The Journal of Shimonoseki University of Fisheries 43(4) 177 − 184 (1995)

Post-mortem Degradation of Arsenobetaine to Inorganic Arsenic in Ordinary Muscle and Liver of Starspotted Shark *Mustelus manazo*[*1]

Ken'ichi Hanaoka,[*2] Kenji Onda,[*2] Shoji Tagawa,[*2]
and Toshikazu Kaise[*3]

A specimen of *Mustelus manazo* was buried in the intertidal sands at a depth of ca. 20 cm. After 40 days, the central part of its dorsal white muscle and the whole liver were taken from the rotten shark and analysed for arsenic compounds derived in them. As the results, the formation of inorganic arsenic(V) via trimethylarsine oxide and methanearsonic acid was confirmed in both the muscle and the liver. From this and other evidence, it was concluded that arsenobetaine, which is bioconverted through the food chain from inorganic arsenic in seawater, is re-converted to original inorganic arsenic.

1. Introduction

Since arsenobetaine [$(CH_3)_3As^+CH_2COO^-$] was isolated and identified in muscle from western rock lobster,[1] many organo-arsenic compounds have been confirmed in marine organisms of various trophic levels, leading a following conclusion concering arsenic bioconversion in marine food chain. Arsenosugars[2-5] are biosynthesized by phytoplanktons or algae as the first arsenic metabolites from seawater and arsenobetaine bioaccumulates in marine animals as their final metabolite after succesive conversion through the food chain.[6-8] In the animals on lower trophic levels, although arsenobetaine accumulated together with another arsenic compounds such as arsenosugars,[9] it is practically the only water-soluble arsenic compound in those in high trophic level.

On the other hand, considering these remarkable progress stated above, the fate of arsenobetaine itself did not draw workers' attention in spite of its importance. In recent years, we have approached the fate of arsenobetaine microbiologically *in vitro* to pursue the arsenic circulation in marine ecosystems. Some kinds of origins of marine microorganisms have been chosen and the degradation experiments of arsenobetaine have been performed with each of them. In every origin of microorganisms, arsenobetaine was degraded, indicating the ubiquitous occurrence of

水産大学校研究業績 第1493号, 1994年12月20日受付.
Contribution from Shimonoseki University of Fisheries, No. 1493. Received Dec. 20, 1994.

*1 Oral presentation at the meeting of IASWS in December 1993, Santa Barbara. (平成5年12月第6回IASWS会議(アメリカ, サンタバーバラ)にて発表)

*2 Department of Food Science and Technology, Shimonoseki University of Fisheries, (花岡研一・恩田健司・田川昭治：水産大学校製造学科生物化学講座)

*3 School of Life Science, Tokyo College of Pharmacy, (貝瀬利一：東京薬科大学環境生命科学科環境衛生化学研究室)

arsenobetaine-decomposing microorganisms. The highest degradation activity was shown with those occurring in the bottom sediments[7, 10-13] and suspended substances,[14] by which arsenobetaine was completely degraded to inorganic arsenic(V)(sediments) or partly to it (suspended substances) via trimethylarsineoxide [$(CH_3)_3AsO$] or dimethylarsinic acid [$(CH_3)_2AsOOH$]. With microorganisms associated with marine macro-algae[15] and those occurring in the intestine of a mollusk,[16] arsenobetaine was degraded to dimethylarsinic acid. These results have led us to a hypothesis: there is an arsenic cycle in marine ecosystems that begins with the methylation of inorganic arsenic in seawater and terminates with the complete degradation of arsenobetaine to inorganic arsenic.[12, 17]

Arsenobetaine contained in the carcass of marine animal must be degraded to inorganic arsenic provided that the above hypothesis is true. In this study, starspotted shark *Mustelus manazo* was chosen as the material because of its high content of arsenobetaine in muscle (30-40 ppm) and liver (ca. 20 ppm), in which it accounted for more than 98 % of water soluble arsenic compounds[18-19]. Thus, an intact *Mustelus manazo* was buried in the intertidal sands for 40 days and it was confirmed that arsenobetaine contained in its muscle or liver was degraded to inorganic arsenic *in situ*.

2. Materials and Methods

2.1 Starspotted shark

A specimen of apparently dead starspotted shark *Mustelus manazo* (814 g, 65 cm), whose heart was still beating, was purchased and buried in the intertidal sands of Yoshimi at a depth of ca. 20 cm. After being left for 40 days from October to November, it was carried back to the laboratory and dissected after washing with water.

