APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2001; **15**: 291–298
DOI: 10.1002/aoc.144

Some characteristics of arsenate transport in a marine cyanobacterium, *Synechococcus* sp.†

Akira Takahashi*, Hidekazu Kawakami, Katsuyuki Iwakiri and Shigeki Matsuto
Tokai University Junior College (Shizuoka), Miyamae-cho 101, Shizuoka 420-8511, Japan

The mechanism of arsenate incorporation in the cells of a unicellular marine cyanobacterium, *Synechococcus* sp., was investigated. A lag period in arsenate incorporation, probably caused by an inhibitory effect of phosphate absorbed on the cell surface, was observed. Although arsenate incorporation into the cells of cyanobacterium was several times faster than that of phosphate, it was readily inhibited by addition of phosphate, also suggesting inhibition caused by phosphate. The Michaelis–Menten type of equation was employed for the simulation of incorporation, and a nonlinear fitting was applied for the estimation of the kinetic parameters in the equation. Analysis based on the assumption that arsenate is incorporated via the phosphate transport system in cells suggested that arsenate competes with phosphate for entry into cells and each species acts as a competitive inhibitor to each other. The affinity of arsenate with the transport system was much less than that of phosphate, but it was not low enough for elucidation of an almost complete inhibition of arsenate incorporation by phosphate, and another mechanism to block the approach of arsenate to the transport system was deduced to exist. The inhibitory effect of phosphate on arsenate incorporation seemed responsible for alleviation of the toxicity of arsenate in the cells.

Keywords: arsenate; phosphate; competitive inhibition; cyanobacteria

Received 9 December 1999; accepted 9 August 2000

* Correspondence to: A. Takahashi, Tokai University Junior College (Shizuoka), Miyamae-cho 101, Shizuoka 420-8511, Japan.
† Based on work presented at the Ninth Symposium of the Japanese Arsenic Scientists' Society (JASS-9), held 21–22 November 1999 at Hiroshima, Japan.

INTRODUCTION

It is generally believed that arsenicals in sea water are the source of arsenic compounds found in first producers and, consequently, those in marine animals. The bioaccumulation and metabolism of arsenic in marine first producers, including cyanobacteria, have been studied from the viewpoint of their ecological importance. Thermodynamic calculations on the equilibrium ratio of the concentrations of arsenate [arsenic(V)] to arsenite [arsenic(III)] in oxygenated sea water at pH 8.1 give a value of about 10^{26}.[1] Indeed, the predominant inorganic arsenic species in sea water is usually arsenate, showing concentrations of 1.0–2.0 ppb, but there also exists a significant amount of arsenite ranging over 10^{-1} to 10 times that of arsenate, far from the equilibrium value.[2] In addition, considerable amounts of monomethyl- and dimethyl-arsenicals have been found in sea water,[3] and it was thought that arsenate and arsenite incorporated into cells of first producers can be transformed to a variety of organic arsenic compounds.[4,5] An epochal finding of arseno-sugar in a brown kelp by Edmonds and Francesconi[6] was succeeded by many reports on other organic arsenic compounds in various organisms, both in sea water[7,8] and in fresh water.[9,10] Various kinds of organism were recognized to have abilities of arsenic accumulation and its conversion to reduced forms or organic compounds. But many problems still remain unclear. One of them is an incomplete comprehension of the detailed mechanism of arsenic incorporation by organisms. It has been generally accepted that arsenate incorporation by cells of first producers occurs readily via their phosphate transport systems located in the cell membrane, despite the lack of details in kinetics or mechanism of incorporation. Some phenomena, in which arsenate behaves as a phosphate analogue in some organisms because of the chemical similarity between them, were observed.[11–14] But relatively few papers on arsenate incorporation in cyanobacteria, exist, and there was some inconsistency

Table 1 Composition of culture media[a]

