after it completely disappeared from the media. In the muscle and liver of the starspotted shark buried in the coastal sands and the muscle of dead chitons suspended in the water column, TMAO and inorganic arsenic (V) were detected as well as in the *in vitro* degradation experiments. The degradation of arsenobetaine to inorganic arsenic (V) in marine animals closes the marine arsenic cycle that begins with the methylation of inorganic arsenic on the route to arsenobetaine.

1 Introduction

Arsenobetaine $[(CH_3)_3As^+CH_2COO^-]$ is considered to be synthesized in marine ecosystems from inorganic arsenic in seawater via arsenosugar(s), arsenocholine $[(CH_3)_3As^+CH_2CH_2OH]$, etc.[1] The occurrence of arsenobetaine was first identified in the tail muscle of western rock lobster (Crustacea) as a naturally occurring organo-arsenical in 1977.[2] Since then arsenobetaine has been found in many other marine animals including blue shark[3] (Chondrichthyes), school whiting[4] (Osteichthyes), sea cucumber[5] (Mollusca) and various kinds of zoo-plankton.[6] We have also reported that arsenobetaine is distributed in marine animals independently of their feeding habits and the trophic levels to which they belong.[7-12] We then interested in the fate of arsenobetaine after the death of marine animals. In our experimental course, the microbial conversion of arsenobetaine has been investigated *in vitro*[12-22] and in a natural environment.[23,24] In this paper, we examined the synthetic arsenobetaine conversion by the microbe-containing sediments, suspended particles, etc., and the derived ar-

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senic compounds were analyzed. After examining the conversion and degradation pattern *in vitro*, a starspotted shark *Mustelus manazo* and chitons *Liorophura japonica* were left in a seashore environment to investigate whether the arsenobetaine that had accumulated in them could be degraded by marine microorganisms in a natural environment through the conversion route shown in the *in vitro* study. As previously reported by us, arsenobetaine is practically the sole arsenic compound occurring in the muscle[10] and liver[11] of the shark, and chitons also accumulate arsenobetaine as a major arsenic compound.[12]

2 Materials and Methods

2. 1 Microbial sources

Sediments, two kinds of macro-algae (the brown alga *Hizikia fujiform* and the green alga *Monostroma nitidum*), the intestine of a mollusk (chiton) and particles suspended in the water were collected from the coastal waters of Yoshimi, Japan. Sinking particles were collected by a sediment trap at a station off Kushiro, Japan at depths of 1,100 and 3,500m.

2. 2 Cultivation

Two culture media were used. These were 1/5 ZoBell 2216E [gdm^{-3} filtered sea water; peptone 1.0; yeast extracts 0.2, pH 7.5] and an aqueous solution of inorganic salts at pH 7.5 [gdm^{-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]. Synthetic arsenobetaine and each microbial source were added to each medium in a flask. The flasks kept at 25℃ in a dark were shaken under an atmosphere of air.

2. 3 Marine animals

A dead starspotted shark was buried in the intertidal sands of Yoshimi for 40 days at a depth of ca. 20cm. Eighteen chitons were collected from the seashore of Yoshimi, killed by stabbing, and wrapped in a net (1 mm in diameter). The net was suspended in seawater (at a depth of ca. 1m) for 30 days.

2. 4 Extraction and purification of arsenic compounds from microorganisms

Water soluble arsenic compounds occurring in the animals or harvested microorganisms from the incubated culture media were extracted with chloroform-methanol (2:1) as described previously.[12] The water-soluble extracts or the filtrates of the incubated culture media were applied to a cation-exchange resin, Dowex 50W-×8 (50-100 mesh, H^+ form) column and eluted with water, 2.0 mol dm^{-3} pyridine and 1.0 mol dm^{-3} HCl, successively. The arsenic-containing fraction was further applied to a Dowex 50W-×2

(200-400 mesh, pyridinium form) column equilibrated with 0.1 mol dm^{-3} pyridine-formic acid buffer (pH 3.1), being eluted with the same buffer and 0.1mol dm^{-3} pyridine.

2. 5 High performance liquid chromatography

Each extract or medium was analyzed by high performance liquid chromatography (Tosoh Co., Ltd., CCP 8000-series) using a ODS 120T column (4.6 × 250mm; Tosoh Co., Ltd.) with a mobile phase of 11.2mmol 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). Fractions were collected at 25-s intervals and each was analyzed for arsenic with a graphite furnace atomic absorption spectrometer. A mixture of authentic arsenic compounds was also fractionated.

2. 6 Confirmation of the metabolite

The purified arsenic metabolites were subjected to thin layer chromatography performed on a cellulose thin layer (Avicel SF, thickness: 0.1mm). FAB mass spectrometry was performed with a JEOL JMS DX-300 mass spectrometer equipped with a fast atom bombardment ion source.