2.2 Extraction and purification of arsenic compounds

The water-soluble arsenic compounds were extracted from each of a part of the white muscle (15.2 g) and the whole liver (9.7 g) with 20 times their volumes of chloroform-methanol (2:1) as described before.[18-19]

The schematic method for the fractionation of arsenic compounds was shown in Fig.1. Each extract was chromatographed with a cation-exchange resin, Dowex 50W-×8 (50-100 mesh, H^+ form) column (2.2 ×18.5 cm), and eluted with 400 ml of water (labelled [50W/water]), 400 ml of 1.5 mol dm^{-3} aqueous ammonia ([50W/NH_3]) and 400 ml of 1.0 mol dm^{-3} HCl ([50W/HCl]), successively. Both the fractions eluted with water and aqueous ammonia were further chromatographed with an anion-exchange resin, Dowex 1-×8 (50-100 mesh, OH^- form) column (2.2 × 18.5 cm) and eluted with 400 ml of water ([50W/water→1/water, 50W/NH_3→1/ water]) and 400 ml of 1.5 mol dm^{-3} acetic acid ([50W/water→1/CH_3COOH, 50W/NH_3 → 1/CH_3COOH]), succecively. The fraction [50W/water → 1/water] was further applied to a Dowex 50W-× 8 (200-400 mesh, pyridinium form) column (1 × 50 cm) equilibrated with 0.1 mol dm^{-3} pyridine-formic acid buffer (pH 3.1) and eluted with the same buffer (200 ml) and 0.1 mol dm^{-3} pyridine (200 ml).

2.3 High performance liquid chromatography

Each arsenic-containing fraction was analysed by high performance liquid chromatography (Tosoh Co., Ltd, CCP 8000-series) using ODS 120T Column (4.6 × 250 mm; Tosoh Co., Ltd) with a mobile phase of 11.2 mmol dm^{-3} solution of sodium heptanesulphonate in water/acetonitril/ acetic acid[20] (95/5/6, by vol.; flow rate, 0.8 cm^3 min^{-1}; sample size, 5 mm^3). Twenty mm^3 of each eluate collected for every 25 s was injected into the graphite furnace atomic absorption spectrometer (GFAA) and analysed as described previously.[7] The mixture of the authentic arsenic compounds (all with 100 mg as As kg^{-1}), which have been detected in the *in vitro* degradation experiments of arsenobetaine so far, were also fractionated.

Some fractions were additionally analysed by

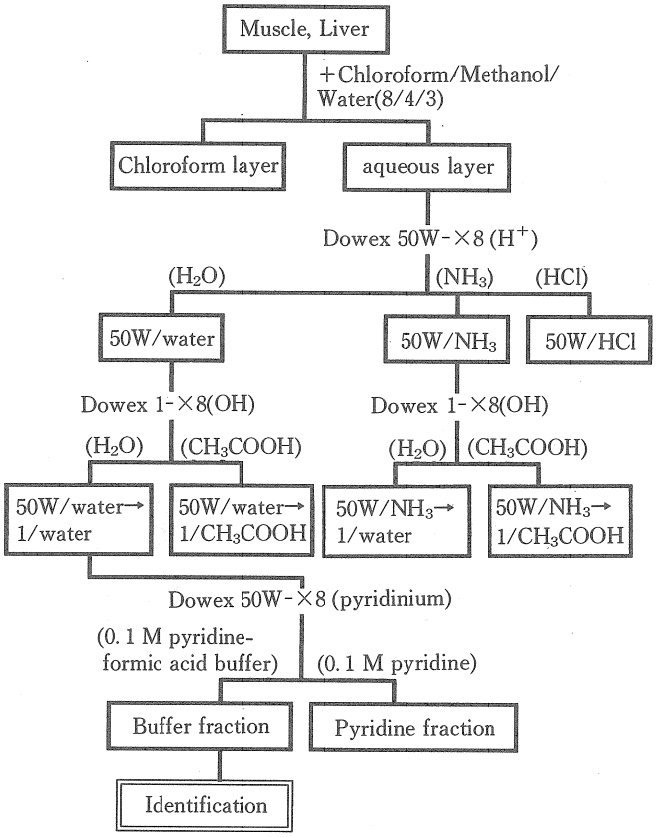

Fig. 1. Scheme for the purification of arsenic compounds.

