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Uptake and Transformation of Arsenosugars in the Shrimp *Crangon crangon*

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The possible role of arsenosugars as precursors to arsenobetaine was investigated by feeding pure arsenosugar compounds to the shrimp, *Crangon crangon*, and monitoring the arsenic metabolites in muscle, midgut gland, gills, and 'remainder' tissues by HPLC–ICP MS. Control shrimps contained arsenobetaine (*ca* 90% of the total As) as the major arsenic compound in all four tissues, and traces of tetramethylarsonium ion and two arsenosugars were also present. Shrimps accumulated only 0.9% of a dimethylated arsenosugar, mostly as unchanged compound, and conversion into arsenobetaine could not be detected. Dimethylarsinate and possibly dimethylarsinoylethanol were present as minor metabolites. Shrimps accumulated 4.2% of a trimethylated arsenosugar, and converted about half into arsenobetaine. The remainder of the arsenic was present as unchanged arsenosugar and several minor unidentified metabolites. The overall accumulation of arsenobetaine from ingested trimethylated arsenosugar was only about 2%, whereas shrimps fed arsenobetaine retained 57% of the dose. The undetectable (dimethylated) to low (trimethylated) conversion of the arsenosugars into arsenobetaine suggests that these compounds do not represent a major source of arsenobetaine for wild *Crangon*.

Keywords: arsenosugars; arsenobetaine; food; accumulation; shrimp

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INTRODUCTION

Arsenic is a common constituent of marine environmental samples, where it can exist in many chemical forms.[1,2] In water and sediment most of the arsenic is inorganic, whereas in biota organoarsenic compounds predominate. The major organoarsenic compounds in marine algae are arsenosugars (arsenic-containing ribosides) which are believed to be biosynthesized by algae from inorganic arsenic in seawater.[3] Dimethylated and trimethylated arsenosugars have been reported in algae, the former being by far the predominant species. In marine animals, the major form of arsenic is arsenobetaine, although several other arsenic compounds are also found, generally as minor constituents. The origin of arsenobetaine is unknown; its apparent absence from seawater, sediments and algae might suggest that it is biosynthesized within animals.

Research to date has focused on two hypotheses for the origin of arsenobetaine. The first proposes that simple methylated arsenic compounds (e.g. dimethylarsinate) are transformed to arsenobetaine by microscopic organisms in seawater. Support for this hypothesis has come from laboratory studies[4,5] with mussels maintained in seawater containing radiolabelled (tritiated CH_3) methylarsonate or dimethylarsinate. Chromatography of extracts from the mussels showed that part of the radiolabel was associated with fractions that matched the chromatographic behaviour of arsenobetaine. Evidence was presented demonstrating that the mussels were accumulating the radiolabelled arsenobetaine from the seawater rather than biosynthesizing the compound themselves. A second hypothesis considers arsenosugars as the precursors of arsenobetaine. This hypothesis has been supported by field studies, for example the recent study[6] investigating the arsenic constituents of a short food chain of herbivorous molluscs grazing on algae. Although the algae contained most of their arsenic as

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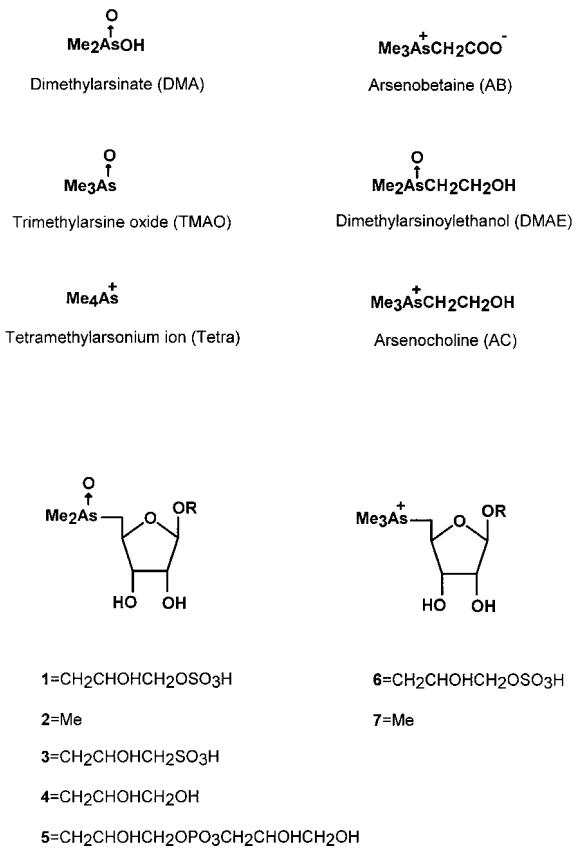

Figure 1 Structures of arsenic compounds relevant to this study.

arsenosugars, and arsenobetaine was not present, the molluscs contained arsenobetaine as their major arsenic metabolite. Hence it appeared that arsenosugars were transformed into arsenobetaine within the molluscs. Laboratory studies, however, have been unable to demonstrate that animals can convert arsenosugars into arsenobetaine. Early studies using radiolabelled organoarsenic compounds (arsenosugars) obtained by culturing algae in seawater containing ^{74}As-arsenate failed to show a transformation into arsenobetaine in crustaceans[7] or molluscs.[8] A comparable recent study[9] with a unicellular alga and copepods (and using HPLC–ICP MS to detect arsenic metabolites) supported that earlier work by demonstrating no transformation of arsenosugars into arsenobetaine. These laboratory studies[7–9] all examined naturally produced arsenic compounds, and, although dimethylated arsenosugars were dominant, inevitably dealt with a mixture of arsenic species. It is possible, and indeed likely, that small quantities of trimethylated arsenosugar(s) were also present in these studies. Any transformations occurring with minor constituents in algae might have been difficult to detect in the presence of large quantities of dimethylated arsenosugars.

