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Identification of arsenobetaine and a tetramethylarsonium salt in the clam *Meretrix lusoria*

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Chemical forms of arsenic were examined in six tissues (gill, mid-gut gland, siphon, foot, mantle and adductor muscle) of the clam *Meretrix lusoria*. The gill was found to contain higher levels of arsenic than the other tissues. Regardless of the nature of the tissues, the presence of arsenobetaine was established by HPLC-ICP; it was a minor arsenic compound in gill but a major one in the other tissues. The major arsenic compound in gill, which was more cationic than arsenobetaine, was obtained in a relatively pure state by ion-exchange chromatography, gel filtration and HPLC. It was positive to the Dragendorff reagent and iodine vapor but negative to ninhydrin reagent. Its ^{1}H-NMR spectrum exhibited only one signal at δ 1.7 (singlet) and its FAB mass spectrum gave a base peak at m/e 135 [$(CH_3)_4As^+$] and two significant peaks at m/e 120 [$(CH_3)_3As$] and 106 [$(CH_3)_2AsH$]. These results suggested that the major arsenic compound in gill exists as a tetramethylarsonium salt $(CH_3)_4As^+ \cdot X^-$. The tetramethylarsonium salt was also found as a minor component in the tissues other than the gill.

Keywords: Clam, arsenic, organoarsenicals, arsenobetaine, tetramethylarsonium salt

INTRODUCTION

High levels of arsenic exist naturally in marine organisms, chiefly as water-soluble organic compounds. Not only from the viewpoint of food hygiene but also to clarify the arsenic cycle in the marine ecosystem, it is particularly important to elucidate the chemical structure of water-soluble organoarsenicals in marine organisms. Along this line, arsenobetaine[1–18] has so far been identified in a variety of marine organisms and arsenosugars,[19–21] arsenocholine[7,17,18,22] and trimethylarsine oxide[23] in a limited number of species. As to bivalves, arsenobetaine is present in soft-tissues of the short-necked clam[18] and the muscle and mid-gut gland of the scallop,[17,18] and arsenosugars in the kidney and adductor muscle of the giant clam.[20] In the course of screening for arsenic compounds in marine organisms by means of high performance liquid chromatography (HPLC) combined with inductively coupled argon plasma emission spectrometry (ICP), we detected a new organoarsenical in the commercially important clam *Meretrix lusoria*. The present paper deals with the identication of this tetramethylarsonium salt, together with arsenobetaine, in *M. lusoria*.

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MATERIALS AND METHODS

Determination of arsenic

Nitric acid (containing 61% HNO_3), perchloric acid (containing 60% $HClO_4$) and sulfuric acid (containing 97% H_2SO_4) used for digestion were of super special grade. For the determination of total arsenic, samples were digested with a mixture of nitric acid, perchloric acid and sulfuric acid (25:5:0.5, v/v/v) (30 cm^3) dissolved in 10, 20 or 25 cm^3 of water and applied to a Jarrell Ash ICP (AtomComp Series 800). The ICP was operated under the following conditions: wavelength, 193.7 nm; rf power, 1.2 kW; observation height, 16 mm; integration time, 20 sec. Calibration of the ICP was carried out using water and disodium arsenate (As 10 $\mu g\,g^{-1}$). In the case of aqueous samples obtained in the extraction and

purification procedure, their arsenic concentrations were directly estimated on the ICP without wet-digestion.

Extraction and fractionation on Dowex 50 of water-soluble arsenic compounds

Immediately after purchase from a retail supplier live specimens of *M. lusoria* were dissected into six tissues (gill, mid-gut gland, siphon, foot, mantle and adductor muscle). These tissues were bulked from at least five specimens. Each tissue (1–5 g) was extracted twice with 5 times its volume of methanol and the extract was evaporated to dryness to remove methanol. The residue was suspended in 10 or 20 cm^3 of water and defatted three times with equal volumes of ether. The aqueous phase, which was regarded as a water-soluble arsenic fraction, was evaporated to dryness and dissolved in 10 cm^3 of water. Eight cm^3 of the water-soluble arsenic fraction was put onto a Dowex 50X2 column (1 × 8 cm, H^+ form) equilibrated with water. The column was first washed with 25 cm^3 of water (unadsorbed fraction) and then eluted successively with 25 cm^3 each of 1 mol dm^{-3} NH_4OH (NH_4OH fraction), water and 1 mol dm^{-3} HCl (HCl fraction).

