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The biotransformation of monomethylarsonate and dimethylarsinate into arsenobetaine in seawater and mussels

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Water-soluble ^{3}H-labeled arsenic compounds were phenol-extracted from mussels (*Mytilus edulis*) and seawater after exposure to [^{3}H]monomethylarsonate (MMAA) and [^{3}H]dimethylarsinate (DMAA). Varying amounts of [^{3}H] arsenobetaine were found in mussels and seawater, depending upon the experimental conditions. The results indicate that arsenobetaine is principally biosynthesized by microscopic organisms in the seawater and that it is bioaccumulated by mussels. Total arsenic concentrations in mussel flesh, byssal threads and shells were also determined, showing concentration increases in all three compartments.

Keywords: Monomethylarsonate, dimethylarsinate, arsenobetaine, mussels, seawater

INTRODUCTION

It has been known for many years that marine organisms contain appreciable quantities of arsenic (up to ~100 ppm).[1] In 1977 Edmonds *et al.* identified arsenobetaine as the major organoarsenical in the tail of the Western rock lobster.[2] Since then the virtual ubiquity of this arsenical in marine animals has been established.[3,4]

The biochemical transformation of arsenic in marine systems has interested a number of investigators. In 1987 Edmonds and Francesconi suggested a possible scheme for the conversion of inorganic arsenate to arsenobetaine.[5] In the proposed scheme arsenate is absorbed by algae and undergoes a series of biotransformations via monomethylarsonate (MMAA) and dimethylarsinate (DMAA) until it is converted to arsenosugars and arsenolipids. The arsenosugars are then converted to arsenobetaine and a number of intermediate compounds have been proposed, although the pathway—and indeed where these transfomations occur—are by no means well established.

Arsenobetaine may be accumulated by marine animals directly from food and/or water, or it may be synthesized from other arsenicals within the animal. Studies in this area have not been fruitful, partly because the analytical techniques available for determining arsenic speciation in seawater are generally not suitable for the detection of arsenobetaine.

In this paper we establish that arsenobetaine is a metabolite of MMAA and DMAA, and report its presence in mussels and seawater.

EXPERIMENTAL

Instrumentation

The ^{3}H activity was measured by using a Packard Tricarb 1900 TR liquid scintillation counter. The HPLC system consisted of a Waters M45 Pump, a Waters U6K injector, the appropriate column [Protein Pak DEAE 5PW column, 7.5 mm i.d. × 7.5 cm (Waters) and a Protein Pak 300SW column, 7.5 mm (i.d.) × 30 cm (Waters)] and an automated fraction collector. Arsenic concentrations were determined by using a Varian Techtron Model AA 1275 atomic absorption spectrometer equipped with a GTA-95 accessory for GF AA spectroscopy and hydride-generation apparatus for HG AA spectroscopy.[6]

Reagents

Arsenobetaine,[7] [^{3}H]MMAA,[8] and [^{3}H]DMAA[9] were prepared by literature methods. The ^{3}H-labeled arsenicals were not fully tritiated.

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Exposure of mussels to radiolabeled arsenicals in seawater

Experiment A

(Approximately 75 mussels (*Mytilus edulis*), ranging in shell length from 2.5 to 5.0 cm, were collected from Point Grey in Vancouver, B.C. They were placed in a wire cage which was submerged in a 15 liter seawater tank. Another 15 liter tank was set up as the control. The seawater sampled from the tank containing mussels will be referred to as the experimental seawater and the seawater sampled from the other tank will be referred to as the control seawater. The water in these tanks was not replenished and initially contained either [^{3}H]MMAA or [^{3}H]DMAA at an activity level of 45 μCi. The mussels were left in the 15-liter tank for four days before being transferred into a third tank which contained 200 liters of seawater. The water in this tank was continually replenished with fresh seawater which was pumped in from the ocean. The seawater in all three tanks originated from the same source and aeration was achieved by the use of compressed air. On days 4 and 7 the experimental seawater, control seawater and mussels were sampled. This sampling procedure involved the collection of 200 ml samples of both the experimental and the control seawater along with 10 medium-sized mussels.

