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Biomethylation of arsenic and its excretion by the alga *Chlorella vulgaris*

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Experimental results in this paper lead to the following conclusions. (1) Cell homogenates of *Chlorella vulgaris* adsorbed the inorganic arsenic compound Na_2HAsO_4 but no methylation of the arsenic occurred *in vitro*. (2) A small part of the arsenic bioaccumulated by *C. vulgaris* was methylated *in vivo*. The quantity of arsenic methylated in the cell increased with an increase of arsenic concentration in the medium. (3) When the arsenic-accumulating cells were transferred into arsenic-free media, the arsenic was excreted and the relative quantity of the methylated arsenic in the excrement was larger than that in the cell. (4) In the growth phase of *C. vulgaris*, a small fraction of the arsenic accumulated in the cell was first transformed to monomethyl and dimethyl-arsenic compounds during the early exponential phase, and after a short time a fraction was transformed to trimethylarsenic species.

Keywords: Arsenic, biomethylation, freshwater alga, *Chlorella vulgaris*, excretion, biotransformation

INTRODUCTION

The mechanism of arsenic resistance of the alga *Chlorella vulgaris* appears to be unlike that of copper resistance, in which copper-resistant *C. vulgaris* has a defense against accumulating copper.[1] Arsenic-resistant *C. vulgaris* seems to have ability both to detoxify the arsenic accumulated in the cell and to excrete it. The detoxification of arsenic by the cell was probably achieved by shielding it with SH-groups of algal components, e.g. proteins, or by methylating it.

Monomethyl- and dimethyl-arsenic compounds were found in the water extract of seaweeds.[2–5] Arsenosugars (dimethylarsenoribose derivatives) have been isolated from *Ecklonia radiata* (macro-algae).[5–7] Arsenobetaine was shown later to be found in some marine algae.[8,9] These methylarsenic compounds were found in marine algae but scarcely in freshwater algae.

This paper describes the methylation of inorganic arsenic by the freshwater alga *C. vulgaris* and its excretion.

EXPERIMENTAL

General procedure for algal culture and analyses fo arsenic

Chlorella vulgaris was stock-cultured, cultured, harvested and analyzed for total arsenic by the general method as mentioned in the previous paper.[10]

Methylated arsenic compounds were determined by the following method. The dry cells (*ca* 10 mg) were digested with 5 cm^3 of 2 mol dm^{-3} NaOH at 90–95 °C for 3 h, using an aluminum heating block. Methylated arsenic compounds in the digest were reduced with sodium borohydride ($NaBH_4$) to the arsine compounds. The arsine gases generated were at once frozen out in a liquid-nitrogen U-trap. The arsines successively borne out of the U-trap upon warming it were passed through a quartz tube atomizer and determined chromatographically using an atomic absorption spectrometer on the basis of the difference in the boiling points of the arsines.[11]

Detection limits for total and methylated arsenic and error limits were 5 ng and 5%, respectively.

Chemicals

Monomethylarsonic acid, dimethylarsinic acid (cacodylic acid) and arsenobetaine were purchased from TRI Chemicals Laboratories, Japan. All other chemicals were of reagent grade and were used without further purification. Chemical solutions and the algal liquid medium were prepared with distilled deionized water.

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RESULTS AND DISCUSSION

Methylation of inorganic arsenic by *Chlorella* homogenate *in vitro*

C. vulgaris was cultured in an arsenic-free modified Dettmer (MD) medium[10] for 20 days under standard conditions[10] and the cells separated and washed by centrifugation were resuspended in pure water and homogenized in ice-cooled water by an ultrasonicator (Kaijoudenki Co. Ltd, T-A-4280, Japan). The homogenated cells (50.2 mg, on dry wt basis) were suspended in 50 cm^3 pH-buffer solution (MES: 2-(*N*-morpholino)ethanesulfonic acid, 0.1 mol dm^{-3}, pH 7) containing 1 μg g^{-1} of arsenic [as elemental arsenic for Na_2HAsO_4, abbreviated as As(V)], the suspension was stirred and incubated at 31–33 °C for 2 and 24 h, then the homogenates were separated by centrifugation, and analyzed for total and methylated arsenic compounds.