HPLC-ICP

The unadsorbed, NH_4OH and HCl fractions were evaporated to dryness, dissolved in 1 or 2 cm^2 of water and passed through a 0.45 μm membrane filter. The separate filtrates were determined for arsenic and then analyzed by the HPLC-ICP system which had been developed in our laboratory.[18] Briefly, a column (0.46 × 25 cm) packed with Nucleosil 10SA (Nagel, strongly acidic cation exchanger; particle size, 10 μm) or Nucleosil 10SB (Nagel, strongly basic anion exchanger; particle size, 10 μm) was connected to a Kyowa Seimitsu HPLC (KHP-010). The solvents were 0.1 mol dm^{-3} pyridine-formate buffer (pH 3.1) for Nucleosil 10SA and 0.02 mol dm^{-3} phosphate buffer (pH 7.0) for Nucleosil 10SB. The flow rate was maintained at 1 cm^3 min^{-1}. The eluate was directly introduced into the nebulizer of the ICP which was operated as described above except that the integration time was shortened to 5 sec. Arsenic concentrations were recorded at 10-second intervals. Analysis of the unadsorbed fraction was performed on Nucleosil 10SB and those of the NH_4OH and HCl fractions on Nucleosil 10SA. Arsenate (disodium), arsenite (sodium), methylarsonic acid (disodium), dimethylarsinic acid, arsenobetaine and arsenocholine were used as standard arsenic compounds.

Purification method of arsenic compounds in gill

Fifty cm^3 of the water-soluble arsenic fraction (containing 250 μg As) prepared from gill was applied to a Dowex 50X2 column (1.8 × 28 cm, H^+ form) equilibrated with water. The column was thoroughly washed with water and then eluted successively with 350 cm^3 each of 0.4 mol dm^{-3} NH_4OH, water and 1 mol dm^{-3} HCl. Fractions of 5 cm^3 were collected. Analysis by the ICP revealed that about 80% of the water-soluble arsenic was eluted with HCl. Only the HCl fractions containing arsenic were combined and evaporated to dryness. The dried material was dissolved in 0.4 mol dm^{-3} NH_4OH and subjected to gel filtration on a Bio-Gel P-2 column (2.0 × 59 cm). Elution was carried out with 0.4 mol dm^{-3} NH_4OH. Fractions of 3 cm^3 were collected at a flow rate of 30 cm^3 h^{-1}. The arsenic-containing fractions were combined, evaporated to dryness, dissolved in water and chromatographed on a Bio-Rex 70 column (1.8 × 28 cm, H^+ form) equilibrated with water. After washing with water, the column was eluted by a linear gradient of 0–0.12 mol dm^{-3} acetic acid (total volume 600 cm^3). Fractions of 5 cm^3 were collected at a flow rate of 30 cm^3 h^{-1}. Final purification was achieved by HPLC on a Nucleosil 10SA column (0.46 × 25 cm) with 0.1 mol dm^{-3} pyridine-formate buffer (pH 3.1). The flow rate was maintained at 1 cm^3 min^{-1} and 0.25 cm^3 fractions were collected.

Thin layer chromatography

Thin layer chromatography was performed on precoated silica gel 60 plates (Merck) with ethanol-acetic acid-water (65:1:34, v/v/v) or on precoated cellulose plates (Funakoshi) with *n*-propanol-water (7:3, v/v). Spots were visualized with the Dragendorff reagent, iodine vapor or ninhydrin reagent.

Spectral analyses

The ^{1}H-NMR spectrum of the purified compound was recorded in D_2O at 100 MHz on a

JEOL FX-100 spectrometer. The fast atom bombardment (FAB) mass spectrum was measured with a JEOL DX-300. Ionization was achieved by xenon atoms with a kinetic energy of 3 kV and glycerol was used as matrix on the target probe.