The experimental seawater samples (200 ml) were filtered through a hydrophilic membrane filter (0.45 μm pore size). The pH of the filtrate was adjusted to 4 by adding a few drops of concentrated HCl. This solution was counted (200 μl) and extracted with phenol (2 × 50 ml). The combined phenol extracts were diluted with diethyl ether (400 ml) and the water-soluble compounds were extracted with deionized water (2 × 50 ml). The aqueous extracts were combined, counted (200 μl), evaporated (rotary) to dryness and redissolved in deionized water (10 ml). This solution was applied to a cation exchange column (Dowex 50W × 8, 1.5 cm × 20 cm in the [H^+] form). The ^{3}H-labeled compounds eluted from the column by using deionized water (70 ml) and 5% ammonium hydroxide (200 ml). These conditions were sufficient to elute all of the ^{3}H activity. Fractions were collected (20 ml) and counted (600 μl samples). the ^{3}H-containing fractions were bulked, (rotary) evaporated to dryness, and redissolved in a minimum amount of deionized water (1 ml). Aliquots (100 μl) of these solutions were examined by using two different types of HPLC columns. The first, a Protein Pak DEAE column, was run by using 5 mM sodium acetate at pH 4.0 and pH 6.67 as the eluent. The second, a Protein Pak 300SW column, was run by using 5 mM sodium acetate at pH 6.67 as the eluent. The flow rate was set at 1 ml min^{-1} and fractions were collected every minute and counted (100 μl samples). Standard solutions of MMAA, DMAA and arsenobetaine in deionized water were run through both the cation-exchange and HPLC columns, and fractions were detected by GF AA spectroscopy.

The control seawater was treated in an identical fashion. The mussels were washed with deionized water and the shells were removed from the flesh, which was then homogenized. The resulting slurry was made up to 200 ml with deionized water. This slurry was treated in an identical fashion to the 200 ml seawater filtrate.

Experiment B

The conditions for this experiment were virtually identical with those described above apart from the following: only labeled MMAA was used, the experiment had a duration of five weeks, the mussels were transferred between tanks every three days (a three-day exposure and three-day wash cycle), and a control group of 75 mussels was set up in the 200 liter tank. The mussels were sampled at the end of the experiment.

The determination of the total arsenic concentration in the mussel flesh, byssal threads, and shells followed the procedures developed by Dodd.[10] Approximately 1 g of accurately weighed mussel flesh or byssal threads was placed in a 250 ml round-bottomed flask. The contents of the flask were digested by using a mixture of 69% nitric acid (3 ml), 98% sulphuric acid (1 ml) and 30% hydrogen peroxide (3 ml). The flask was fitted with a modified condenser[6] and the solution was heated to a gentle reflux for 3 h. This solution was then transferred into a 100 ml volumetric flask and made up to the mark with deionized water.

The shells were washed with deionized water and ground to a fine powder by using a mortar and pestle. Hydrochloric acid (2 M) was added to the powder in a ratio of 15 ml to every gram. After an hour, the resulting suspension was filtered; the filtrate was transferred to a 100 ml volumetric flask and made up to the mark with deionized water. The digestate from the shells of both the experimental and the control groups was counted.

All digestions described above were performed in duplicate. The total arsenic was determined in the samples by using HG AA spectroscopy.[6]

RESULTS AND DISCUSSION

The biotransformation of [^{3}H]MMAA and [^{3}H]DMAA to [^{3}H]arsenobetaine in seawater and mussels

Experimental seawater

[^{3}H]MMAA or [^{3}H]DMAA was added to seawater which contained mussels (*M. edulis*). After four days the mussels were transferred into another tank. The seawater from the tank which contained the mussels was examined on days 4 and 7. The seawater was filtered, counted, extracted with phenol, back-extracted with deionized water, and then counted again. The chromatographic results and identification of the ^{3}H-labeled compounds phenol-extracted from the seawater are described below.

It is important to note that there is no possibility of proton exchange *in situ* for the compounds which are being investigated. Any casual exchange of the label between compounds would require a cleavage of the methyl–arsenic bond. Also, the various ^{3}H-labeled compounds in the seawater will all have different extraction efficiencies into phenol; therefore, the ratio of compounds in the extracts will be different from the actual ratio of compounds in the seawater. Both DMAA and MMAA have extraction efficiencies of about 10%, whereas the extraction efficiency for arsenobetaine is closer to 100%.