The study reported here was carried out to augment the previous laboratory studies by working with synthetic arsenosugars. The ideal compounds for such work were the dimethylated arsenosugar **1** (Fig. 1) and its trimethylated analogue **6**, since both are natural products.[10,11] However, because these compounds were not available in sufficient quantities, we chose the arsenosugars **2**[12] and **7**[13] as model compounds. Arsenosugar **2**, a minor natural constituent of algae, has been chemically synthesized[12] and **7** can be readily synthesized[13] from **2**. The assumption made is that the various dimethylated arsenosugars found in algae behave similarly in natural systems, at least in relation to their transformations and resultant major arsenic-containing metabolites. Previous studies[14,15] indicate that this is a valid assumption since the four major arsenosugars in algae (compounds **1, 3, 4, 5**), in addition to **2**, all produce the same arsenic metabolite on anaerobic degradation. Similarly, arsenic metabolites resulting from **7** are assumed to be representative of trimethylated arsenosugars.

The present investigation examines the fate of a dimethylated and a trimethylated arsenosugar (**2** and **7**) following ingestion by the shrimp *Crangon crangon*. The use of pure compounds facilitated the detection and quantification of transformation products.

EXPERIMENTAL

Collection and maintenance of *Crangon crangon*

Common shrimps, *Crangon crangon* (L.) (Crustacea, Decapoda), were caught at Kerteminde, on the Island of Funen, Denmark, in early July 1997 and transported in seawater to the aquarium room at Odense University (14 ± 0.5 °C; light–dark cycle, 12:12). They were held in a glass aquarium containing 100 litres of aerated Kerteminde seawater, and allowed to acclimatize to laboratory conditions for six days during which they were fed every third day with chicken muscle tissue. After this acclimatization period, 10 individuals were randomly sampled and frozen (−80 °C); these animals constituted the control Day 0 group.

Preparation of arsenic-dosed food

Undosed and arsenic-dosed food was prepared from chicken muscle and gelatin as previously described.[16] A known amount (1.13–1.33 mg As dissolved in 50 µl water) of each of the three arsenic compounds was added to weighed portions of the chicken–gelatin mixture to give a nominal arsenic concentration of 500 µg As g^{-1} wet wt. These portions were poured into moulds and immediately frozen (−20 °C). Once solid, the slabs of food were trimmed to leave a piece of 1.9 g wet wt which was cut into 190 pellets weighing *ca* 10 mg (dimethylated arsenosugar, 10.7 ± 0.6 mg; trimethylated arsenosugar, 10.7 ± 0.6 mg; arsenobetaine, 9.1 ± 1.0 mg; mean ± SD, $n = 10$). Arsenic concentrations (µg As g^{-1} wet wt, mean ± SD, $n = 5$) measured in pellets taken from different locations on the food slabs were: dimethylated arsenosugar, 521 ± 14; trimethylated arsenosugar, 491 ± 6; and arsenobetaine 498 ± 16. The control pellets contained 0.3 ± 0.2 µg As g^{-1} wet wt (mean ± SD, $n = 5$).

Experimental design

Four groups (10 individuals in each) of *Crangon* were fed undosed chicken muscle (controls), or chicken muscle dosed with one of the two arsenosugars, or with arsenobetaine. For 19 days, shrimp were fed daily with a single pellet of food equivalent to approximately 2% of their body weight. In the few instances when individuals refused to feed (typically the day after they had moulted), the food was removed, refrozen, and fed to the animal at a later date. Total quantities of arsenic consumed by individual shrimps fed the compounds for 19 days were 99 µg (dimethylated arsenosugar), 93 µg (trimethylated arsenosugar), and 95 µg (arsenobetaine). Leaching of the arsenic compounds from the food pellets was not measured, but is considered to have been minimal as the pellets were typically consumed within seconds of their addition. Shrimps were then maintained for a further two days without food before being sacrificed. To ensure that they received and ate the same number of meals, and that no individuals were cannibalized after moulting, shrimps were held individually in polypropylene beakers containing *ca* 700 ml of seawater. The seawater in each beaker was gently aerated, and changed daily within an hour after feeding. Natural seawater (14 ± 0.5 °C, 15 ± 0.5 psu) was used throughout the study. All experimental animals were females; mean weights of the ten shrimps ranged from 0.55 to 0.61 g wet wt for each of the four groups.

Sampling and dissection of shrimp

The shrimps were removed and frozen (−80 °C) on Day 21 (48 h after their last meal). Length, wet weight, sex and the presence of eggs were recorded for each animal. The tail muscle, midgut gland, gills, and 'remainder' (primarily consisting of the exoskeleton (cuticle and hypodermis) and musculature of the appendages) were dissected from each individual. For tail muscle and 'remainder' tissues, samples from individual shrimps were processed separately; for midgut gland and gill tissues, material was pooled for each tissue type within each group. The wet weight and dry weight (following freeze-drying) of each sample were recorded to the nearest 0.1 mg, and the freeze-dried samples were stored in a desiccator until analysed. The control Day 0 shrimps were treated in an identical manner.