The experimental results were as follows. Total arsenic concentration of the homogenates after incubation for 2 and 24 h were 422 and 376 μg As g^{-1} dry weight, respectively. No mono-, di- or tri-methylated arsenic was detected in either homogenate. In our previous papers,[12] arsenic accumulated by *C. vulgaris in vivo* was found to be methylated in the cell. This experimental result meant that the algal cell possessed enzymes catalyzing the methylation of inorganic arsenic accumulated in the cell. However, the enzymes in the homogenate did not methylate inorganic arsenic *in vitro*.

Accumulation and methylation of inorganic arsenic by *C. vulgaris in vivo*

C. vulgaris (9.1 mg dry weight per dm^3 medium) pre-cultured in an arsenic-free MD medium was inoculated in 2 dm^3 MD medium containing 0, 10, 100 and 1000 mg As(V) dm^{-3} and cultured for 38 days under the general conditions but with an illumination of 10 000 lux.

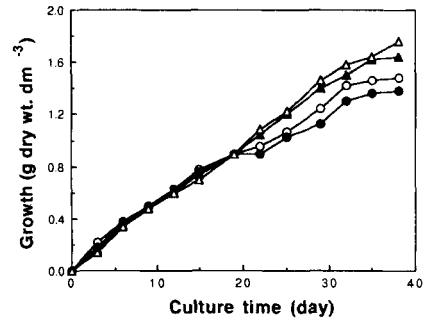


Figure 1 Growth curves of *C. vulgaris* cultured in MD media containing (○) 0, (●) 10, (▲) 100 and (△) 1000 mg As(V) dm^{-3}.

Experimental results on the growth curves and analyses for arsenic are summarized in Fig. 1 and Table 1, respectively.

The growth of the alga in Fig. 1 is generally three times that obtained under a standard illumination of 4000 lux. The higher the arsenic concentration in the medium, the greater was the growth at 38 days' culture. Table 1 shows that arsenic accumulation increased with an increase in arsenic concentration in the medium. These results agreed with the data in previous papers.[10,13]

Table 1 also shows that a part of the arsenic accumulated was methylated and the quantities of methylated arsenic compounds (monomethyl arsenic and dimethylarsenic compounds, abbreviated as MMA and DMA, respectively) increased with an increase in arsenic concentration in the medium, but the relative concentration of the methylated arsenic compounds to the total decreased from 1.8% to 0.4%. Trimethylarsenic was detected in only trace amounts. These results show that when *C. vulgaris* accumulated inorganic arsenic compounds from the aqueous

Table 1 Accumulation of arsenic(V) and its methylation by living *C. vulgaris*

As(V) level (mg As dm^{-3})	Cell growth (g dm^{-3})	Arsenic accumulation, μg As g^{-1} dry wt (%)				
		Total	IA[a]	MMA[a]	DMA[a]	TMA[a]
0	1.47	0	0	0	0	0
10	1.38	270 (100)	265 (98.2)	0.6 (0.2)	4.3 (1.6)	tr[a]
100	1.64	1400 (100)	1400 (98.7)	6.5 (0.5)	11.2 (0.8)	tr
1000	1.76	8700 (100)	8700 (99.6)	15.5 (0.2)	18.0 (0.2)	tr

[a] In all the tables, IA, MMA, DMA and TMA, and tr represent inorganic, monomethyl-, dimethyl- and trimethyl-arsenic compounds, and a detectable but trace amount, respectively. IA = Total − (MMA + DMA + TMA).

Table 2 Excretion of arsenic accumulated by living *C. vulgaris*

Incubation medium	As in alga[a] before excretion (μg As)	Arsenic excreted in aqueous phase[b], μg As (% of total As excreted)				
		Total	IA	MMA	DMA	TMA
MD medium	35	0.62 (100)	0.15 (24)	0	0.47 (76)	tr
	182	5.17 (100)	4.53 (88)	0	0.64 (12)	tr
Pure water	35	2.09 (100)	1.55 (74)	0	0.54 (26)	tr
	182	41.8 (100)	34.1 (81.6)	0	7.7 (18.4)	tr

[a] 130 mg dry weight; [b] 100 cm^3 medium.

phase, the quantity of the arsenic accumulated increased with an increase in aqueous arsenic concentration, and a part of the arsenic was methylated in the cell but the quantity of methylated arsenic did not increase in proportion to that of the total arsenic accumulated. The alga seemed therefore to have a limited methylation capacity.