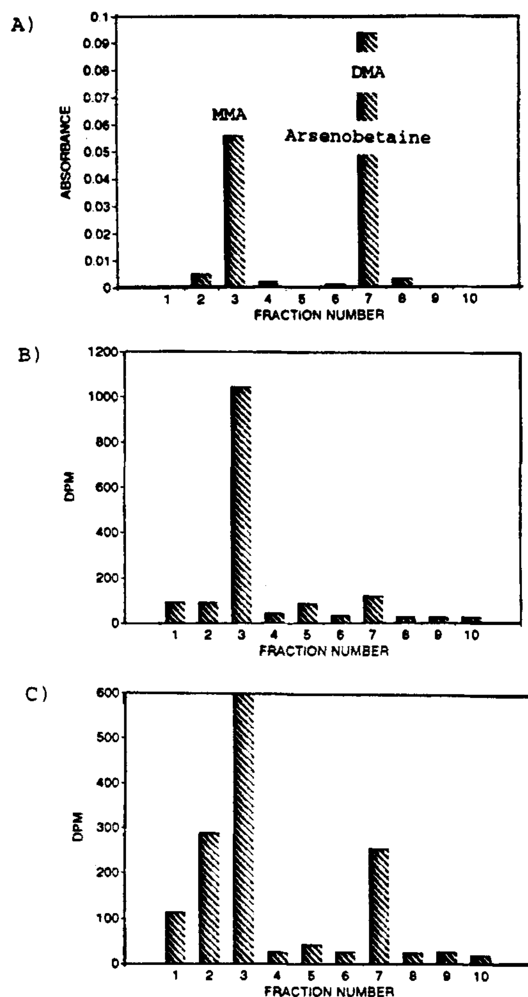


Figure 1 The cation exchange chromatograms for the standards (A) and the phenol extract from the experimental seawater on days 4 (B) and 7 (C) after [^{3}H]-MMAA exposure.

[^{3}H]MMAA

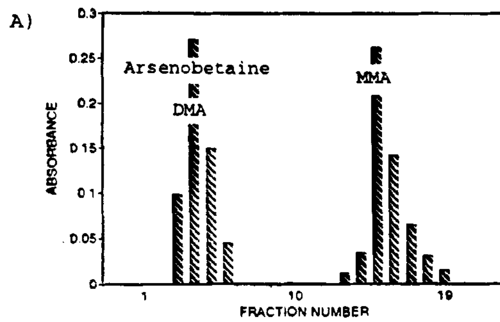
The cation- and anion-exchange chromatograms of the standards and of the ^{3}H-containing compounds extracted from the experimental seawater on days 4 and 7 are displayed in Figs 1 and 2.

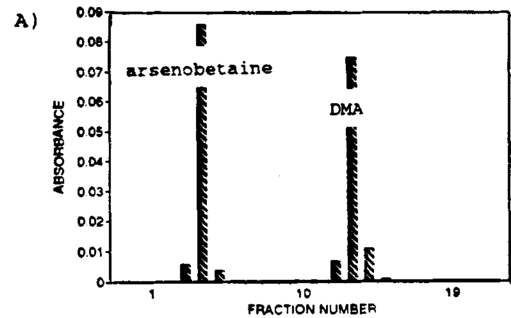
The compound eluting in fraction 3 from the cation exchange column (Fig. 1) is identified as MMAA on the basis of its retention time, which is identical with that of the standard. This identification is confirmed by the chromatograms (Fig. 2) for the elution profiles from the anion-exchange (pH 4.0) and size-exclusion HPLC columns.

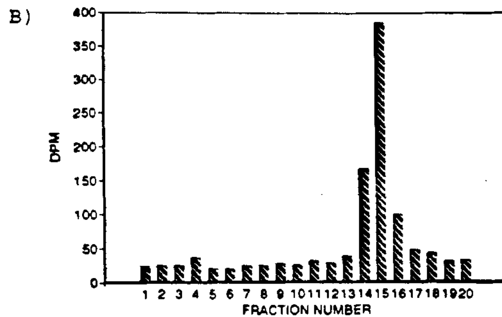
The compound eluting in fraction 7 from the cation-exchange column (Fig. 1) is identified as arsenobetaine on the basis of its retention time, which is identical with that of the standard. This identification is confirmed by the chromatograms (Fig. 3) for the elution profiles from the anion-exchange (pH 6.67) and size-exclusion HPLC columns.