high performance liquid chromatography using Nucleosil 10SB Column (4.6 × 250 mm; Wako-Junyaku-Kogyo Co.) with a mobile phase of 0.02 mmol dm^{-3} phosphate buffer[21] (pH 6.8; flow rate, 1.0 cm^3 min^{-1}; sample size, 5 mm^3). In order to eliminate the interference in the GFAA of phosphate contained in the mobile phase, the arsenic compound in each eluted fraction (0.4 cm^3) was precipitated by addition of both 0.1 cm^3 of aqueous ammonia (25%) and 20 mm^3 of 0.1 mol dm^{-3} $Fe(NO_3)_3$. The precipitate resulted was centrifuged for 10 min at 3000 rpm and dissolved in 0.1 cm^3 of 4 mol dm^{-3} HNO_3 solution after the supernatant was discarded. Twenty mm^3 of each solution was analysed by GFAA.

2.4 Confirmation of inorganic arsenic

The purified arsenic metabolite from arsenobetaine was subjected to the following analyses. Thin layer chromatography was performed on cellulose thin layer (Avicel SF, thickness: 0.1 mm, Funakoshi Yakuhin Co., Ltd.). $SnCl_2$-KI reagent[22] was used to indicate the position of the metabolite. Ion chromatography was carried out usingAS4A column (Dionex Co., 4.6 × 250 mm). An aqueous solution containing 1.8 mmol dm^{-3} Na_2CO_3 and 1.7 mmol dm^{-3} $NaHCO_3$ was used as a mobilephase at a flow rate of 1.0 cm^3 min^{-1}. Absorption spectra of the molybdoarsenate-Malachite Green aggregate in 2-methoxyethanol[23] were measured on a U-1100 Spectrophotometer (Hitachi Co., Ltd.).

3. Results

3.1 Fractionation of arsenic compounds extracted from the *Mustelus manazo* left in the sands

The white muscle of the *Mustelus manazo* left in the sands was considerably fragile. In order to avoid the contamination with another tissues or organs, white muscle (15.2 g) was taken from the central part of dorsal white muscle of the shark. On the other hand, as the liver of the shark had still kept a solid structure (9.7 g), the whole was used.

The extracted arsenic compounds from the muscle and the liver were fractionated column chromatographically. Arsenic compounds were detected in four fractions after fractionation with Dowex 50W- × 8 (H^+) and Dowex 1- × 8 (OH^-). These fractions were separated by HPLC with a ODS column. The fraction [50W/NH_3→1/water] showed two arsenic peaks, while the other three fractions ([50W/water → 1/water], [50W/water → 1/CH_3COOH] and [50W/HCl]) showed a single peak in both muscle and liver.

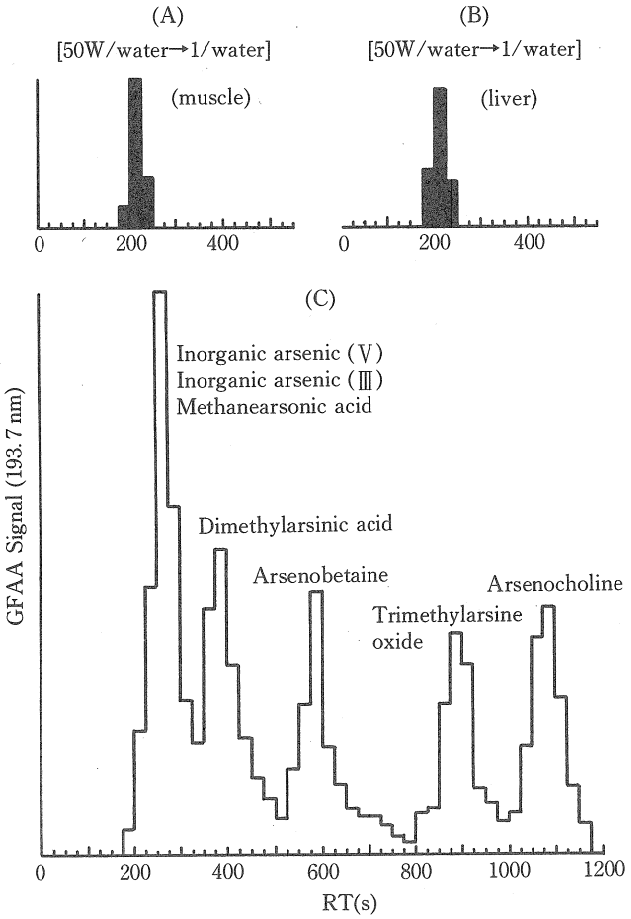

Fig. 2. High performance liquid chromatograms (column: ODS 120T) of partially purified arsenic compound in [Dowex 50W/water→Dowex 1/water] derived from arsenobetaine in the muscle and the liver of the *Mustelus manazo* left in coastal sands.