RESULTS

Analyses of arsenic compounds by HPLC-ICP

With respect to both the arsenic concentration (Table 1) and the behavior of water-soluble arsenic compounds on Dowex 50 (Table 2), the gill exhibited significantly different patterns from the other five tissues. The arsenic concentration in gill (15–40 $\mu g\,g^{-1}$) was relatively higher than those in the other tissues (below 10 $\mu g\,g^{-1}$). After chromatography on Dowex 50, 74% of the total arsenic in gill was recovered in the HCl fraction, suggesting that the major arsenic compound in gill is strongly cationic. On the other hand, the major arsenic compound in the other tissues was less cationic because it was recovered not in the HCl fraction but in the NH_4OH fraction.

Table 1 Arsenic concentration in six tissues of *M. lusoria*

	As ($\mu g\,g^{-1}$, wet weight basis)			
	Experiment			
Tissue[a]	1	2	3	4
Gill	15.4	17.1	21.5	41.3
Mid-gut gland	7.3	5.3	5.4	8.4
Siphon	4.0	3.4	4.1	4.8
Foot	4.8	3.9	2.8	3.4
Mantle	3.4	2.7	2.3	3.0
Adductor muscle	3.6	2.1	1.8	2.7

[a]Each tissue was bulked from 30, 5, 100 and 5 specimens in experiments 1, 2, 3 and 4, respectively.

When analyzed by HPLC-ICP, the unadsorbed fraction from gill gave one arsenic peak which did not coincide with arsenate, arsenite, methylarsonic acid or dimethylarsinic acid (Fig. 1). This arsenic compound also apparently differed from either arsenobetaine or arsenocholine, both of which are never obtained in the unadsorbed fraction.[18] The unadsorbed fractions from other tissues exhibited the same HPLC pattern as that from gill. In the case of the NH_4OH fraction, the same HPLC pattern was also observed in common with the six tissues. A typical example is shown in Fig. 2. Only one arsenic compound was found and its retention time coincided well with that of arsenobetaine. It is known that arsenobetaine is quantitatively recovered in the NH_4OH fraction.[18] Preliminary tests also showed that like arsenobetaine, the arsenic compound in the NH_4OH fraction from mid-gut gland was adsorbed neither by Dowex 2X8 (OH^- form) nor by Amberlite CG-50 (H^+ form) and that it was eluted from Bio-Gel P-2 at the same position as arsenobetaine. Thus, the arsenic compound in the NH_4OH fraction from each tissue was reasonably identified as arsenobetaine. Among the standards used, only arsenocholine can be recovered in the HCl fraction.[18] However, analysis by HPLC-ICP revealed that the HCl fraction from gill did not contain arsenocholine (retention time, 9.5 min) but instead an unknown compound at a retention time of 11.5 min (Fig. 3A). Identification of this

Table 2 Fractionation on Dowex 50 of the water-soluble arsenic in six tissues of *M. lusoria*

		Water-soluble As ($\mu g\,g^{-1}$, wet weight basis)		
Tissue[a]	Total As ($\mu g\,g^{-1}$, wet weight basis)	Unadsorbed[b]	NH_4OH[b]	HCl[b]
Gill	21.5	1.8 (8)[c]	1.1 (5)[c]	15.9 (74)[c]
Mid-gut gland	5.4	1.7 (31)	2.6 (48)	0.2 (4)
Siphon	4.1	0.7 (17)	1.7 (41)	1.1 (27)
Foot	2.8	0.3 (11)	1.8 (64)	0.9 (32)
Mantle	2.3	0.2 (9)	0.8 (35)	0.6 (26)
Adductor muscle	1.8	0.2 (11)	1.3 (72)	0.3 (17)

[a]The tissues are the same as those used in experiment 3 in Table 1.
[b]The water-soluble arsenic was chromatographed on Dowex 50 and separated into three fractions (unadsorbed, NH_4OH and HCl fractions).
[c]The ratio (%) to the total As is given in each parenthesis.