NH_4Cl*	0.5 g l^{-1}
$CaCl_4{\cdot}2H_2O$*	0.5 g l^{-1}
$MgSO_4{\cdot}7H_2O$*	0.37 g l^{-1}
Na_2–EDATA*	3 mg l^{-1}
$H_3BO_4^*$	34.26 mg l^{-1}
$MnCl_2{\cdot}4H_2O$*	4.32 mg l^{-1}
$ZnCl_2^*$	316 μg l^{-1}
$CuSO_4{\cdot}5H_2O$*	3 μg l^{-1}
$CoCl_2{\cdot}6H_2O$*	14 μg l^{-1}
Conc.HCl*	0.83 μl l^{-1}
$FeSO_4^*$	38.9 mg l^{-1}
Vitamin B_{12}^*	0.2 mg l^{-1}
$NaMo_4{\cdot}2H_2O$*	5 mg l^{-1}
NaCl*	18 g l^{-1}
$KH_2PO_4^{**}$	13.61 mg l^{-1}
$KH_2AsO_4^{***}$	18 mg l^{-1}

[a] Standard medium contains components marked with * and ** in table. Arsenate medium contains components marked with * and *** in table. Phosphate-free medium contains only components marked with * in table.

between their findings. For example, Thiel reported a noncompetitive inhibition of phosphate transport into the cells of fresh water cyanobacterium by arsenate.[15] On the other hand, Grillo and Gibson reported a highly specific transport system of phosphate in cells of another kind of fresh water cyanobacterium, in which neither phosphate entry nor cell growth was affected by a 100-fold excess of arsenate.[16] A comprehensive view for the mechanism of arsenate incorporation in cyanobacteria has not been obtained, and, for that matter, the comprehension of phosphate incorporation in cyanobacteria is at almost the same level. Very little is known about the physiology of phosphate incorporation in cyanobacteria, despite the obvious importance of its nutrition for their growth.[16–20] Previously we reported an alleviation effect from phosphate against arsenic toxicity on the physiology and growth of cells of the marine cyanobacterium, *Phormidium* sp.[21]

Cyanobacteria are very good materials for surveying their physiology because of their primitive cell structure, and the relationship between cyanobacteria and arsenate seemed worthy of investigation, because they, as well as algae, are one of the most common first producers and an entry of various elements and compounds into the food web. In addition, because of its extremely large biomass all over the world, the responsibility of cyanobacteria for arsenic circulation in the ocean is also relevant and deserves to be investigated, especially with respect to phosphate circulation.

In this study, we investigate arsenate incorporation and conversion to arsenite in the cells of a marine unicellular cyanobacterium, *Synechococcus* sp., cultured in artificial sea water containing arsenate instead of phosphate. The time course of the conversion of arsenate to arsenite was observed. The observations were compared with those for phosphate incorporation. The Michaelis–Menten-type model for incorporation of arsenate and phosphate was employed to analyze the time courses, and this suggested that arsenate competes with phosphate for the same transport system in the cell membrane and each species acts as a competitive inhibitor to each other. Some discussion will also be given on the toxicity of arsenate on cells and alleviation of it by phosphate.

MATERIALS AND METHODS

Organism and culturing conditions

The organism used in the study was a unicellular marine cyanobacterium, *Synechococcus* sp., isolated from the coastal water of Sagami Bay, Kanagawa, Japan. The stock culture was maintained axenically in the same medium as described before,[21] but the composition of the medium was sometimes subject to some modification as needed (Table 1). 'Standard medium' refers to a medium containing phosphate but no arsenate; 'arsenate medium' refers to a medium containing arsenate but not phosphate; 'phosphate-free medium' refers to a medium containing neither phosphate nor arsenate. The medium pH was first adjusted to around 8–8.5 and monitored all through the experiments, because pH change may affect the physiology of cells, chemical forms of polyvalent ions, and, consequently, the kinetics of the incorporation of arsenate or phosphate.