Chemical syntheses

Chemical syntheses have been reported previously for the arsenic compounds used in this study, namely arsenobetaine,[17] a dimethylated arsenosugar (compound **5**, methyl 5-deoxy-5-(dimethylarsinoyl)-β-D-riboside)[12] and a trimethylated arsenosugar (compound **7**, methyl 5-deoxy-5-(trimethylarsonio)-β-D-riboside).[13] The source of other arsenic compounds used as chromatographic standards has been described previously. K. A. Francesconi, W. Goessler, S. Panutrakul and K. J. Irgolic, *Sci. Tot. Environ.*, **221**, 139 (1998).

Determination of total arsenic

Samples were digested by heating to 500 °C with $MgO/Mg(NO_3)_2$,[18] and analysed for total arsenic by hydride-generation–atomic absorption spectrophotometry (AAS) using a Perkin-Elmer MHS-20 mercury/hydride system coupled to a Perkin-Elmer 2380 AAS. The standard reference material DORM-1 (certified arsenic concentration 17.7 ± 2.1 mg kg^{-1}) was analysed with each set of samples; the mean arsenic concentration (± SD) found was 18.0 ± 0.8 mg kg^{-1} ($n = 12$).

Determination of arsenic compounds

Preparation of extracts

Pooled samples

Separate pooled samples of tail muscle and 'remainder' were prepared by combining 10 mg from the ten individuals within each group. A sample (50.0 mg for tail muscle or 'remainder', or 2–4 mg weighed to ±0.01 mg for midgut gland or gills) of pooled tissue was transferred to a test-tube and extracted with water (2.0 ml) in a sonicator (MSE, amplitude 22, for 30 s). The homogenate was centrifuged (5000 *g*), and the supernatant transferred to a clean test-tube. The pellet was re-extracted with water (2.0 ml, Whirlymix) and the mixture centrifuged (5000 *g*). The second supernatant was added to the first, the combined volume was measured, and a sample (1.00 ml) removed for the determination of total arsenic. The pellet was freeze-dried and, for muscle and remainder, a portion (~10 mg) was prepared for total arsenic determination; difficulties associated with handling small quantities of material precluded the analysis of the pellet from midgut gland and gill samples. The remaining supernatant was evaporated to dryness at 30 °C on a centrifugal lyophilizer (Maxi Dry Lyo, Heto Holten). The ensuing dry residue was dissolved in water (1.00 ml) and the small amount of insoluble material was separated by centrifugation. Samples (2 × 0.40 ml) of the supernatant were separately dried (as above) and stored (−20 °C) before analysis by high-performance liquid chromatography using an inductively coupled plasma mass spectrometer as the arsenic-specific detector (HPLC-ICP MS). Immediately before HPLC, the extracts were reconstituted in water (1.00 ml) and diluted to give an arsenic concentration of 10 to 100 ng As ml^{-1}.

Individual muscle tissue

Six individual muscle tissue samples from each of three groups (control, dimethylated arsenosugar, and trimethylated arsenosugar) were extracted as follows. The dry tissue (*ca* 20 mg weighed to ± 0.01 mg) was extracted in a sonicator (MSE, amplitude 22, for 30 s) with water (5.0 ml). The mixture was centrifuged (50000 *g*), the supernatant was removed and the pellet was re-extracted with water (2 × 5.0 ml, Whirlymix followed by 50000 *g* centrifugation). The combined supernatants were evaporated to dryness at 30 °C (Maxi Dry Lyo, Heto Holten). The residue was redissolved in water (3.00 ml) and the small quantity of insoluble material was separated by centrifugation (50000 *g*). A sample (0.50 ml) of the supernatant was removed for total arsenic determination, and 2 × 1.00 ml samples were separately evaporated and stored (−20 °C) for HPLC analysis.

Chromatographic separation, identification and quantification of arsenic compounds

The HPLC system consisted of a Hewlett-Packard 1050 solvent delivery unit (Hewlett-Packard, Waldbronn, Germany) and a Rheodyne 9125 six-port injection valve (Rheodyne, Cotati, USA) with a 100-mm^3 injection loop. Separations were performed on a Supelcosil LC-SCX cation-exchange column at 40 °C, or on a Hamilton PRP X-100 anion-exchange column at 40 °C. For the pooled tissue samples, the cation-exchange column was eluted with 10 mM pyridine, pH 5.0, at 1.5 ml min^{-1}; and for the individual muscle tissue samples, elution was with 20 mM pyridine, pH 2.6, at 1.5 ml min^{-1}. The anion-exchange column was eluted with 20 mM $NH_4H_2PO_4$, pH 5.6. The outlet of the HPLC column was connected via a 800-mm, 1/16-inch (1.6 mm.) PEEK (polyether-ether-ketone) capillary tubing (0.13 mm i.d.) to the Babington-type nebulizer of a Hewlett-Packard 4500 inductively coupled plasma mass spectrometer (Hewlett-Packard, Waldbronn, Germany). The ion intensities at *m/z* 75 and 77 were monitored. The ICP MS signal was optimized with a solution of the mobile phase containing 20 μg As l^{-1} to give maximum response on the arsenic signal (*m/z* 75). Chromatograms were converted and integrated with software written in-house.[19] Arsenic compounds in the samples were quantified against standard solutions of arsenobetaine or arsenocholine chromatographed under identical conditions; the detector response following HPLC was the same for these two compounds. For complete HPLC recovery of the extracted arsenic, the sum of individual compounds should match the total arsenic in the extract. In our experiments, recovery ranged from 60 to 112% (mean 89%, $n = 16$) for the pooled samples, and 85–88% (mean 86%, $n = 18$) for the individual muscle samples. The quantities of arsenic reported here are based on the values for individual compounds obtained in the chromatography.