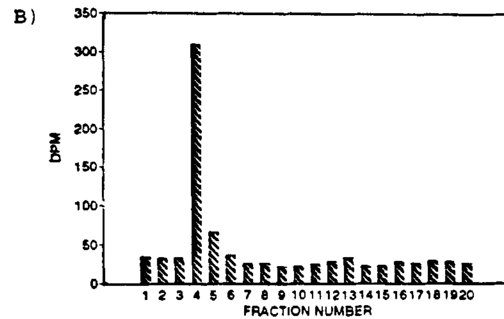
These results describe for the first time the isolation of arsenobetaine from seawater. Francesconi had speculated that arsenobetaine might be present in seawater, but was unable to verify its presence.[6] He estimated that if arsenobetaine was present in seawater its concentration would be less than 0.5 µg As liter^{-1}. The isolation of arsenobetaine from the present experiments may have been facilitated by the use of elevated arsenic concentrations and by the use of a radioactive label to allow easier detection.

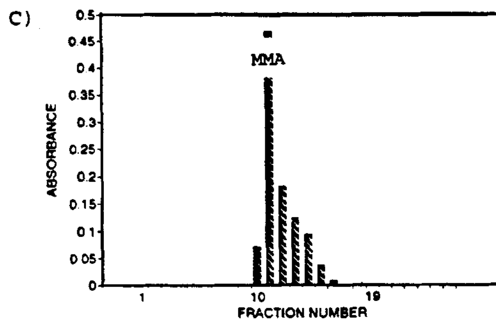
The chromatograms in Fig. 1 show a change in

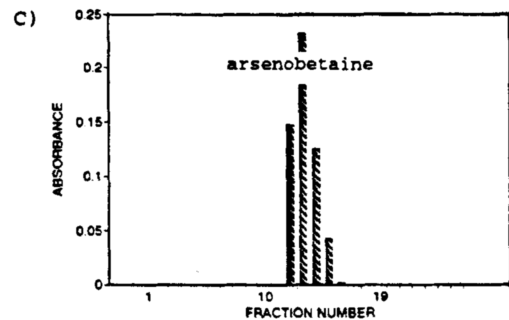


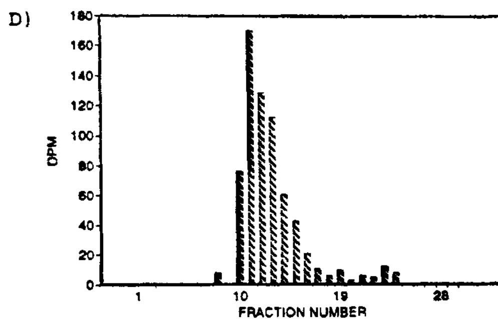












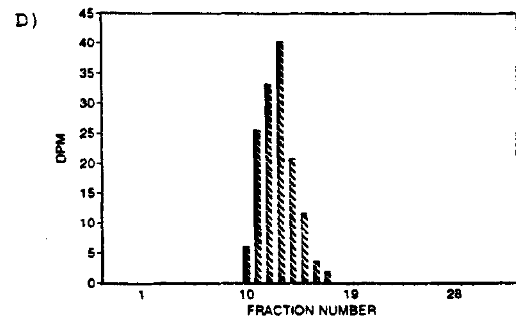


Figure 2 HPLC chromatograms; (A) arsenobetaine, MMAA and DMAA on the anion exchange column at pH 4, (B) the compound eluting in fraction 3 [Figure 1(B)] on the anion exchange column at pH 4, (C) MMAA on the size exclusion column, and (D) of the compound eluting in fraction 3 [Figure 1(B)] on the size exclusion column.