The cells collected from the preliminary cultures in the standard medium or in the phosphate-free medium were concentrated and washed with the phosphate-free medium by centrifugation three times before inoculation. The culturing vessel was a 3 l flask containing 1 l of medium of suitable composition for the purpose of the experiment. All procedures for inoculation were under sterilized conditions. The vessels were set under a 40 W white fluorescent lamp and the light intensity on the surface of the media was about 1700 lux. The temperature of the culture was kept at 26 ± 3 °C and the media were aerated by air passed through a

sterilized cotton plug and dilute sulfuric acid at a flow rate of about 2.0 $dm^3\,min^{-1}$ during the experiments. The growth of cells was determined as dry weight change of biomass in unit volume of culture medium, which was estimated based on a linear relationship between the dry weight and optical density of the culture at 620 nm. The correlation was almost 0.990.

Reagents and determination of PO_4 and arsenic(V)

All the chemicals used in the study were of reagent grade purchased from Waco Pure Chemical, Tokyo, Japan. Deionized water was used for all experimental procedures. Glassware was first cleaned with detergent, then soaked in dilute nitric acid for over 24 h and finally rinsed with water.

Incorporation of arsenate into cells was determined by two methods. One method was a direct determination of total arsenic in cells, and the other was an indirect determination by tracing the concentration changes of the species in media; phosphate incorporation was also determined by the indirect method.

For the direct estimation of arsenic accumulation in cells, the cells were first collected and rinsed three times with water by centrifugation; then the arsenic in the cells was extracted with 9 N HCl after measurement of the dry weight of cells and was determined using atomic absorption spectrophotometry.

A system composed of a high-performance liquid chromatography and an atomic absorption spectrophotometer, as reported by Matsuto *et al.*,[8] was used for detection of not only inorganic arsenic, but also the monomethylated, dimethylated and more complicated forms of arsenic compound in media; however, only conversion of arsenate to arsenite by cells was detected with this system. Thus, the more convenient method mentioned by Murphy and Riley[22] (with a small modification) was used for selective determination of arsenate, arsenite and phosphate in media, in which absorbances of arsenate– and phosphate–molybdenum blue complexes were measured. After filtration of an aliquot of culture on a GC-50 glass-fiber filter (Advantec Toyo), the filtrate was subjected to selective determinaton of the species. The filtrate was first divided into three portions. One of the portions was used for the direct determination of the sum of phosphate and arsenate concentrations in the aliquot; the second was for the selective determination of only phosphate by reducing arsenate to arsenite, which does not form the molybdenum blue complex; the third was for the determination of total concentrations of arsenate, arsenite and phosphate by oxidizing arsenite to arsenate. For reducing arsenate, a slight excess of sodium thiosulfate was added to the second part of the aliquot after adjusting the pH of the aliquot to 6.5–7 with diluted sulfuric acid; for oxidizing arsenite, a potassium permanganate solution (5 g/100 ml) was added to the third part until it showed a pale pink color of excess potassium permanganate. The molybdenum blue complexes of phosphate and arsenate formed in the filtrate were then extracted in *n*-amyl alcohol and the absorbance of the extract was measured at a wavelength of 742 nm, which is the isobestic point of both molybdenum blues in *n*-amyl alcohol.

Model analysis

Statistical methods for the estimation of kinetic parameters in model equations of, for example, the growth of microorganisms, and their incorporation and excretion of various compounds have been widely used in many fields.[15,23] In this study, cell growth was simulated with polynominals with respect to time, and the incorporation kinetics of PO_4 and arsenic(V) was simulated with the Michaelis–Menten-type equation. The incorporation rate V_X of individual species X (PO_4 or arsenic(V)) is given by:

$$V_X = -\frac{1}{W}\frac{d[X]}{dt} = -\frac{V_{max}[X]}{K_X + [X]} \qquad [1]$$

Here, W (g-dry weight.l^{-1}) is the dry weight of cells in a unit volume of medium expressed with a polynominal, [X] (μmol l^{-1}) is the concentration of X, V_X and V_{max} (units of both variables are μmol h^{-1} (g-dry weight)$^{-1}$) are the incorporation rate of species X and its maximum rate respectively. K_X (μmol l^{-1}) is a parameter, the inverse of which indicates an affinity between X and the transport system of X embedded within the cell membrane matrix. The best fitted estimations of V_{max} and K_X were obtained by repetition of step-wise re-tuning of both parameters according to non-linear fitting of the result of numerical integration of Eqn [1] to observed values in the time course of X concentration change, using the Marquardt algorithm[24] according to Yamaoka and Nakagawa.[25]