Data treatment and statistics

Statistical analyses were performed on log-transformed data using Systat software for PC use. A one-way ANOVA/Tukey test was used to deter-

Table 1 Total arsenic concentrations (μg As g^{-1} dry wt) in shrimp tissues following ingestion of arsenic compounds[a]

Tissue	Control group	Me_2 As-sugar group	Me_3 As-sugar group	Arsenobetaine group
Tail muscle	9.2 ± 2.6	16 ± 7	31 ± 8	610 ± 140
Midgut gland	20	34	250	1300
Gills	24	45	140	1300
'Remainder'	6.6 ± 1.5	15 ± 8	38 ± 16	430 ± 150

[a] Values (recorded to two significant figures) represent mean ± SD for 10 individuals.

mine the influence of the form of ingested arsenic on arsenic concentrations in the tail muscle and 'remainder' tissues, and to compare the contribution of the midgut gland to the overall body weight of the shrimps. The *t*-test was used to determine whether arsenic concentrations in the tissues of the control shrimps sampled at the beginning (Day 0) and end (Day 21) of the experiment were significantly different, and to compare the mean wet weights of the animals at the start and end of the experiment. To determine retention efficiencies for the compounds, arsenic concentrations were converted ([As] × dry wt) to arsenic contents, which were adjusted to account for the slightly different amounts of arsenic (93–99 μg) consumed. As it was necessary to pool the gill and midgut gland samples from the individuals constituting each group, no significance values are attached to the changes in arsenic concentrations in these tissues.

RESULTS

Feeding, condition and behaviour

No mortality or change in behaviour (e.g. willingness to feed or reduced activity) was noted in any of the groups. Some shrimps moulted during the study: control (6 moults), dimethylated arsenosugar (2), arsenobetaine (8) and trimethylated arsenosugar (8). Within each group, the mean whole wet weight of the shrimp at the end of the study was not significantly different ($P \geq 0.41$) from the initial mean weight. The contribution of the midgut gland to overall body weight [(midgut dry wt)/(whole animal wet wt)], which is a better indicator of the animals' condition, fell significantly ($P < 0.001$) in the control group during the experiment (i.e. Day 0; cf. Day 21 animals). There were, however, no significant differences ($P = 0.79$) in the ratio of (midgut dry wt)/(whole animal wet wt) between any of the four groups (control and treatments) sampled on Day 21.

Total arsenic concentrations in shrimp

There were no significant differences in arsenic concentrations between control shrimps sampled at Day 0 and at Day 21. Consequently, only the results for control Day 21 animals were used in the data analysis. Mean arsenic concentrations in shrimps fed the arsenic compounds were all significantly higher ($P < 0.001$) than those in the control group (Table 1). The arsenic concentrations in the three arsenic-dosed groups were also significantly different from each other ($P < 0.001$) with the relative concentrations being arsenobetaine ≫ trimethylated arsenosugars > dimethylated arsenosugars. These differences were apparent for both tail muscle and 'remainder' (gills and midgut were pooled samples and hence were not amenable to statistical analysis). The arsenic distribution patterns within animals were similar for control shrimp and for shrimp receiving arsenobetaine or the dimethylated arsenosugar (Table 1). Within each of these three groups, arsenic concentrations were comparable for tail muscle and 'remainder', and 2–3-fold higher in the midgut gland and gills. The distribution of arsenic in the group receiving trimethylated arsenosugar, however, was quite different, with the midgut gland showing a much higher concentration (2–8-fold) than the other tissues. On a whole animal basis, the retention efficiency of arsenic for the three compounds was arsenobetaine (57%), trimethylated arsenosugar (4.2%) and dimethylated arsenosugar (0.90%) (Table 2).

Extraction of arsenic compounds and their determination by HPLC–ICP MS

From pooled samples

Aqueous extraction with sonication removed most of the arsenic from the dried tissues. Extraction efficiencies were calculated in two ways: (i) by comparing the relative quantities of arsenic in the

Table 2 Retention (%) of the three arsenic compounds in individual tissues and whole shrimp fed 95 μg As (normalized; see Experimental section) over 19 days[a]

Tissue	Me_2As-sugar group	Me_3As-sugar group	Arsenobetaine group
Tail muscle	0.32 (0.355)	1.08 (0.258)	27.6 (0.488)
Midgut gland	0.02 (0.022)	0.40 (0.096)	2.1 (0.038)
Gills	0.03 (0.035)	0.12 (0.029)	1.1 (0.019)
'Remainder'	0.53 (0.588)	2.58 (0.617)	25.7 (0.455)
Whole animal[b]	0.90 (1.00)	4.2 (1.00)	57 (1.00)

[a] The proportion of arsenic body burden in each tissue is shown in parentheses. Values for midgut gland and gills are based on arsenic contents of pooled tissues.
[b] Not including haemolymph and other fluids lost during dissection. Values have been rounded to two significant figures.