3 Results

3. 1 Arsenic compounds as metabolites from arsenobetaine in the filtrates of the medium

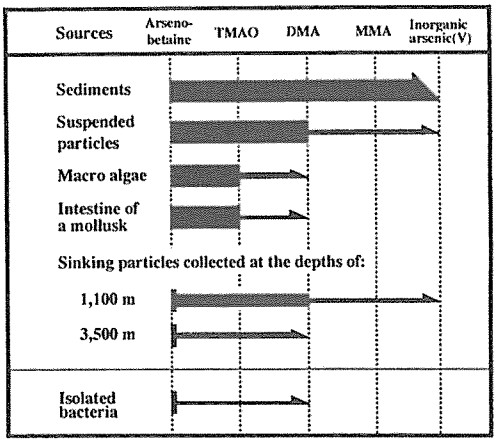

Fig. 1. Degradation of arsenobetaine by the microorganisms occurring in various marine sources.
TMAO: trimethylarsine oxide, DMA: dimethylarsinic acid, MMA: methanarsonic acid.

In each incubated medium containing arsenobetaine and a microbial source, arsenobetaine was degraded to some extent by microorganisms. Fig. 1 schematically shows the extent or ability of arsenobetaine decomposion for each source. Trimethylarsine oxide [$(CH_3)_3AsO$] and dimethylarsinic acid [$(CH_3)_2AsOOH$] were always detected as metabolites in the incubation mixtures. The highest degradation activity was shown by the sediments where all of these metabolites were further degraded to inorganic arsenic (V) as a complete degradation product. Suspended substances and sinking particles (from a depth of 1,100m) showed a high activity in which a part of the arsenobetaine was degraded to inorganic arsenic (V). With the sinking particles, however, there were great seasonal differences: for particles collected at 1,100 m in autumn, arsenobetaine was most degraded. Fig. 2

shows a typical degradation pattern of arsenobetaine in the medium containing the sediments, which showed the highest activity. With the disappearance of arsenobetaine, trimethylarsine oxide appeared and the trimethylarsine oxide further degraded to inorganic arsenic (V). A small amount of dimethylarsinic acid also appeared and finally disappeared.

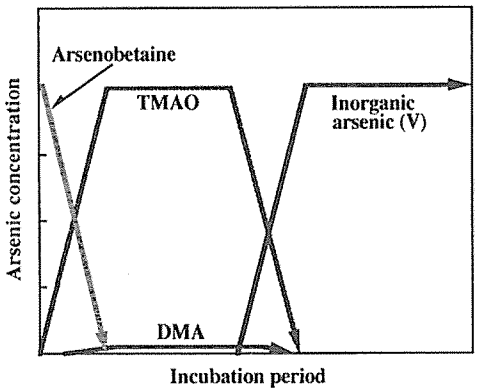

Fig. 2. Schematic degradation pattern of arsenobetaine to inorganic arsenic (V) *in vitro* by sedimentary microorganisms. With the disappearance of arsenobetaine within 2-3 days, trimethylarsine oxide, dimethylarsinic acid and inorganic arsenic (V) appeared in sequence. For abbreviations, see Fig. 1.

3. 2 Arsenic compounds within the bodies of the microorganisms

We investigated whether arsenobetaine is taken into the bodies of microorganisms themselves and, if this occurs, the structure(s) of the arsenic compound accumulating in them. Sediments were used as the microbial source because of their high activity in degrading arsenobetaine. After 50 days of incubation in the ZoBell medium, that is at the final stage of degradation, only arsenic compound present in the filtrate of broth was inorganic arsenic(V), in contrast the major arsenic compounds extracted from the microorganisms cells was arsenobetaine. HPLC analyses showed the conversion of arsenobetaine to inorganic arsenic(V) in the medium, on the other hand arsenobetaine taken in the microorganisms kept in this form during the incubation periods.

3. 3 Post-mortem formation of inorganic arsenic from arsenobetaine in animals under natural conditions

In the shark buried in the coastal sands, three new arsenic containing metabolites were found in the muscle and liver in addition to arsenobetaine. The arsenic compounds and the percent of the total arsenic that they represent in the muscle and liver were: arsenobetaine (63.3% and 83.8%), trimethylarsine oxide (3.8% and 3.9%), methanearsonic acid [$(CH_3)AsO(OH)_2$] (20.0% and 7.9%) and inorganic arsenic(V) (12.6% and 4.4%). The dead chitons that were suspended in the water column also had these four compounds plus dimethylarsinic acid and tetramethylarsonium ion [$(CH_3)_4As^+$]. Arsenic was distributed in these compounds as follows: arsenobetaine 17.1%; trimethylarsine oxide 25.3%; methanearsonic acid 11.8%; inorganic arsenic(V) 9.8%; dimethylarsinic acid 12.0%; tetramethylarsonium ion 24.0%.