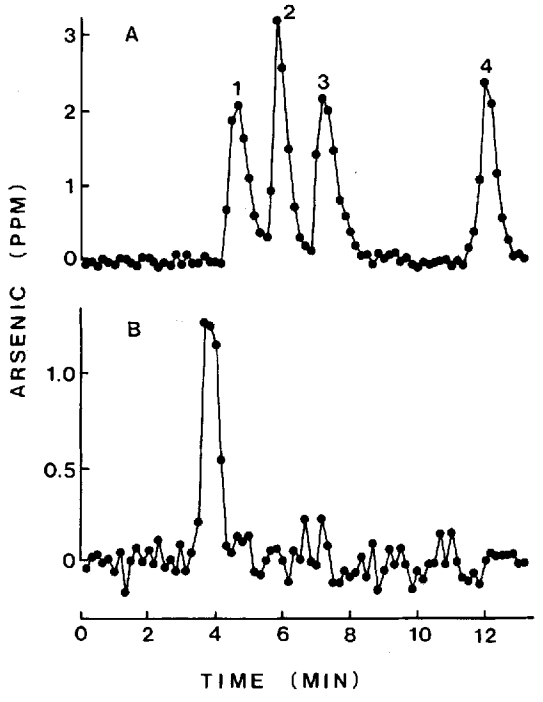

Figure 1 HPLC of standard arsenic compounds (A) and the unadsorbed fraction from gill (B) monitored by ICP. Column, Nucleosil 10SB (0.46 × 25 cm); solvent, 0.02 mol dm^{-3} phosphate buffer (pH 7.0); flow rate, 1 cm^3 min^{-1}. The standard arsenic compounds are: 1, arsenite; 2, methylarsonic acid; 3, dimethylarsinic acid; and 4, arsenate. Injection volume: A, 60 mm^3 (containing 1.5 μg As for each standard compound); B, 100 mm^3 (containing 0.86 μg As).

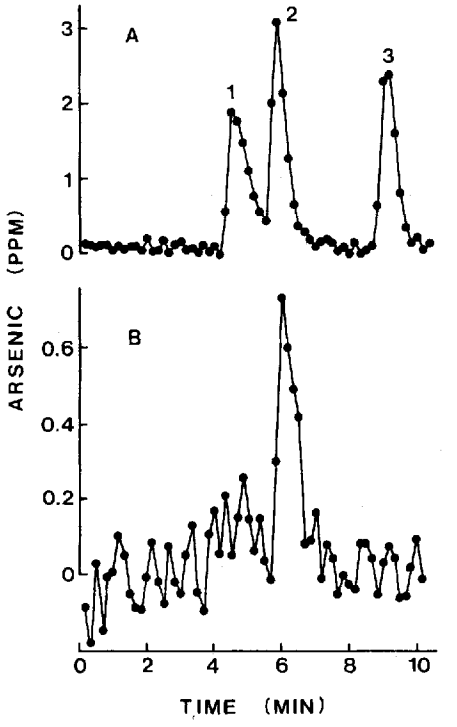

Figure 2 HPLC of standard arsenic compounds (A) and the NH_4OH fraction from gill (B) monitored by ICP. Column, Nucleosil 10SA (0.46 × 25 cm); solvent, 0.1 mol dm^{-3} pyridine-formate buffer (pH 3.1); flow rate, 1 cm^3 min^{-1}. The standard arsenic compounds are: 1, dimethylarsinic acid; 2, arsenobetaine; and 3, arsenocholine. Injection volume: A, 45 mm^3 (containing 1.5 μg As for each standard compound); B, 100 mm^3 (containing 0.55 μg As).

unknown compound is described later. The HCl fraction from the other tissues afforded a similar HPLC pattern to each other. As a typical example, the result with HCl fraction from siphon tissue is illustrated in Fig. 3B. Together with the unknown compound observed for the HCl fraction from gill, another unknown arsenic compound was invariably detected at a retention time of around 4 min.