Figure 3 HPLC chromtograms; (A) arsenobetaine and DMAA on the anion exchange column at pH 6.67, (B) the compound eluting in fraction 7 [Figure 1(B)] on the anion exchange column at pH 6.67, (C) arsenobetaine on the size exclusion column, and (D) of the compound eluting in fraction 7 [Figure 1(B)] on the size exclusion column.

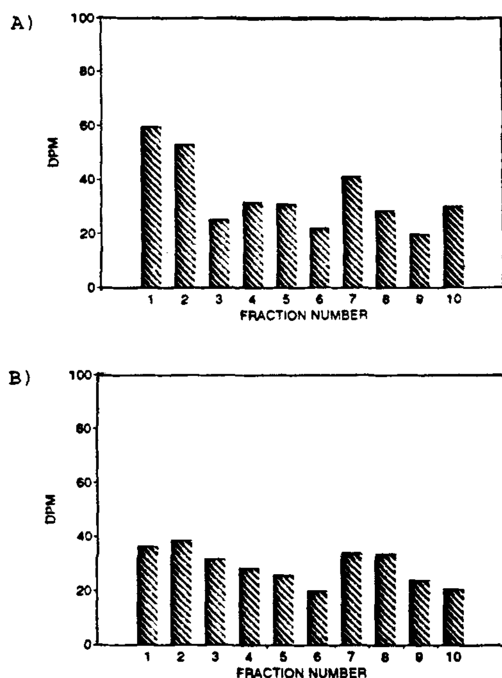


Figure 4 The cation exchange chromatograms for the experimental seawater on days 4 (A) and 7 (B) after [^{3}H]-DMAA exposure.

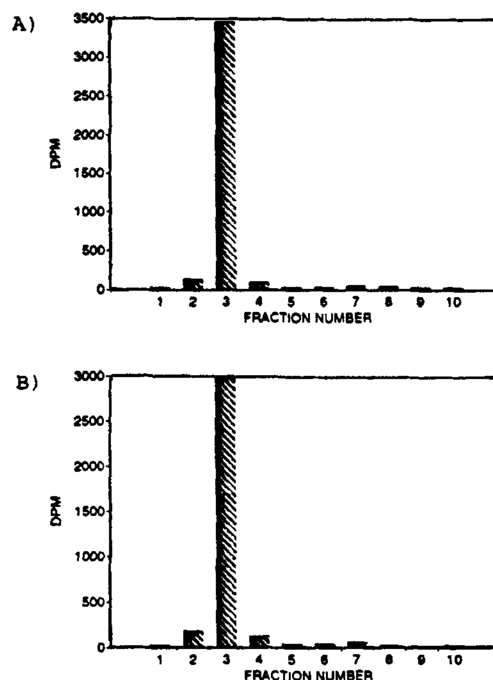


Figure 6 The cation exchange chromatograms for the phenol extract from the control seawater on days 4 (A) and 7 (B) after [^{3}H]-MMAA exposure.

the relative amounts of arsenobetaine and MMAA on days 4 and 7. The amount of arsenobetaine increased in the absence of mussels; this indicates that it is being biosynthesized, at least in part, by other organisms which are present in seawater.

[^{3}H]DMAA
The cation-exchange chromatograms of the ^{3}H-containing compounds extracted from the experimental seawater are displayed in Fig. 4. The most notable feature of these chromatograms is the low level of activity, which is barely above the background. Arsenobetaine is tentatively identified in fraction 7 (Fig. 4) by the chromatographic technique described above. The elution profile from the anion-exchange (pH 6.67) HPLC column for the compound(s) eluting in fraction 7 [Fig. 4(B)] from the cation exchange column is displayed in Fig. 5.

Control seawater
The seawater from the control 15-liter tank was filtered, counted, extracted with phenol, back-

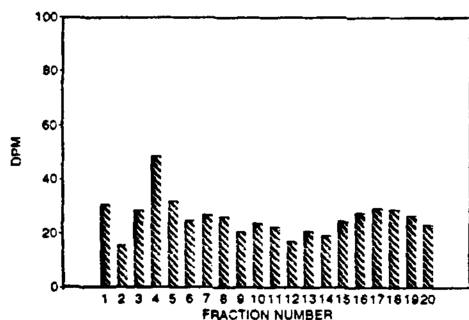


Figure 5 The HPLC chromatogram for the compound eluting in fraction 7 [Figure 4(A)] on the anion exchange column at pH 6.67.