supernatant and pellet [100 $S/(S + P)$], and (ii) by comparing supernatant arsenic with the quantity in the initial material determined by analysis of a separate subsample of freeze-dried tissue (100 S/initial). We prefer the first calculation method because it is internally consistent and avoids 'false' recovery values of more than 100%. When applied to the muscle and 'remainder' samples, extraction efficiencies were 89.9–99.2% (mean 93.5%), and 86.4–103% (mean 93.1%) for methods (i) and (ii), respectively. Extraction efficiencies could not be calculated by method (i) for the midgut gland and gill samples because the 'pellets' obtained following centrifugation of the homogenates were too small to process. Based on calculation method (ii), extraction efficiencies were 75–135% (mean 96%). The greater spread of results for these samples probably reflects difficulties in handling small amounts of material.

Analysis of the muscle of control shrimps (Fig. 2a) showed that most of the native arsenic was present as arsenobetaine (89.9–92.9% of total arsenic, depending on the tissue), with the remainder accounted for by a broad peak eluting near the solvent front (*ca* 2.0 min), an unknown peak at 3.45 min and tetramethylarsonium ion (10.03 min). The tetramethylarsonium ion accounted for about 1% of the total arsenic in both muscle tissue and 'remainder'; it was also detected in the gills and midgut, but could not be quantified (the estimated level of quantification was 0.2% of the total). Shrimps fed arsenobetaine accumulated this compound unchanged (Fig. 2b). Traces (less than 0.2% of total arsenic) of the other minor arsenic compounds present in control shrimps were detectable, but no other arsenic compounds were found (the estimated detection limit was 0.05% of the total).

HPLC–ICP MS analysis of the extracts of tail muscle from shrimps fed the dimethylated arsenosugar showed, in addition to arsenobetaine and other native arsenic compounds, a broad peak at 4–5.5 min (Fig. 2c). This broad peak appeared to contain two arsenic compounds but the identity of these compounds could not be established. Although the retention time and peak shape matched that for unchanged starting material, both dimethylarsinoylethanol and trimethylarsine oxide chromatographed in a similar manner and could not be eliminated as candidates. Biotransformation of dimethylated arsenosugars to dimethylarsinoylethanol is a facile process under certain conditions,[14] and its presence as a metabolic product in the current experiment would not be unexpected. Trimethylarsine oxide may be considered a less likely metabolite. The pattern of arsenic compounds was similar for the four tissues although small differences were apparent. For example, the ratio of arsenic associated with the peak at 4–5.5 min and that present as arsenobetaine varied as follows: tail muscle (1.0), 'remainder' (1.4), midgut gland (0.7), gills (0.7).

The chromatogram (Fig. 2d) of the extract of shrimp muscle from the trimethylated arsenosugar group showed two major peaks assigned to arsenobetaine (2.98 min) and unchanged trimethylated arsenosugar (9.55 min). Arsenocholine was not detected in any of the tissues. Smaller unassigned peaks was also present, including a broad peak at 4–5 min. Initially, this peak was thought to contain trimethylarsine oxide but chromatography of individual muscle samples under different conditions suggested otherwise (see below). In contrast to the other groups, the pattern of compounds showed large differences depending on the tissue type, with the ratio of trimethylated arsenosugar to arsenobetaine varying as follows: tail muscle (0.6), 'remainder' (2.1), midgut gland (4.0) and gills (1.9).

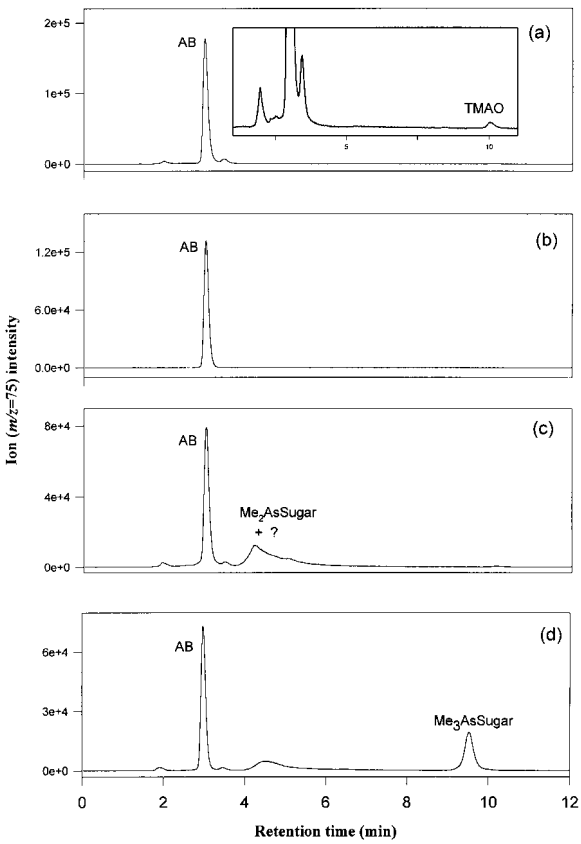

Figure 2 HPLC–ICP MS chromatograms (cation-exchange) for pooled muscle tissues from *Crangon*: (a) control (inset: same chromatogram with expanded intensity scale showing tetramethylarsonium ion at 10.03 min); (b) arsenobetaine-fed *Crangon;* (c) dimethylated arsenosugar-fed *Crangon;* (d) trimethylated arsenosugar-fed *Crangon.* Separations were performed on a Supelcosil LC-SCX cation-exchange column with 10 mM pyridine, pH 5.0, as mobile phase. Retention times for standards under these conditions were: arsenobetaine (2.98 min), dimethylated arsenosugar (4–6 min, broad), dimethylarsinoylethanol (4–6 min, broad), trimethylarsine oxide (4–6 min, broad), arsenocholine (8.40 min), trimethylated arsenosugar (9.53 min) and tetramethylarsonium ion (10.00 min).