4 Discussion

By comparing the extent of degradation of arsenobetaine shown by sedimentary microorganisms with the extents of degradation by other microbial sources (Fig. 1), one may expect that complete degradation of arsenobetaine is also attainable with any other microbial sources by providing the sufficient incubation period. No further degradation, however, occurred with longer incubation period (unpublished data), indicating that the incompleteness of degradation of arsenobetaine could not be attributed merely to a delay in the formation of inorganic arsenic. The microflora in the sources is considered to be the most important factor affecting the extent of degradation. Differences in the conversion rate between the ZoBell and inorganic salt media have been consistently found in the degradation experiments so far. The results of these experiments, however, have not indicated the superiority of one medium over the other with respect to the microbial degradation, i.e., we could not predict in which medium a microbial source would show a higher degradation activity. Thus, we always used both types of media. Whether the occurrence of abundant organic matter is favorable for the degadation of arsenobetaine by microorganisms may also depend again the microflora. The occurrence of microorganisms that can degrade arsenobetaine by one way or another has been clearly demonstrated in various places in the marine environment including the deep sea.

Throughout the *in vitro* degradation of arsenobetaine in the microbial sources, arsenobetaine remained the major arsenic compound within the microbes themselves. This fact may mean that when marine microorganisms degrade arsenobetaine, they take in arsenobetaine, cleave some useful groups such as the carboxymethyl moiety, which is a possible starting material for the synthesis of fatty acids, and discard the dangerous arsenic-containing residue. The accumulation of arsenobetaine in the microorganisms is very interesting with respect to understanding the contribution of microorganisms to arsenic circulation through the food chain.

In the muscle and liver of the starspotted shark and in the muscle of chitons, trimethylarsine oxide, dimethylarsinic acid and inorganic arsenic (V) were detected as well as in the *in vitro* degradation experiments so far. The *in vitro* degradation experiments used in this study may also prove

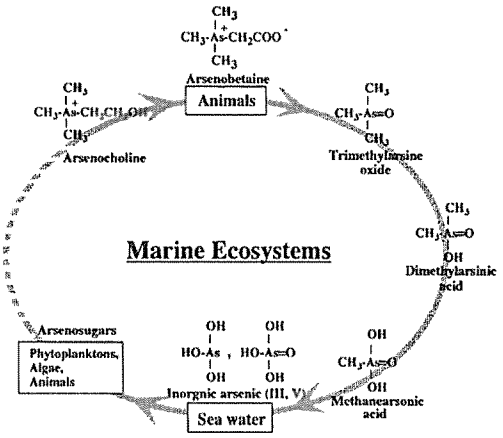

Fig. 3. A tentative arsenic cycle in marine ecosystems

to be useful in investigating the arsenic circulation in marine ecosystems. From these results, we conclude that arsenobetaine that is bioconverted through the food chain from inorganic arsenic in seawater is re-converted to the original inorganic arsenic via trimethylarsine oxide. Thus, the arsenic cycle that we previously proposed to operate in the marine ecosystem[12, 18] appears to be confirmed by the present results (Fig. 3).

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海産動物に存在するアルセノベタインの微生物分解

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海産動物に普遍的に存在する有機ヒ素化合物であるアルセノベタインの微生物分解について，*in vitro* および自然条件下で研究した。培地に添加された合成アルセノベタインは，底泥や懸濁物等，本研究で用いたすべての微生物源により分解された。微生物源のうち，最も高い活性を示した底泥においては，アルセノベタインはトリメチルアルシンオキシド（TMAO）およびジメチルアルシン酸を中間代謝物として完全に無期ヒ素（V）にまで分解された。しかし，培地中のヒ素化合物がすべて無期ヒ素（V）に変換された後でも，微生物自身の体内に保持されている主要なヒ素化合物はアルセノベタインであった。一方，海岸の砂中に埋めたホシザメの筋肉および肝臓，あるいは即殺後に水中に吊したヒザラガイの筋肉中においても TMAO および無機ヒ素（V）が生成した。すなわち，海洋生態系におけるヒ素循環を解明する方法として，本研究で行った *in vitro* 実験の有効性が示された。また，以上の結果から海洋生態系には無機ヒ素の有機化に始まり，アルセノベタインの合成を経て，元の無機ヒ素に回帰するヒ素サイクルが存在すると結論した。