Excretions of methylated arsenic compounds into the MD medium and pure water

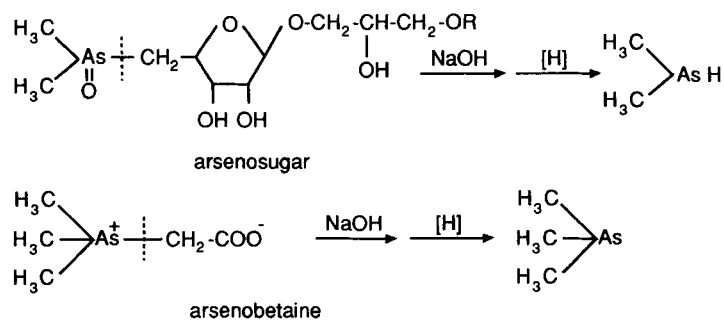
The algal cells which had accumulated arsenic from MD media containing 10 and 1000 mg As(V) dm^{-3} shown in Table 1 were harvested, washed, transferred to arsenic-free MD media or to pure water and incubated for three days at cell concentrations of about 130 mg dry weight in 100 cm^3 media, and then the arsenic excreted in the aqueous phase was analyzed. The experimental results are summarized in Table 2.

On calculation of the mass balance of the arsenic in the four arsenic excretion experiments shown in Table 2, the excretion rates of total arsenic were 1.7, 2.8, 6.0 and 23%, respectively. These data and Table 2 show that arsenic accumulated in *C. vulgaris* was preferably excreted to pure water rather than to the MD medium. This experimental result agreed with the results obtained in the previous paper,[10] that accumulated arsenic was readily excreted under undesirable conditions for multiplication of the alga.

In the algal cell, more than 98% of accumulated arsenic was present in the inorganic form, as shown in Table 1. But in the excrement, the relative concentration of methylated arsenic increased and the methylated arsenic was found to be only in the dimethylarsenic form. No monomethylarsenic compound was detected in the excrement. The dimethylarsenic compound was found to be the most preferable chemical form for excretion from *C. vulgaris*

The concentrations of arsenic excreted in the aqueous phase as shown in Table 2 were determined directly by use of the aqueous sample, i.e. without NaOH digestion of the aqueous samples. If dimethyl and trimethyl-arsenic compounds existed in the aqueous phase in free chemical forms such as cacodylic acid and trimethylarsine oxide, respectively, these would be completely determined without NaOH digestion. However, arsenosugar and arsenobetaine in the aqueous phase would not be detected without NaOH digestion as shown in Scheme 1.

Table 3 shows corrected data corresponding to the fourth set of experimental results in Table 2, obtained by NaOH digestion. Compared with data obtained without NaOH digestion the



Scheme 1

Table 3 NaOH digestion of excreted methylated arsenic compounds

NaOH digestion	Arsenic excreted in aqueous phase, μg As (% of total As excreted)				
	Total	IA	MMA	DMA	TMA
No	41.8 (100)	34.1 (81.6)	0	7.7 (18.4)	tr
Yes	41.8 (100)	32.5 (77.8)	0	8.0 (19.1)	1.3 (3.1)

dimethylarsenic species increased by 0.3 μg (0.7%) and trimethylarsenic was found at 1.3 μg (3.1%) in the aqueous phase with NaOH digestion. These differences caused by NaOH digestion (including differences in IA) mean that methylated arsenic compounds were excreted, not only in free forms such as cacodylic acid and trimethylarsine oxide but also in complex forms such as arsenosugar and arsenobetaine.