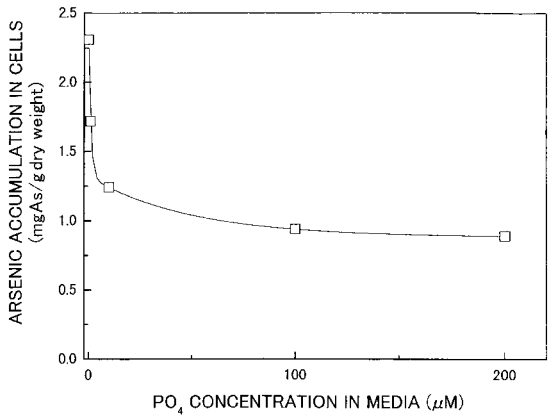

Figure 1 Effect of phosphate concentration on arsenic accumulation in cells.

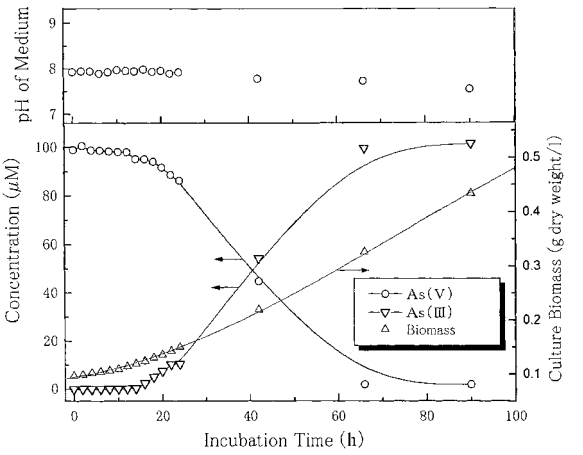

Figure 2 Conversion of arsenate to arsenite in a culture of *Synechococcus* sp. The conversion shows a lag period of almost 16 h in the early stage of incubation.

RESULTS

Incorporation of arsenate and phosphate

Cells (g-dry weight.l^{-1}) were cultured for 12 days in media containing 1.3 mM arsenate together with various concentrations of phosphate and collected for estimation of arsenic accumulation in cells (Fig. 1). Even the slightest concentration of phosphate (1 or 10 μM) was sufficient to reduce arsenic accumulation, and this suggested an inhibitory effect of arsenic accumulation in cells by phosphate.

The concentration change of arsenate (Fig. 2) and that of phosphate (Fig. 3) in media were individually traced to monitor the incorporation of the species into cells. The growth rate of cells in arsenate medium (Fig. 2) was rather faster than that in standard medium (Fig. 3) and there was no apparent difference in color of either culture. The pH values in both media were so stable that they could not cause disturbance in incorporation and growth. Arsenate incorporation in the arsenate media was always faster than that of phosphate in the standard media, as can be seen in Figs 2 and 3. However, incorporation during the first 10–20 h was nearly zero, and then an extremely rapid incorporation started after that period. Such a lag in starting incorporation was often seen in arsenate media, but the lag period was indefinite. As arsenate concentration went down, that of arsenite increased, showing a rapid conversion of arsenic(V) to arsenic(III) in cells of *Synechococcus* sp.

After preincubation in phosphate-free medium for 1 week, however, a rapid incorporation of arsenate and its conversion to arsenite with no lag was seen (Fig. 4); there was also a negative growth, although slight, probably caused by the toxic effect of arsenate. In the experiment, perturbation by pH change was also ignorable. The parameters in Eqn [1] were estimated on the arsenate incorporation