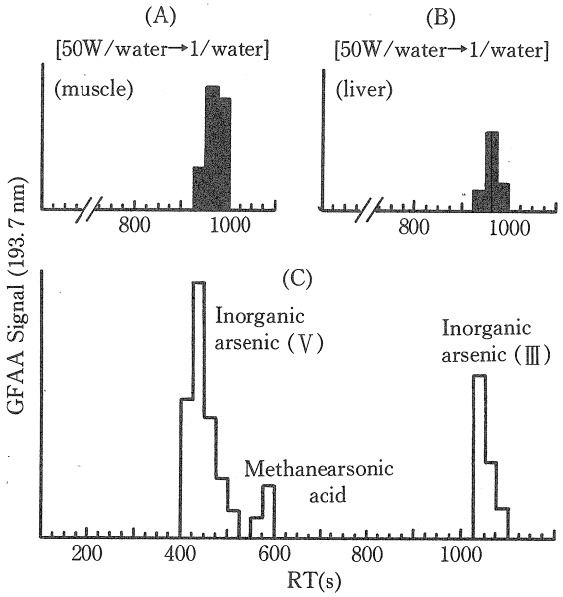

Fig. 3. High performance liquid chromatograms (column: Nucleosil 10SB) of partially purified arsenic compound in [Dowex 50W/water→Dowex 1/ water].

Their retention times (RTs) agreed with those of arsenobetaine and trimethylarsine oxide in [50W/NH_3→1/water], inorganic arsenic(Ⅴ) both in [50W/water→1/water] (Fig. 2) and in [50W/HCl], and methanearsonic acid [$(CH_3)AsO(OH)_2$] and/or inorganic arsenic (Ⅲ) in [50W/water → 1/CH_3COOH]. The small amount of inorganic arsenic(Ⅴ) detected in [50W/HCl] was attributed to that which unavoidably contaminated the hydrochloric acid, and was ignored. Because of the mutually close RT of inorganic arsenic(Ⅴ), inorganic arsenic(Ⅲ) and methanearsonic acid, [50W/water → 1/water] and [50W/water → 1/CH_3COOH] were rechromatographed with Nucleosil 10SB. The RT of arsenic compound in each of [50W/water → 1/water] and [50W/water → 1/CH_3COOH] agreed with those of inorganic arsenic(Ⅴ) (Fig. 3) and methanearsonic acid, respectively. The relative distribution of these arsenic compounds, arsenobetaine, trimethylarsine oxide, methanearsonic acid and inorganic arsenic(Ⅴ), was 63.6, 3.8, 20.0 and 12.6 % in the muscle and 83.8, 3.9, 7.9 and 4.4 % in the liver, respectively.

3.2 Purification and identification of inorganic arsenic(Ⅴ)

The fraction of [50W/water→1/water] was further subjected to an ion-exchange chromatography in order to confirm the formation of inorganic arsenic. The fraction [50W/water→1/water] was applied to a Dowex 50W-× 2 (pyridinium form) column (1 × 50cm). Arsenic was eluted with the pyridine-formic acid buffer only. Arsenic-containing fractions were collected, concentrated and subjected to some analyses.

The each purified arsenic compound was chromatographed on cellulose thin layer. Its Rf values on TLC with 5 solvent systems were shown in Table 1 with the authentic arsenic compounds. The Rf value of each purified compound agreed with that of inorganic arsenic(Ⅴ) with all solvent systems. Ion chromatography using AS4A column was performed with the purified arsenic compound and authentic inorganic arsenic(Ⅴ). The compound showed the same RT (muscle: 559 s, liver: 562 s) essentially as that of in-

Table 1. Rf values on TLC of the arsenic compounds isolated from the muscle and the liver of a starspotted shark left in the coastal sands for 40 days