Effect of pH on excretion of methylated arsenic into pH-buffer solutions

The following experiment was carried out to study variation in methylarsenic excretion over a range of pH values (9.0–5.3) found in the aqueous environment.

Six algal samples (114 mg each, dry weight) had bioaccumulated arsenic at a concentration of 1300 μg As g^{-1} dry weight were transferred to three different pH-buffers [Bicine: *N*,*N*-bis(2-hydroxyethyl)glycine] solutions (100 cm^3 each; pH 9.0, 7.1 and 5.3) and the cultures were incubated by shaking for four days at room temperature with and without illumination (3000 lux). The cells were separated and the supernatants were analyzed for arsenic after NaOH digestion. The experimental results are summarized in Table 4.

The effect of pH on the arsenic excretion without illumination was larger than that with illumination. In the former case, 5.6% (8.3 μg) of arsenic accumulated in the cells (148 μg) was excreted to the aqueous phase at pH 9.0 and 2.0% (2.9 μg) was excreted at pH 5.3. In the latter case, 3.8% (5.6 μg) and 3.3% (4.9 μg) of the arsenic were excreted to the aqueous phase at pH 9.0 and pH 5.3, respectively. The relative concentration of methylated arsenic in the excreted arsenic increased with a decrease in pH of the aqueous phase.

In the previous experiments,[10] arsenic excretion was found to be increased under undesirable conditions for the multiplication of the alga. In this experiment, the buffer solution did not contain any nutrient, so there was no algal growth during the excretion. The differences in the quantities of excreted arsenic were therefore independent of the suitability for multiplication.

The reasons for the effects of pH both on quantity of arsenic excteted and the relative concentration of methylated arsenic excreted are unknown at the present stage.

Change in methylated arsenic species, both in algal cells and the water phase, during the growth

C. vulgaris was cultured in the MD medium (20 dm^3) containing 9 mg As dm^{-3} for 20 days and methylated arsenic compounds accumulated in the cell and excreted in the medium were determined every two days during 20 days' culture. Both the algal and the medium samples were analyzed for arsenic after hot-NaOH digestion.

Table 4 Effect of pH and illumination on excretion of methylated arsenic accumulated by *C. vulgaris* into pH-buffer solutions

pH	Arsenic species excreted in pH-buffer solution (μg)									
	With illumination					Without illumination				
	Total	IA	MMA	DMA	TMA	Total	IA	MMA	DMA	TMA
9.0	5.6	4.7	0	0.9 (16%)	0	8.3	8.0	0	0.3 (4%)	0
7.1	5.9	4.5	0	1.4 (24%)	0	6.1	5.3	0	0.8 (13%)	0
5.3	4.9	2.5	0	2.4 (45%)	0	2.9	1.3	0	1.6 (55%)	0

Table 5 Arsenic compounds accumulated in *Chlorella* cells and excreted in the medium

Culture time (days)	Arsenic in *Chlorella* (μg As g^{-1} dry wt)					Arsenic in medium (μg As dm^{-3} medium)			
	Total	IA	MMA	DMA	TMA	Total	MMA	DMA	TMA
0	0	0	0	0	0	9000	0	0	0
2	1140	1100	15	20	tr	8300	0	0	0
4	2290	2270	tr	16	0.1	7900	0	0	0.3
6	3230	3200	12	15	3.9	7700	0	0	1.5
8	3410	3380	10	13	5.0	8200	0	0	4.9
10	3750	3720	9	11	6.1	7600	0	0	13.4
12	3510	3480	9	11	5.4	6900	0	tr	tr
14	2600	2570	6	11	4.6	6700	tr	0.3	0.2
16	2520	2500	tr	10	4.6	7000	0	0	tr
18	1550	1530	2.2	9	5.3	7200	tr	0.7	0
20	1950	1930	3.5	9	4.6	8000	tr	0.5	tr