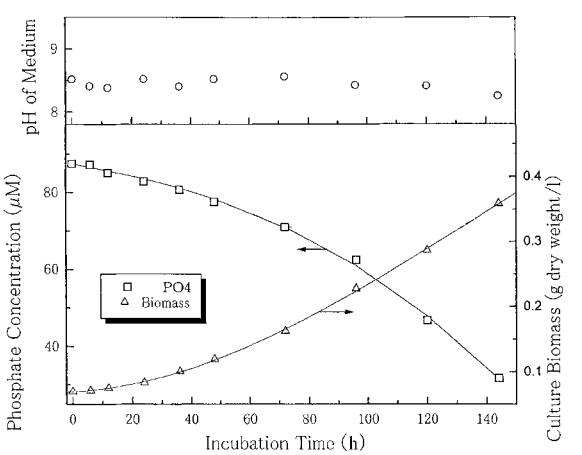

Figure 3 Incorporation of phosphate in cells of *Synechococcus* sp. The fitted curve based on Eqn [1] was added to the time course of phosphate uptake in the figure.

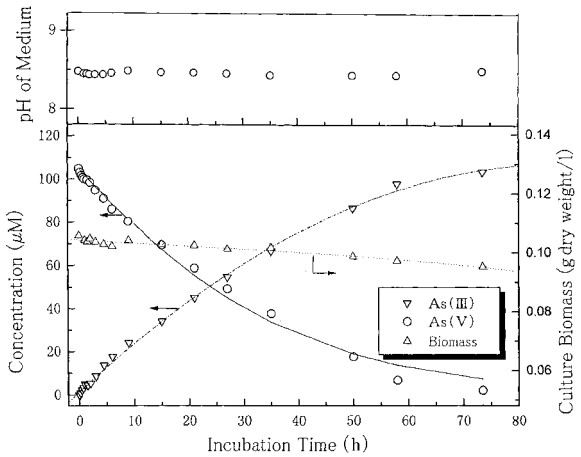

Figure 4 Conversion of arsenate to arsenite in a culture of *Synechococcus* sp. preincubated in phosphate-free medium. After preincubation of cells in phosphate-free medium for 1 week, there was no lag in the conversion. The fitted curve based on Eqn [1] was added to the time course of arsenate incorporation in the figure.

using the data in Fig. 4 and on phosphate using the data in Fig. 3, and are shown in Table 2.

Competition between arsenate and phosphate for entry into cells

Phosphate was added to a culture in the arsenate medium 18 h after inoculation to make its concentration about 40 μM (Fig. 5). As soon as the phosphate had been added, the incorporation of arsenic(V) and excretion of arsenic(III) stopped and only phosphate incorporation was seen thereafter for almost 40 h. After exhaustion of the phosphate, conversion of arsenic(V) to arsenic(III) started again. In the culture, cell growth was not inhibited at all. The pH change in the medium was small.

DISCUSSION

Cells, cultured for 12 days in media containing 1.3 mM of arsenate, incorporated and accumulated arsenic, but there was an inhibitory effect of phosphate on aresenic accumulation (Fig. 1), suggesting competition between both species for entry into cells.

Individual incorporation of arsenate and phosphate (Figs 2–4) and their competition (Fig. 5) were investigated by tracing the concentration changes of the species. Arsenate incorporation was very slow during the early stage of the culturing period, but it became extremely rapid after that stage (Fig. 2), and finally became faster than that of phosphate (compare with Fig. 3). The lag period in the early stage was indefinite, ranging from 5 to 20 h, and the cause of the indefiniteness was not clear. The medium pH was rather stable all through the experiment and seemed to have no effect on incorporation. The cell growth in the arsenate medium was almost the same as that in the standard medium (Fig. 3), and thus arsenate apparently seemed not to be toxic to cells. Grillo and Gibson reported a highly specific transport system of phosphate in the cells of a fresh water cyanobacterium, in which neither phosphate entry nor cell growth was affected by a 100-fold excess of arsenate,[16] and this was similar to our result reported previously, in which we reported an alleviation effect by phosphate against arsenic toxicity on the physiology and growth of cells of the marine cyanobacterium *Phormidium* sp.[21] These observations suggested an inhibitory effect

Table 2 Estimation of K_m, V_{max} and incorporation rates of phosphate and arsenate

			Estimated rates of incorporation[a] (μmol h^{-1} (g-dry weight)$^{-1}$)	
	V_{max} (μmol h^{-1} g^{-1})	K_m (μmol l^{-1})	Individual incorporation	Competitive incorporation
Phosphate	2.22	1.81	2.14	2.12
Arsenate	64.6	143	16.7	0.78

[a] Estimated based on [As] = 50 μmol l^{-1} and [P] = 50 μmol l^{-1}.