Sample	Rf value				
	Solvent system				
	1	2	3	4	5
Isolated arsenic compound;					
from muscle	0.32	0.00	0.22	0.00	0.21
from liver	0.32	0.00	0.22	0.00	0.22
Inorganic arsenic (Ⅴ)	0.31	0.00	0.22	0.01	0.21
Inorganic arsenic (Ⅲ)	0.22	0.30	0.40	0.07	0.35
Methanearsonic acid	0.55	0.22	0.50	0.02	0.50
Dimethylarsinic acid	0.80	0.78	0.61	0.23	0.71
Arsenocholine	0.53	0.87	0.54	0.56	0.62
Arsenobetaine	0.73	0.84	0.49	0.35	0.55

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

organic arsenic(Ⅴ) (561 s). Aggregates were formed in dilute sulfuric acid by mixing the purified compound with a mixture of ammonium molybdate and marachite green, showing an typical absorption spectrum in 2-methoxyethanol with a higher peak at 627 nm and a lower peak at 430 nm.[23] This fact indicates the compound to be inorganic arsenic (Ⅴ) because no other arsenic compound aggregates with the reagents.

On the basis of the results from HPLC, TLC, ion chromatography, and spectrophotometric analyses, the purified metabolite was confirmed to be inorganic arsenic(V).

4. Discussion

We concluded that arsenobetaine contained in the muscle or the liver of the shark degraded to inorganic arsenic *in situ* under a natural environment: arsenobetaine which is bioconverted through the food chain from inorganic arsenic in seawater was re-converted to the original inorganic arsenic via trimethylarsine oxide and methanearsonic acid. Thus, the arsenic cycle which we have previously proposed[18-19] has applied to the marine ecosystems. On the other hand, in the marine environments, the greater part of organic matters is present as particulate matters such as suspended substances which may be more easily attacked by the microorganisms than the round body. The fact that considerable amount of arsenobetaine contained in the round shark was degraded to inorganic arsenic within 40 days, suggests a relatively rapid degradation of arsenobetaine or other organo-arsenic compounds to inorganic arsenic. The degradation of arsenobetaine or other arsenic compounds contained in particulate matters, for example, suspended substances and setting matters is now under investigation.

In the in vitro degradation experiments with arsenobetaine so far, methanearsonic acid has been never detected as a microbial degradation product: trimethylarsine oxide and/or dimethylarsinic acid have been detected or isolated. The fact that methanearsonic acid was derived in this *in vivo* study instead of dimethylarsinic acid was interesting for pursuing the arsenic circulation in marine ecosystems, because it indicates the difference in the degradation behaviour between natural condition and laboratory

one. The difference in temperature, nutrients, etc. may affect on the degradation activity of microorganisms, although a clear explanation for this can not be made at the present stage.

The *in vitro* degradation experiments of arsenobetaine so far have shown the suitability of an aerobic condition for the microbial degradation of it: while no or little arsenobetaine has been degraded by marine microorganisms from any origins investigated under an anaerobic condition, it has been done in every origin under an aerobic condition only. It was the reason why we buried the shark shallowly in the intertidal coastal sands where a constant supply of oxygen-rich seawater was ensured. The fact that the degradation has occurred indicates that the place of leaving fish was properly selected, and that this degradation could not occur if the shark was buried more deeply in the sediments. In other words, in the natural environment, arsenobetaine in residues or particles originated from organisms may be hard to be suffered degradation when they are in anaerobic condition such as deep seewater or the inside of the sediments of reduction conditions.

Acknowledgement

We would like to express our sincere thanks to Dr. Bon Kimura, Tokyo University of Fisheries, and Dr. M. Murakami, Shimonoseki University of Fisheries, for their helpful microbiological advice.

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ホシザメ普通筋および肝臓におけるアルセノベタインの無機ヒ素への分解

花岡研一*1・恩田健司*1・田川昭治*1・貝瀬利一*2

仮死状態のホシザメを水産大学校前海岸の砂浜に埋めた(深さ，約20cm)。40日間放置した後これを研究室に持ち帰り，その普通筋および肝臓を取出し，クロロホルム-メタノール(2：1)によりヒ素化合物を抽出した。その結果，これらの組織に含有されていたアルセノベタインの一部が，トリメチルアルシンオキシドおよびジメチルアルシン酸を経て無機ヒ素にまで分解された。このことから，海洋の食物連鎖を通じて海水中の無機ヒ素から生合成されたアルセノベタインは，微生物分解により元の無機ヒ素に回帰すると結論した。

*1 水産大学校製造学科
*2 東京薬科大学環境生命科学科