Purification of arsenic compounds in gill

In Dowex 50 column chromatography experiments, about 80% of the water-soluble arsenic was obtained in the HCl fraction. Although the unadsorbed and NH_4OH fractions contained small amounts of arsenic, they were discarded. The arsenic compound recovered in the HCl fraction gave only one and symmetrical arsenic peak in gel filtration on Bio-Gel P-2. The peak was observed between fractions 47 and 50 while synthetic arsenobetaine was eluted between fractions 45 and 48, suggesting that as to molecular size, the arsenic compound is almost equivalent to or rather smaller than arsenobetaine. When chromatographed on a weakly acidic cation exchanger (Bio-Rex 70), the arsenic compound was adsorbed by the exchanger and eluted as one arsenic peak at the position corresponding to 0.10 mol dm^{-3} acetic acid. The elution profile in HPLC on Nucleosil 10SA was the same as shown in Fig. 3A. Thus, the purified preparation containing 53 μg As was obtained but the weight could not be determined due to scarcity. Judging from the behavior in the above purification procedure, it was assumed that the HCl fraction from gill contained only one arsenic compound.

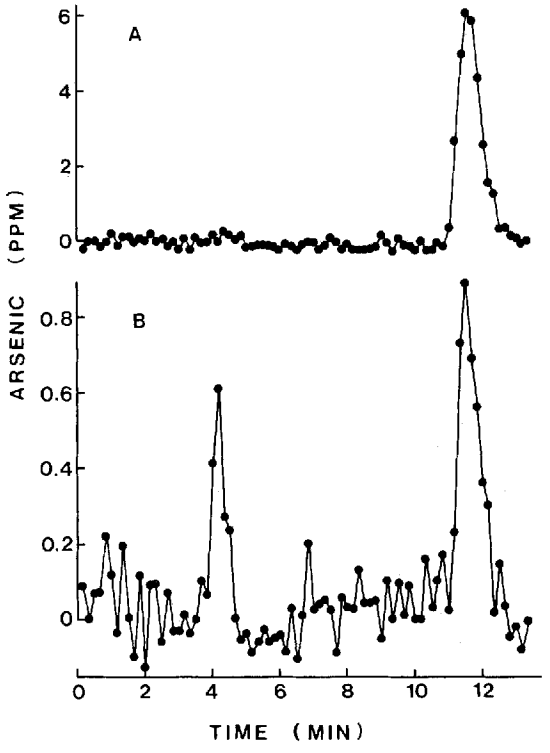

Figure 3 HPLC of the HCl fractions from gill (A) and siphon (B) determined by ICP. Column, Nucleosil 10SA (0.46 × 25 cm); solvent, 0.1 mol dm^{-3} pyridine-formate buffer (pH 3.1); flow rate 1 cm^3 min^{-1}. Injection volume: A, 100 mm^3 (containing 3.2 μg As); B, 100 mm^3 (containing 0.84 μg As).

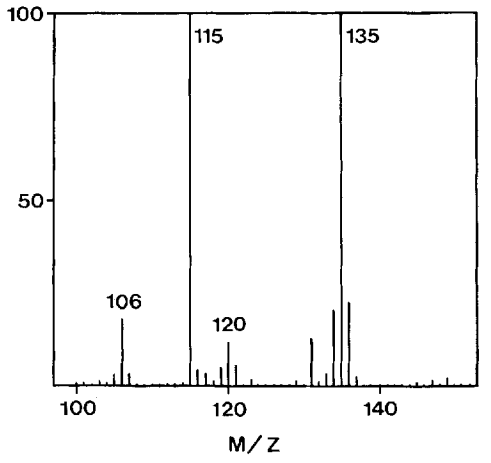

Figure 4 FAB mass spectrum of the purified arsenic compound from gill. The peaks at m/e 135, 120, 106 and 115 were assignable to $(CH_3)_4As^+$, $(CH_3)_3As$, $(CH_3)_2As$ H and glycerol $+Na^+$, respectively.

Properties of the purified preparation from gill

Thin layer chromatographic analyses showed that the purified preparation contained one major compound and another minor one. On a silica gel plate the major compound appeared at Rf 0.38 and the minor one at Rf 0.90 while the former at Rf 0.54 and the latter at Rf 0.59 on a cellulose plate. Following visualization with iodine vapor, each portion of the silica gel plate corresponding to both compounds was scraped off, homogenized in 2 cm^3 of water and centrifuged. Determination of arsenic in the supernatant revealed that only the major compound contained arsenic. This arsenical was positive to the Dragendorff reagent and iodine vapor but negative to ninhydrin reagent, indicating that it has a tertiary arsine or a quaternary arsonium moiety.