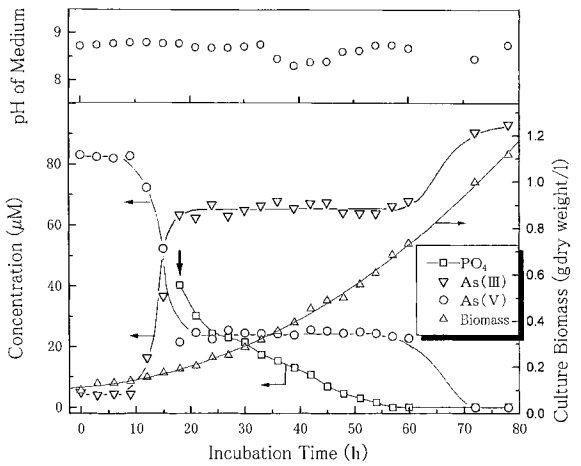

Figure 5 Effect of phosphate addition on conversion of arsenate to arsenite in a culture of *Synechococcus* sp. Almost 40 μM of phosphate was added to the culture in arsenate medium 18 h after the inoculation of cells. The addition is marked with ↓ in the figure.

of phosphate on arsenate incorporation in cells of cyanobacteria.

On the other hand, preincubation of cells in a phosphate-free medium for 1 week removed such a lag (Fig. 4) and even a slight negative growth was seen, probably caused by the toxic effect of arsenate. According to those observations, it was most likely, as pointed out by Falkner *et al.*,[17] that the amount of phosphate bound to the surface of cells was such that it inhibited arsenate incorporation; thus, it could be a cause of such a lag in arsenate incorporation, as seen in Fig. 2, and consequently be a cause of alleviation of growth inhibition by arsenate. Although, unfortunately, we had no means for estimation of phosphate adsorbed on the cell surface, the experimental results shown in Fig. 5 readily verified the inhibitory effect of phosphate on arsenate incorporation. Phosphate added 18 h later to the culture in the arsenate medium immediately stopped the conversion of arsenic(V) to arsenic(III), and the conversion resumed after the exhaustion of phosphate in the medium. In addition, there was no inhibitory effect of arsenic(V) on cell growth (Fig. 5). Probably, phosphate added later was preferentially incorporated in cells, and, as a consequense, inhibited the arsenate incorporation and alleviated the toxic effect of arsenate.

The observation in Fig. 5, however, was apparently inconsistent with those in Figs 2 or 4 and Fig. 3, i.e. the incorporation rate of arsenate alone was faster than that of phosphate, but it was completely reversed in media containing both species: arsenate incorporation was readily inhibited by phosphate (Fig. 5). We have employed the model shown in Eqn [1] to simulate the kinetics of individual incorporation of phosphate and arsenate; now, taking those observations into account, we use another assumption in which arsenate and phosphate in the same medium compete for a common transport system in the cell membrane; consequently, each species acts as a competitive inhibitor to the other. We propose the following set of equations for the kinetics of incorporation of both species with their mutual inhibiton:

$$V_{\mathrm{P}}^{\mathrm{comp}} = -\frac{1}{W}\frac{\mathrm{d[P]}}{\mathrm{d}t} = -\frac{K_{\mathrm{As}}V_{\mathrm{max}}^{\mathrm{P}}[\mathrm{P}]}{K_{\mathrm{P}}K_{\mathrm{As}} + K_{\mathrm{As}}[\mathrm{P}] + K_{\mathrm{P}}[\mathrm{As}]} \quad [2]$$