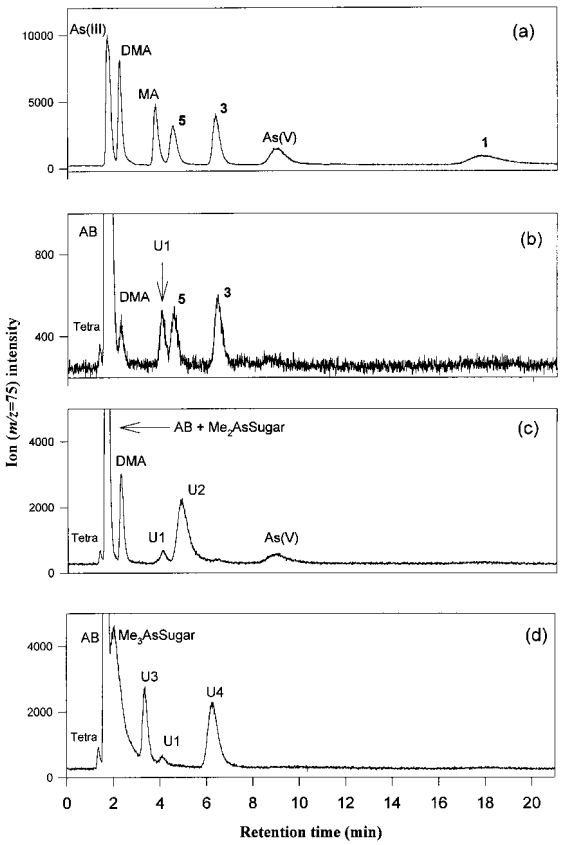

Figure 3 HPLC–ICP MS chromatograms (anion-exchange) for seven standard arsenic anions and pooled tissues from *Crangon*: (a) seven standard arsenic anions; (b) control *Crangon* gills; (c) dimethylated arsenosugar-fed *Crangon* muscle; (d) trimethylated arsenosugar-fed *Crangon* muscle. Separations were performed on a Hamilton PRP X-100 anion-exchange column with 20 mM $NH_4H_2PO_4$, pH 5.6, as mobile phase.

The quantities of arsenobetaine present in each of the samples were determined from the HPLC chromatograms (Table 3). There were no clear differences between the arsenobetaine content of the control group and the dimethylated arsenosugar group. The trimethylated arsenosugar group, however, contained increased arsenobetaine concentrations in all tissues (a factor of 1.5 to 1.9 depending on tissue).

Anion-exchange chromatography was also carried out on the pooled tissue samples (Fig. 3). Although this chromatography gave little information on the cations, since they all eluted near the solvent front, it did provide information on the small quantities of anions in the extracts. There were slight differences in the pattern of arsenic species between the tissues for these minor constituents. For example, in control shrimps arsenosugar concentrations (compounds **3** and **5**) were higher in gill and midgut gland than in muscle and 'remainder'. Control *Crangon* was also shown to contain traces of dimethylarsinate and an unknown compound, U1. Dimethylated arsenosugar-fed *Crangon* contained (in addition to the

Table 3 Arsenobetaine concentrations (μg As g^{-1} dry wt) in shrimp tissues following ingestion of arsenic compounds[a]

Tissue	Control group	Me_2As-sugar group	Me_3As-sugar group	Arsenobetaine group
Tail muscle	5.0	4.9	9.5	320
Midgut gland	13	11	23	730
Gills	14	14	23	760
'Remainder'	4.5	4.2	6.8	280

[a] Values represent a single HPLC–ICP MS analysis from pooled tissue samples from 10 individual shrimps within each group (recorded to two significant figures).

native arsenic compounds) arsenate, an unknown arsenic compound (U2), and increased quantities of dimethylarsinate. Trimethylated arsenosugar-fed *Crangon* contained two unknown arsenic compounds, U3 and U4. Although the data clearly show that compounds U2–U4 were arsenic metabolites from the ingested arsenosugars, possible structures possessing anionic character are not readily apparent.

From individual muscle tissue samples

Six individual muscle samples from the control group and the two arsenosugar groups were analysed to allow statistical comparison of arsenobetaine concentrations. The method used to process these individual samples was slightly different, and more efficient, than that used for the pooled samples. In addition, cation-exchange HPLC was performed with 20 mM pyridine at pH 2.6 (instead of 10 mM pyridine at pH 5.0) since trimethylarsine oxide chromatographs as a less broad peak under these conditions. K. A. Francesconi, W. Goessler, S. Panutrakul and K. J. Irgolic, *Sci. Tot. Environ.*, **221**, 139 (1998). The chromatograms for the individual muscle tissues showed differences from the chromatograms of the pooled samples (Fig. 4 shows typical chromatograms). For control samples, the small peak that formerly appeared at 3.45 min was now eluting at 6.10 min (Fig. 4b). This behaviour is similar to that of an unknown arsenic compound that we have noticed in several different samples, including the standard reference material DORM-1.