The purified preparation exhibited only one significant signal at δ 1.7 (singlet) in the ^{1}H-NMR spectrum. Judging from the ^{1}H-NMR spectra reported for arsenobetaine,[1,7,8] arsenocholine[7] and trimethylarsine oxide,[23] the signal at δ 1.7 was attributable to a methyl group attached to arsenic. The positive color reaction to the Dragendorff reagent strongly suggested the presence of three or four methyl groups. The FAB mass spectrum of the purified preparation is shown in Fig. 4. Aside from a peak at m/e 115 (corresponding to glycerol $+Na^+$), the base peak at m/e 135 and two characteristic peaks at m/e 120 and 106 were observed. The peaks at m/e 135, 120 and 106 were assignable to $(CH_3)_4As^+$, $(CH_3)_3As$ and $(CH_3)_2AsH$, respectively.

DISCUSSION

As in the cases of short-necked clam (*Tapes japonica*),[18] and scallop (*Patinopecten yessoensis*),[17,18] arsenobetaine was detected in the clam *M. lusoria* by HPLC-ICP. It was a major arsenic constituent in all tissues except for gill. The significance of this study was a finding of a more cationic organoarsenical than arsenobetaine. The strongly cationic organoarsenical, which was a major component in the gill but a minor one in the other five tissues, was obtained in a relatively pure state and its ^{1}H-NMR and FAB mass spectra conformed to tetramethylarsonium ion $(CH_3)_4As^+$. In this connection, a nitrogenous compound, tetramine (tetramethyl-

arsonium ion), in which the arsenic of the tetramethylarsonium ion is replaced by nitrogen, has also been detected in sea anemones[24] and in the salivary glands of some marine gastropods.[25,26] Recently tetramine was reported to exist in vivo in the form of the chloride.[27] It is, therefore, very likely that the strongly cationic organoarsenical in *M. lusoria* is present as one or more tetramethylarsonium salts $(CH_3)_4As^+ \cdot X^-$, e.g. chloride and hydroxide. In this study, however, its precise form has not been deduced. This appears to be the first report concerning the presence of a tetramethylarsonium salt in nature.

Arsenobetaine and arsenocholine are major arsenic species in marine organisms. Metabolic experiments using mammals have shown that arsenobetaine is rapidly excreted unchanged[28] and that arsenocholine is also excreted following rapid biotransformation to arsenobetaine.[29] Furthermore, arsenobetaine has no acute toxicity in mammals.[30] Therefore, it is generally thought that elevated levels of arsenic in marine food do not necessarily evoke a serious problem to human health. However, tetramine is known to be the causative compound for numerous intoxications in Japan due to ingestion of whelks such as *Neptunea arthritica*. In those intoxications the principal symptoms were intense headaches, dizziness, nausea and vomiting.[31] Also, tetramine chloride is lethal to mice at a minimum dose of 0.43 mg per 20 g mouse.[27] It is very important to clarify whether the tetramethylarsonium salt, which is structurally and chemically similar to tetramine, exhibits toxic effects and how it is metabolized in animals.

The clam *M. lusoria* is a typical plankton-feeder and would therefore incorporate arsenic compounds from plankton. It is possible that arsenobetaine and/or the tetramethylarsonium salt are contained in plankton. Another possibility is that these compounds are produced by the clam from unknown arsenicals in plankton. Based on the result that the gill contained arsenic at remarkably high levels as compared with the other tissues and the major arsenic species in gill was the tetramethylarsonium salt (whereas that in the other tissues was arsenobetaine), one or both of arsenobetaine and the tetramethylarsonium salt seems to be a metabolite in the clam from the tetramethylarsonium moiety. It is interesting to conclude that biotransformation of arsenic compounds in clam tissues, especially in the gill, appears to play a special role in arsenic metabolism.

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