$$V_{\mathrm{As}}^{\mathrm{comp}} = -\frac{1}{W}\frac{\mathrm{d[As]}}{\mathrm{d}t} = -\frac{K_{\mathrm{P}}V_{\mathrm{max}}^{\mathrm{As}}[\mathrm{As}]}{K_{\mathrm{P}}K_{\mathrm{As}} + K_{\mathrm{As}}[\mathrm{P}] + K_{\mathrm{P}}[\mathrm{As}]} \quad [3]$$

which are same as the equations for enzyme reaction with a competitive inhibitor. Under competition between the very similar concentrations of both species ([As], [P]), we observe

$$V_{\mathrm{P}}^{\mathrm{comp}} > V_{\mathrm{As}}^{\mathrm{comp}}$$

as seen in Fig. 5. After some simple operations on Eqns [2] and [3] considering [As] ≒ [P], we have

$$\frac{V_{\mathrm{max}}^{\mathrm{As}}}{V_{\mathrm{max}}^{\mathrm{P}}} < \frac{K_{\mathrm{As}}}{K_{\mathrm{P}}} \quad [4]$$

Furthermore, the individual rate of incorporation of each species follows the relation

$$V_{\mathrm{P}}^{\mathrm{ind}} < V_{\mathrm{As}}^{\mathrm{ind}}$$

as seen in Figs 2 or 4 and Fig. 3. From Eqn [1] with [As] ≒ [P], we have:

$$1 < \frac{V_{\mathrm{max}}^{\mathrm{As}}}{V_{\mathrm{max}}^{\mathrm{P}}} \quad [5]$$

Then the apparent lack of coherence between results in Figs 2 and 3 and Figs 4 and 5 becomes consistent under the following condition:

$$1 < \frac{V_{\mathrm{max}}^{\mathrm{As}}}{V_{\mathrm{max}}^{\mathrm{P}}} < \frac{K_{\mathrm{As}}}{K_{\mathrm{P}}} \quad [6]$$

The estimation of parameters in Eqn [1] for both

species (Table 2) satisfied Eqn [6]. The estimated rate of individual incorporation (Eqn [1]) of each arsenate and phosphate, together with those of the competitive incorporation of both species (Eqns [2] and [3]), are also shown in Table 2. As the inverse of K_X is a measure of the affinity between the transport system in cells and species X, and V_{max} is that of the dissociation tendency of a complex between X and the transport system, then Eqn [6], together with the estimation in Table 2, seems a reasonable explanation for the apparent inconsistency mentioned above. Larger values of K_m and V_{max} of arsenate than those of phosphate mean that arsenate is harder to combine with the transport system and easier to dissociate from the system into cytoplasm than phosphate, and this might be the cause of the result by Grillo and Gibson, which excluded the possibility of error in the recognition of arsenate with phosphate.[16]

Thus, the estimation of parameters in Table 2 met the criterion in Eqn [6], but it was not good enough to explain the decisive inhibition of arsenate incorporation by phosphate, such as that seen in Fig. 5. According to the estimation (Table 2), phosphate incorporation in competition with arsenate is only several times faster than that of arsenate, and it is apparently not large enough to stop the arsenate incorporation, as seen in Fig. 5. There probably exists another mechanism that makes arsenate difficult to approach to the transport system in the cell membrane. For example, absorption of phosphate on the cell surface, as reported by Falkner *et al.*,[17] may make the phosphate concentration near the cell surface higher than that remote from cells; if so, it could make it more difficult for arsenate to approach the transport system. In addition, there may exist two or more successive transport systems, such as described by Eqns [1]–[6], on the pathway of phosphate entry into cells. As reviewed by Silver and Walderhaug,[26] the pathway for phosphate movement from the external environment into the cytoplasm of *Escherchia coli* consists of three steps. If such a route also exists in cyanobacteria, and the mechanism expressed by Eqns [2] and [3] works at each step, the inhibition of arsenate incorporation by phosphate could be so intense that a strict inhibition of arsenate incorporation, such as seen in Fig. 5, would be possible. Details have to be clarified after this.