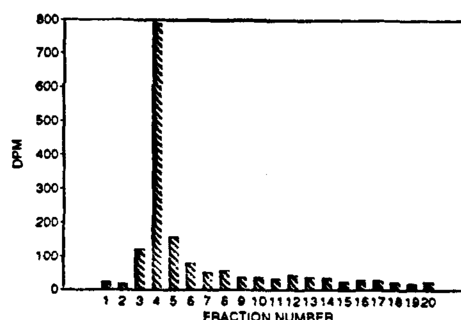


Figure 7 The HPLC chromatogram for the compound eluting in fraction 7 [Figure 6(B)] on the anion exchange column at pH 6.67.

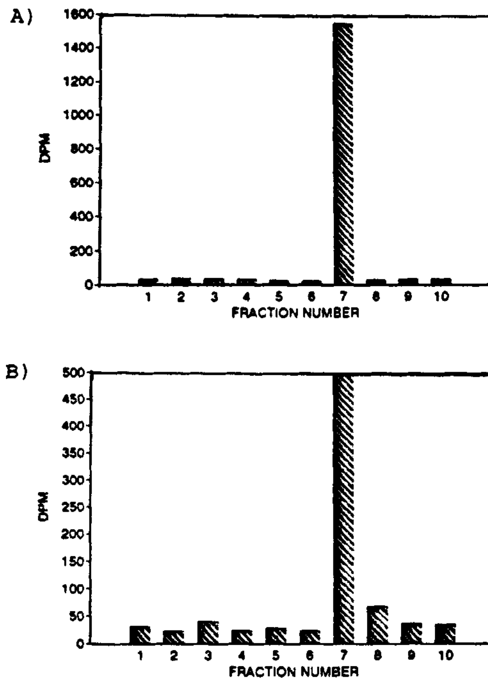


Figure 8 The cation exchange chromatograms for the phenol extract from the control seawater on days 4(A) and 7(B) after [^{3}H]-DMAA exposure.

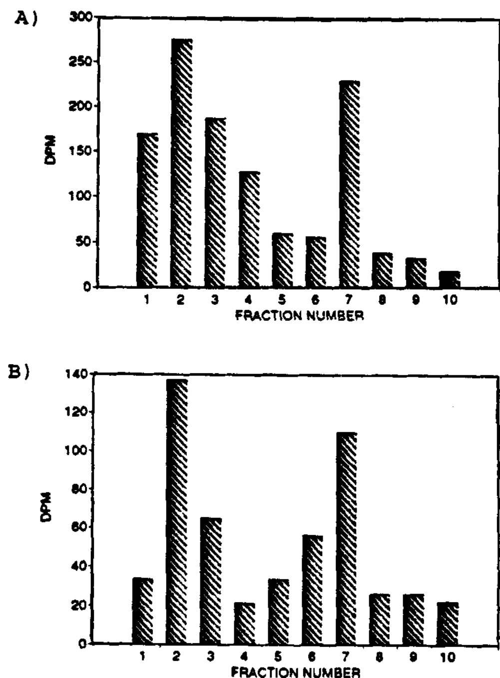


Figure 10 The cation exchange chromatograms for the phenol extracted compounds from the mussel flesh on days 4 (A) and 7 (B) after [^{3}H]-MMAA exposure.

extracted with deionized water, and then counted again as described above. The chromatographic results and identification of the ^{3}H-labeled compounds extracted from the seawater are described below. Either [^{3}H]MMAA or [^{3}H]DMAA was added to the seawater at the beginning of the experiment.

[^{3}H]MMAA

The cation- and anion-exchange chromatograms of the ^{3}H-containing compounds extracted from the control seawater are displayed in Figs 6 and 7.

The compounds eluting in fractions 3 and 7 from the cation-exchange column (Fig. 6) were identified as MMAA and arsenobetaine by using the chromatographic techniques described above.

Arsenobetaine is confirmed by the chromatogram in Fig. 7 for the elution profile from the anion-exchange HPLC column at pH 6.67.