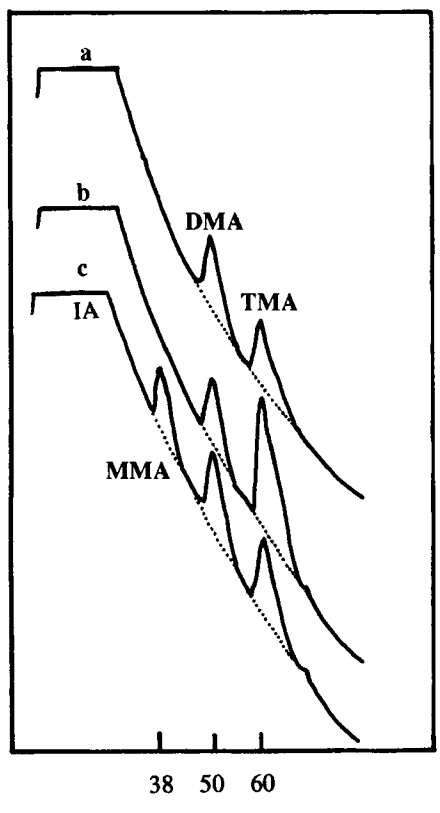


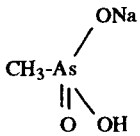
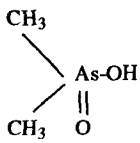
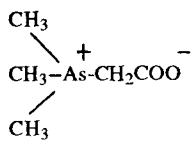
Figure 2 Chromatographic atomic absorption spectrophotometry traces of methylated arsenic compounds in the presence of large excesses of inorganic arsenic: a, 0.5 cm^3 medium containing 10 μg As(V), 10 ng TMA and 10 ng MMA; b, a + 10 ng TMA; c, a + 10 ng MMA.

Following hot-base digestion, the methylated arsenic compounds are not degraded into inorganic arsenic.[14] Arsenobetaine[15,16] and dimethylarsenoribose derivatives[15] are quantitatively converted to trimethylarsine oxide and dimethylarsinate on hot-base digestion, and hydride-generated to trimethylarsine and dimethylarsine on treatment with borohydride[17] respectively. Arsenobetaine and the arsenosugars have been found generally in marine animals and plants, respectively.

On the other hand, arsenocholine, tetramethylarsonium ion and some other methylated arsenic compounds were not quantitatively converted to the corresponding methylarsenic compounds on hot-base digestion.[15,16] These compounds were found in nature but not widely. The authors therefore assumed that most methylarsenic compounds present in the freshwater alga in this paper are measured by the hot-base digestion–hydride generation method.

The experimental results are summarized in Table 5. Preliminary experiments were carried out on the determination of methylated arsenic in the presence of a large excess of inorganic arsenic. Figure 2(a) shows an atomic absorption spectrophotometry trace of the fractional determination of methylated arsenic in a medium sample (0.5 cm^3) containing about 10 μg As(V) and excreted arsenic compounds of 10 ng TMA and 10 ng DMA. The absorption peaks of excreted DMA and TMA were detected on the shoulder of the IA peak. The retention times of both DMA and TMA coincided with those obtained for the individual determinations. Figures 2(b) and (c)

Table 6 Metabolism of methylated arsenic by *C. vulgaris* cultured for 7 days in the MD medium containing 10 mg methylated arsenic compounds per dm^3

Methylarsenic species *in MD*	Arsenic compounds accumulated in cell, μg^{-1} dry wt (% of total arsenic)				
	Total	IA	MMA	DMA	TMA
	50.9	0	24.0 (47.2%)	26.9 (52.8%)	tr
	60.0	0	0	60.0 (100%)	0
	58.1	0	0	tr	58.1 (100%)

show the traces obtained when TMA and MMA (10 ng each) were added to the same medium sample (0.5 cm^3), respectively. In Fig. 2(b), the TMA peak only increased and the increment corresponded to the quantity of TMA added. In Fig. 2(c), the third peak was detected and the retention time coincided with that obtained on a single determination of MMA.

It was found from preliminary experiments that methylated arsenic compounds were able to be determined in the presence of a large quantity (100-fold or more) of inorganic arsenic.

Table 5 shows that monomethyl- and dimethyl-arsenic compounds were produced in the algal cell in the initial stage of the growth and that both monomethyl- and dimethyl-arsenic concentrations fell with growth time. The production of trimethylarsenic compounds was found to follow a few days after those of monomethyl- and dimethyl-arsenic compounds and the concentration of trimethylarsenic in the cell become constant at the end of the exponential growth phase.