The growth inhibition, such as that seen in Fig. 4, cannot be attributed only to the arsenate, because we could not eliminate the effect of shortage of phosphate on growth. However, since the conversion was not observed in media without cells (data not shown), it was clear that cells were the cause of the conversion, and then, at least partially for that reason, arsenate could be toxic to the cell physiology of cyanobacteria. Though the mechanism of conversion in cells was not clarified, it was most likely that the conversion might be dependent on photosynthesis, directly or indirectly. It was supposed that the overwhelming predominance of phosphate for entry into cells was the cause of the alleviation effect of the arsenate toxicity.

SUPPLEMENT

Interestingly, the cells preincubated in a phosphate-free medium for over 4 weeks showed an extremely rapid incorporation of phosphate (data not shown). The K_m value observed on such phosphate-starved cells seemed rather small compared with that in Table 2, suggesting an occurence of another type of phosphate transportation in cells for compensation of phosphate shortage. In such cells, the rate of individual incorporation of arsenate also became faster, but it was not as fast as that of phosphate, i.e. a completely opposite phenomenon to the case of phosphate replete cells was observed. It was suggested that a new phosphate transporter induced in phosphate starved cells may be specific for phosphate.

REFERENCES

1. Sillen LG. In *Oceanography*. American Association for the Advancement of Science: Washington, 1961; 574.
2. Tsunogai S, Sase T. *Deep Sea Res.* 1969; **16**: 489.
3. Hasegawa H. *Appl. Organomet. Chem.* 1996; **10**: 733.
4. Yamaoka Y, Takimura O, Fuse H, Murakami K. *Appl. Organomet. Chem.* 1999; **13**: 489.
5. Cullen WR, Harisson LG, Li H, Hewitt G. *Appl. Organomet. Chem.* 1983; **8**: 313.
6. Edmonds JS, Francesconi KA. *Nature* 1981; **289**: 602.
7. Shiomi K, Shinagawa A, Azumi M, Yamanaka H, Kikuchi T. *Comp. Biochem. Physiol. C* 1983; **74**: 393.
8. Matsuto S, Stockton RA, Irgolic J. *Sci. Total Environ.* 1986; **48**: 133.
9. Maeda S, Kusakabe K, Arima H, Oki A, Naka K. *Appl. Organomet. Chem.* 1992; **8**: 303.
10. Cullen WR, Li H, Hewitt G, Reimer J, Zalunard N. *Appl. Organomet. Chem.* 1994; **8**: 303.
11. Meharg AA, Macnair MR. *J. Exp. Bot.* 1992; **43**: 519.
12. Gresser MJ. *J. Biol. Chem.* 1987; **256**: 7954.

13. Kenney LJ, Kaplan JH. *J. Biol. Chem.* 1988; **263**: 7954.
14. Slooten L, Nuyten A. *Biochim. Biophysic. Acta* 1983; **725**: 49.
15. Thiel T. *J. Bacteriol.* 1987; **170**: 1143.
16. Grillo J, Gibson J. *J. Bacteriol.* 1988; **140**: 508.
17. Falkner G, Horner WF, Simonis W. *Planta (Berlin)* 1980; **149**: 138.
18. Rigby C, Craig SR, Budd K. *J. Phycol.* 1980; **16**: 389.
19. Budd K, Kerson GW. *Can. J. Bot.* 1987; **65**: 1901.
20. Wagner F, Falkner R, Falkner G. *Planta (Heidelberg)* 1995; **197**: 147.
21. Takahashi A, Kawakami H, Bada A, Okonogi Y, Matsuto S. *Appl. Organomet. Chem.* 1990; **4**: 269.
22. Murphy J, Riley JP. *Anal. Chim. Acta* 1962; **27**: 31.
23. Furihata T, Suzuki M, Sakurai H. *Plant Cell Physiol.* 1992; **33**: 1151.
24. Marquardt DW. *J. Soc. Ind. Appl. Math.* 1963; **11**: 431.
25. Yamaoka K, Nakagawa T. *J. Pharm. Dyn.* 1983; **6**: 596.
26. Silver S, Walderhaug M. *Microbiol. Rev.* 1992; **56**: 195.