The results confirm tht MMAA can be converted to arsenobetaine in natural seawater. Mussels were never present in the tank so the

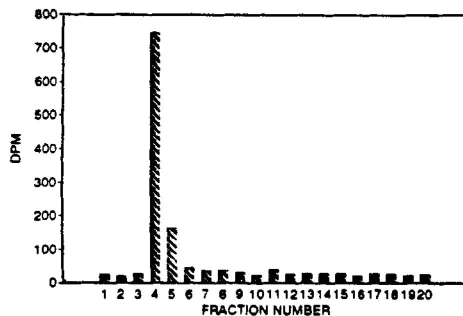


Figure 9 The HPLC chromatogram for the compound eluting in fraction 7 [Figure 8(B)] on the anion exchange column at pH 6.67.

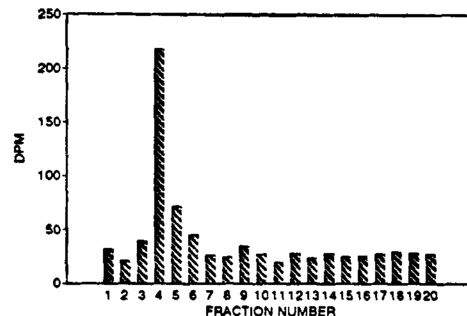


Figure 11 The HPLC chromatogram for the compound eluting in fractions 2 and 3 [Figure 10(B)] on the anion exchange column at pH 4.

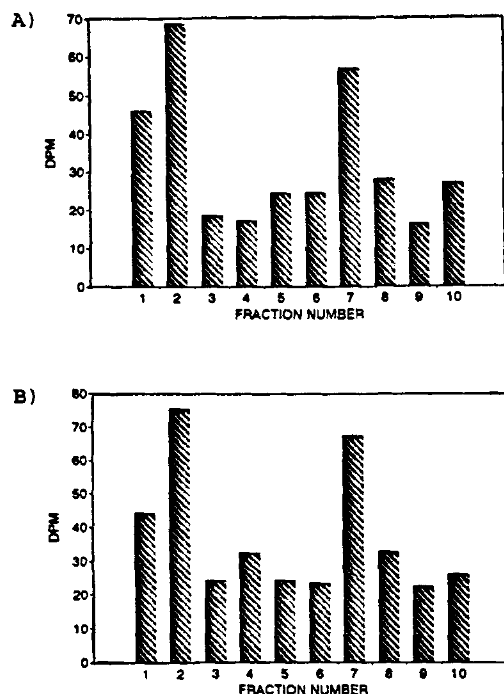


Figure 12 The cation exchange chromatograms for the phenol extracted compounds from the mussel flesh on days 4 (A) and 7 (B) after [^{3}H]-DMAA exposure.

transformation must be accomplished by organisms at lower trophic levels. The ratio of arsenobetaine to MMAA present in the control (Fig. 6) is much less than that found in the experimental seawater (Fig. 1). This could mean that arsenobetaine is being synthesized by mussels and/or other microscopic organisms that are symbiotic or otherwise present in seawater with the mussels. It is impossible to distinguish between the contributions of the organisms associated with the mussels and those of the mussels in the current set of experiments.

[^{3}H]DMAA

The cation- and anion-exchange chromatograms of the ^{3}H-containing compounds extracted from the control seawater are displayed in Figs 8 and 9.

The compound eluting in fraction 7 from the cation exchange column (Fig. 8) is identified as arsenobetaine by using the chromatographic techniques described above. Arsenobetaine is confirmed by the chromatogram in Fig. 9 for the elution profile from the anion-exchange HPLC column at pH 6.67.

It seems that DMAA is more easily biotransformed to arsenobetaine than MMAA. This conclusion is supported by our recent work which indicates that DMAA passes more freely through model cell membranes than MMAA.[8,11] Thus, it would be more readily available for biotransformation.

Table 1 Total arsenic concentration (ppm) in mussels

	Control group	Experimental group
Flesh	0.45	0.87
Shells	0.24	0.32
Byssal threads	4.2	6.3

Table 2 ^{3}H activity in shells (dpm)

	Control group	Experimental group
Shells	22.15, 22.95	32.43, 31.26

Mussel flesh

After exposure of the mussels to the labeled arsenicals, the flesh was homogenized, and the ^{3}H-containing compounds were extracted from the flesh with phenol and then back-extracted into water.