On the other hand, methylated arsenic compounds were also found in the medium; these were excreted by the alga during growth. The predominant methylated arsenic species in the medium was trimethyl arsenic. The concentration of excreted trimethylarsenic reached a peak on the tenth day and then decreased. This drop in trimethyl arsenic concentration in the medium suggested re-accumulation by *C. vulgaris*.

Metabolism of methylated arsenic by *C. vulgaris*

C. vulgaris which had been cultured in an arsenic-free MD medium was inoculated in an MD medium containing 10 mg As dm^{-3} of methylated arsenic compounds (MMA as methylarsonic acid, DMA as sodium cacodylate and TMA as arsenobetaine) at a cell concentration of about 0.12 g dry weight per dm^3 medium, cultured for seven days and harvested under standard conditions, and analyzed for methylated arsenics.

This experiment was performed to determine if methylated arsenic compounds were accumulated, whether they were further methylated and whether they were demethylated by *C. vulgaris*.

The experimental results are summarized in Table 6. Table 6 shows that methylarsonic acid, cacodylic acid and arsenobetaine were similarly taken up by *C. vulgaris*. About half of the monomethylarsenic compound was transformed to dimethylarsenic species in the algal cells, but no demethylation occurred. When the cells were exposed to cacodylate, only dimethylarsenic was

accumulated. This means that neither methylation nor demethylation occurred during dimethylarsenic accumulation. In a the case of arsenobetaine accumulation, no demethylation occurred.

These experimental results revealed that *C. vulgaris* took up not only inorganic arsenic compounds but also methylated arsenic compounds and that methylarsenic compounds taken up were further biomethylated but not demethylated. Dimethylarsenic species seemed to be the most stable arsenic forms in the algal cells.

REFERENCES

1. Foster, P L *Nature (London)*, 1977, 269: 322
2. Kaise, K *Bull. Hokkaido Hyg. Inst.*, 1983, 33: 21
3. Tagawa, S *Bull. Jap. Soc. Sci. Fish.*, 1980, 29: 563
4. Yamauchi, H and Yamamura, Y *Jap. J. Publ. Health*, 1980, 27: 647
5. Edmonds, J. S, Francesconi, K A and Hansen, J A *Experimentia*, 1982, 38: 643
6. Edmonds, J S and Francesconi K A *Nature (London)*, 1981, 289: 602
7. Edmonds, J S and Francesconi, K A *J. Chem. Soc. Perkin Trans. I*, 1983, 2375
8. Kaise, T, Watanabe, S and Ikeda, H *J. Fd. Hyg. Soc. Jap.*, 1980, 21: 58
9. Kaise, T, Watanabe, S, Itoh, K, Hanaoka, K, Tagawa, S, Hirayama, T and Fukui, S *Chemosphere*, 1987, 16: 91
10. Maeda, S, Kusadome, K, Arima, H, Ohki, A and Naka, K *Appl. Organomet. Chem.*, 1992, 6: 395
11. Maeda, S, Ohki, A, Tokuda, T and Ohmine, M *J. Appl. Organomet. Chem.*, 1990, 4: 251
12. Maeda, S, Wada, H, Kumeda, K, Onoue, M Ohki, A, Higashi, S and Takeshita, T *J. Appl. Organomet. Chem.*, 1987, 1: 465
13. Maeda, S, Nakashima, S, Takeshita, T and Higashi, S *Sep. Sci. Technol.*, 1985, 20: 153
14. Yamauchi, H and Yamamura, Y *Toxicol. Appl. Pharmacol.*, 1984, 74: 134
15. Kaise, T, Yamauchi, H, Hirayama, Y and Fukui, S *Appl. Organomet. Chem.*, 1988, 2: 339
16. Norin, H and Christakopoulos, A *Chemosphere*, 1982, 11: 287
17. Yamauchi, H and Yamamura, Y *Jap. J. Ind. Health*, 1979, 21: 47