For muscle from dimethylated arsenosugar-fed shrimp (Fig. 4c), peaks at about 2.6 min and at 5.7 min corresponded to dimethylarsinate and unchanged dimethylated arsenosugar. A small peak at 6.0 min matched the retention time of dimethylarsinoylethanol, but because the peak was broad, this assignment was tentative. For shrimp fed trimethylated arsenosugar, the broad peak at about 4–5 min obtained at pH 5.0 (Fig. 2d) had virtually disappeared, and signals at 2.8 min and at 4.1 min became significant peaks (Fig. 4d). This behaviour was not compatible with the presence of trimethylarsine oxide in these samples.

At pH 2.6, arsenobetaine was well separated from the other arsenic compounds, and could be easily quantified in the extracts. The data on the individual muscle tissues from each of the three groups agreed well with those obtained on the pooled samples. There was no significant difference in the mean arsenobetaine concentration of muscle tissue for the control (6.3 ± 2.8 μg As g^{-1}) and dimethylated arsenosugar (5.9 ± 2.3 μg As g^{-1}) groups (mean ± SD) whereas the levels of arsenobetaine in the group fed trimethylated arsenosugar (11.5 ± 3.0 μg As g^{-1}) were significantly ($P < 0.02$) higher than those in the other two groups. The slightly higher arsenobetaine values obtained for the individual muscle tissues compared with the pooled muscle samples (Table 3) probably result from the more efficient process used to prepare the individual extracts.

DISCUSSION

Accumulation and distribution of arsenic in *Crangon*

Arsenobetaine was included in the experimental design as a positive control since *Crangon* is known to accumulate this compound readily from food.[16] The efficiency of retention was even higher in the present study (57%, compared with 42%), possibly as a consequence of the lower concentration of arsenobetaine in the food (500 μg As g^{-1}, compared with 1000 μg As g^{-1}). The retention efficiencies of total arsenic for the dimethylated arsenosugar (0.90%) and the trimethylated arseno-

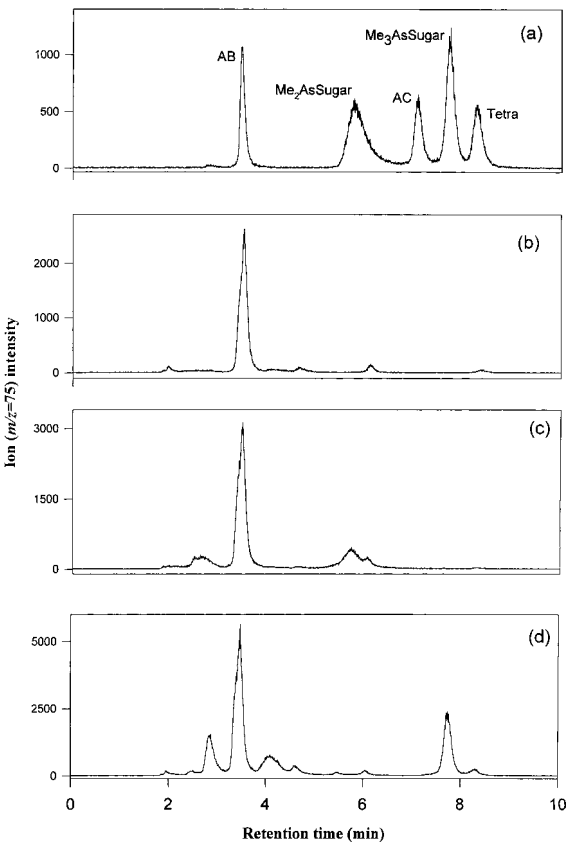

Figure 4 HPLC–ICP MS chromatograms (cation-exchange) for standard arsenic cations and for muscle tissues from individual *Crangon*: (a) five standard arsenic cations; (b) control *Crangon;* (c) dimethylated arsenosugar-fed *Crangon;* (d) trimethylated arsenosugar-fed *Crangon*. Extraction and dilution conditions for (b)–(d) were identical so the quantities are directly comparable. Separations were performed on a Supelcosil LC-SCX cation-exchange column with 20 mM pyridine, pH 2.6, as mobile phase.

sugar (4.2%) were much lower than that for arsenobetaine (57%). They were, however, comparable with or higher than those found for arsenate and trimethylarsine oxide (1.2% and 1.6%, respectively) in the earlier study with *Crangon*.[16] Uptake experiments with fish have also reported low retention of arsenate,[20–22] dimethylarsinate[22] and trimethylarsine oxide,[22] and negligible retention of dimethylarsinoylethanol and two related organoarsenic compounds.[23]

The tissue distribution patterns of arsenic in shrimps receiving arsenobetaine or dimethylated arsenosugar were both similar to that for control shrimps (Table 1). The data matched those reported[16] for arsenate, trimethylarsine oxide and arsenobetaine in *Crangon*. The trimethylated arsenosugar, however, behaved differently by showing high accumulation in the midgut gland relative to the other tissues. These data suggest that the midgut gland readily takes up the trimethylated arsenosugar from the gastrointestinal tract.