[^{3}H]MMAA

The cation-exchange and anion-exchange chromatograms of the ^{3}H-containing compounds phenol-extracted from the mussel flesh after four days' exposure to the label and after the full seven-day cycle (four days exposure, three days wash) are displayed in Figs 10 and 11.

The compound eluting in fraction 7 from the cation-exchange column (Fig. 10) is identified as arsenobetaine by the chromatographic techniques described above.

The chromatographic behaviour of the compound in fraction 2 of Fig. 10 does not match that of any of the available standards. Also, the elution profile for this compound from the anion-exchange HPLC column shown in Fig. 11 does not compare with that of the MMAA standard shown in Fig. 2. Its chromatographic behavior indicates that it is neutral, as it elutes in the void volume from both the cation- and anion-exchange columns. Arsenic is present in the fraction (detected by GF AA) and it is possible that the arsenical is a neutral arsenosugar such as 2′,3′-

dihydroxypropyl 5-deoxy-5-dimethylarsinyl-riboside, which has been recently found in a *M. edulis* standard.[12]

The two chromatograms in Fig. 10 are quite similar in that the ratio of arsenobetaine to the unknown is almost the same. Between days 4 and 7 the mussels were exposed to continuously circulating seawater (from the ocean) and any ^{3}H-labeled compounds outside the mussels would be quickly washed away. Thus, the chromatograms indicate that, within the experimental uncertainty, the mussels are not biosynthesizing arsenobetaine from any of the compounds they have accumulated while being exposed to labeled MMAA. This finding supports the hypothesis that arsenobetaine in mussels is accumulated from the surroundings.

[^{3}H]DMAA

The flesh of the mussels exposed to labeled DMAA was examined in a similar manner. The compound eluting in fraction 7 from the cation-exchange column (Fig. 12) is identified as arsenobetaine by the chromatographic techniques described above.

The compound in fraction 2 appeared to be the same unidentified arsenical as was isolated from the MMAA exposure experiment. Again, as in Fig. 10, the two cation-exchange chromatograms (Fig. 12) are quite similar. This supports the idea that mussels selectively accumulate arsenicals, as was shown by Francesconi in controlled uptake experiments where mussels readily bioaccumulated arsenobetaine and arsenocholine, but do not bio-accumulate trimethylarsine oxide or 2-dimethylarsinylethanol.[6]

The results indicate that either MMAA or DMAA can act as a precursor to arsenobetaine and both are part of the same biosynthetic pathway.

The accumulation of arsenicals in the byssal threads, flesh and shells of mussels

The mussels were transferred into and out of the [^{3}H]MMAA-containing tank on a three-day cycle. After five weeks they were washed, shucked, and digested, as outlined in the Experimental section. The total arsenic concentrations in the mussel flesh, byssal threads and shells of both the experimental and the control groups were determined and are summarized in Table 1.

The natural arsenic concentration in the seawater that was being pumped into the tanks was determined by using HG AA spectroscopy to be 0.0054 ppm. The initial arsenic concentration of the ^{3}H-labeled MMAA to which the organisms were exposed was 2 ppm.

There was an increase in the total arsenic concentration in each of the three parts. The digestion procedure for both the flesh and the byssal threads would have decomposed any organoarsenicals which may have been present; therefore, counting techniques were not employed. The procedure used to dissolve the shells (2 M HCl) would not have led to the complete degradation of any organoarsenical that may be present. In particular, the [^{3}H]methyl–arsenic bond would not have been cleaved, so this solution was counted to yield the results displayed in Table 2.

Compounds may be either adsorbed onto the shell from the surrounding medium or incorporated into the shell by the mussel. The activity increase shows that the arsenicals in the shells are methylated. This finding is consistent with the findings of low levels of methyl- and dimethylarsenicals in the shells of some costal bivalves of British Columbia.[13]

In conclusion, feeding experiments of this type yield interesting information which aids in the understanding of how arsenic cycles in the ocean. In particular, the results indicate that arsenobetaine is principally biosynthesized by microscopic organisms found in the seawater and that it is bioaccumulated by mussels.

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