Metabolism of arsenosugars in *Crangon*

A chromatographic system capable of separating specific arsenic compounds was required to monitor possible transformations in the shrimp. The compounds of interest included (i) those present in control shrimps (arsenobetaine and tetramethylarsonium ion); (ii) those administered to the shrimp (arsenobetaine, dimethylated arsenosugar **2** and trimethylated arsenosugar **7**); and (iii) possible metabolites (dimethylarsinoylethanol, arsenocholine, arsenobetaine, trimethylarsine oxide and dimethylarsinate). Schemes for the generation of these metabolites are depicted in Fig. 5. Although most of the arsenic compounds were resolved under the various ion-exchange chromatographic conditions tested (e.g. Figs 3a and 4a), we were unable to separate fully the dimethylated arsenosugar standard from standards of dimethylarsinoylethanol and trimethylarsine oxide. In addition, several metabolites were produced in the experiments that did not match any of the available standards. In view of the relatively low uptake of the arsenosugars, these metabolites are unlikely to be significant in terms of arsenic biotransformation.

Crangon retained very little of the ingested dimethylated arsenosugar (0.90%), and that portion

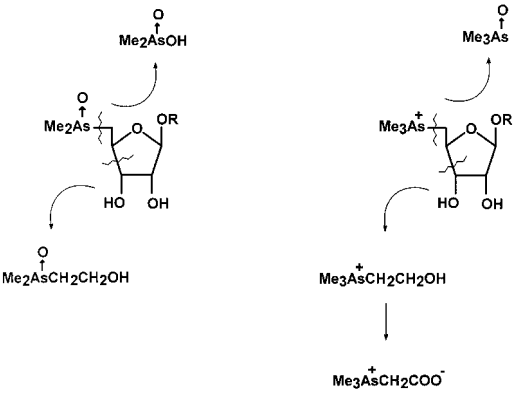

Figure 5 Schemes for possible degradation pathways and metabolites from arsenosugars.

that was retained appeared to be present predominantly as unchanged starting material. There was no detectable transformation of the dimethylated arsenosugar into arsenobetaine. Minor metabolites were dimethylarsinate and possibly dimethylarsinoylethanol, although the presence of the latter compound was not conclusively demonstrated. These results are consistent with those reported for experiments, where crustaceans[7,9] or molluscs[8] fed algal arsenic compounds (predominantly dimethylated arsenosugars) did not accumulate arsenobetaine.

In contrast to the dimethylated arsenosugar, about half the accumulated trimethylated arsenosugar was metabolized within the shrimp, and the major metabolite was arsenobetaine. Several minor metabolites were produced that did not match the chromatographic behaviour of the available arsenic standards, and the remainder was present as unchanged arsenosugar. The quantity of arsenobetaine produced within *Crangon* represented about 2% of the ingested arsenic. It is of interest that arsenocholine was not detected in the shrimp, since this compound is a known metabolite of trimethylated arsenosugars.[23] Probably, arsenocholine was formed within the shrimp, but was then rapidly metabolized to arsenobetaine, as demonstrated in fish[21,22] and mussels.[24]

The data reported here demonstrate that conversion of a trimethylated arsenosugar to arsenobetaine occurs within a marine animal. Whether or not this transformation is significant in natural systems remains to be established, although an estimation of its significance can be made based on current reported levels of trimethylated arsenosugars in marine algae and the uptake/transformation data. We may assume an average arsenic concentration in algae of 10 μg g^{-1} wet wt, and that the trimethylated arsenosugar content is about 1% of this value.[10,11] A daily food intake of 5% of body weight consumed as algae for a 1 g *Crangon* represents ingestion of 0.005 μg As as trimethylated arsenosugar per day. Results from the experiment reported here suggest that about 2% of the ingested trimethylated arsenosugar might be transformed and retained as arsenobetaine. This quantity is equivalent to only 0.0001 μg As g^{-1} d^{-1}. Clearly, this proposed source is insufficient to account for the concentrations of arsenobetaine (about 2 μg As g^{-1} wet wt) found in wild *Crangon*.

There are several factors that may influence the parameters used in the above estimation. First, the possibility exists that the uptake of trimethylated arsenosugars may be inversely related to dose, as seen for the tetramethylarsonium ion, B. Bachmann, D. A. Hunter and K. A. Francesconi, *Appl. Organometal. Chem*, **13**, 771–776 (1999), any may be much higher at environmentally relevant levels (in the current experiment, shrimps were fed trimethylated arsenosugar at 5000-fold the estimated natural levels). Second, the transformation into arsenobetaine may also be more efficient at lower doses; the high concentrations of trimethylated arsenosugar in the midgut compared with muscle tissue might be seen as support for this view. Third, marine animals that eat primarily algal material may carry out this transformation more efficiently than the omnivorous *Crangon*. And finally, natural sources (detritus?) richer in trimethylated arsenosugars than the macroalgae so far examined are also possible, although we consider this improbable because processes for methylating dimethylated arsenosugars outside algae are unlikely to proceed while maintaining the sugar ring intact.

The inability of *Crangon* to metabolize dimethylated arsenosugars to arsenobetaine is further evidence questioning the possible role of these compounds in the origin of arsenobetaine in marine animals. Nevertheless, the field data supporting such a conversion cannot be discounted and they call for continued efforts to attempt to elicit the proposed transformations in the laboratory. These processes are likely to involve interactions with microorganisms, and future arsenic transformation studies should profit from using well-defined microbial conditions.

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