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Effects of phosphate on arsenate inhibition in a marine cyanobacterium, *Phormidium* sp.

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The effect of arsenate on cells of a marine cyanobacterium, *Phormidium* sp. preliminarily starved for phosphate for a week was studied. Cells were harvested and cultured in artificial seawater containing various concentrations of arsenate and phosphate. Arsenate at concentrations above 30 mg As dm^{-3} inhibited biosynthesis in the cells and consequently, growth when incubated without phosphate in the medium. On the contrary, phosphate at 50 μmol dm^{-3} was sufficient for apparently complete cancellation of the inhibitory. effects of arsenate at concentrations up to 150 mg As dm^{-3}. Study of the carbohydrate metabolism revealed an intense inhibition by arsenate on turnover of carbohydrate to other cell components in the phosphate-depleted cells. This resulted in a color change of the cells from blue–green to yellowish. The synthesis of carbohydrate itself was also inhibited by arsenate. Arsenate incorporation into cells was clearly inhibited by phosphate in the medium, suggesting that arsenate competes with phosphate for entry into cells. In addition, arsenate incorporated in cells could not inhibit the incorporation of phosphate and subsequent growth of cells on phosphate. These observations indicate that arsenate can act as a poisonous substitute for phosphate in the cells but, once incorporated into the phosphate-replete cells, it no longer has an inhibitory effect. The inhibitory effects of arsenate seem to be mainly related to ATP synthesis in the photosynthetic system.

Keywords: arsenate, phosphate starvation, growth, cyanobacterium, *Phormidium* sp., seawater

INTRODUCTION

The fate of arsenic in the natural environment has been a problem of interest for many years because of its severe toxicity. It has been well established that arsenic has a wide range of distribution, in both the aquatic and terrestrial environments.[1] A large number of arsenic compounds, both organic and inorganic, have been found in living tissues of marine organisms, in rather high concentration in some cases.[2–6] These observations suggest an important participation of marine biota in arsenic circulation in marine environments. Among those, bioaccumulation and metabolism of arsenic in primary producers (i.e. algae, phytoplankton and other microorganisms) seem important, because they are the first steps in the entry of arsenic into the food chain, which may lead the element, often in high concentrations, to incorporation by human beings. Cyanobacteria, a group of common primary producers, have been studied in their relation to the element[2,3,7–10] and, in such studies, some attention has been paid to the possibility of confusion between phosphate and arsenate (which may come from the chemical analogy between the elements), in the incorporation or metabolism of phosphate in algae or phytoplankton.[2,3,7,9,10] In fact, there are some enzyme-reactions in which arsenate behaves as a poisonous substitute for phosphate.[11,12] However, there is no unique interpretation with respect to such a confusion of the elements in phytoplankton. It should be attributed mainly to the variety of strains used, but also at least partially, to the differing experimental conditions used. There are many factors fixing the characteristics of aqueous solutions, for instance composition or concentrations of solutes, pH, total salinity, redox potential, temperature, partial pressures or composition of gases and possibly others, and they are often responsible not only for physiological conditions in organisms but for the chemical forms of polyvalent anions such as carbonate, phosphate or arsenate. The most likely chemical forms of CO_2[13] and PO_4[14] (which is dependent mainly on

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pH), for incorporation into cyanobacterial cells, has been well discussed. pH also affects the incorporation of different forms of arsenate into phytoplankton. Furthermore, change in salinity by addition or elimination of arsenate and phosphate should be important, especially for a survey using the strains in fresh water.

We previously reported an extremely high accumulation of arsenic in the cells of the marine cyanobacterium, *Phormidium* sp., which showed a tolerance to very high concentrations of inorganic arsenic.[15] In this paper, we focus our attention on the competition between arsenate and phosphate for entry into cells or into metabolic pathways in cells of cyanobacteria. *Phormidium* sp., with the characteristics mentioned above, was suitable for the purpose, and an intense inhibition of carbohydrate metabolism in phosphate-depleted cells by arsenate was revealed.

MATERIALS AND METHODS

Organism and culture conditions

The organism used in this study was one of the marine cyanobacteria, *Phormidium* sp., collected and isolated from the coastal waters of Suruga Bay, Shizuoka, Japan. The basic composition of the growth medium was as follows: NaCl, 18.0 g dm^{-3}; Na_2-EDTA, 30 mg dm^{-3}; $FeSO_4 \cdot 7H_2O$, 3.89 mg dm^{-3}; $MgSO_4 \cdot 7H_2O$, 5.0 g dm^{-3}; KCl, 0.6 g dm^{-3}; $CaCl_2 \cdot 2H_2O$, 0.37 g dm^{-3}; KH_2PO_4, 13.6 mg dm^{-3}; H_3BO_3, 34.26 mg dm^{-1}; $CuSO_4 \cdot 5H_2O$, 3 μg dm^{-3}; $CoCl_2 \cdot 6H_2O$, 14 μg dm^{-3}; $MnCl_2 \cdot 4H_2O$, 4.32 mg dm^{-3}; $ZnCl_2$, 0.316 mg dm^{-3}; Na_2MoO_4, 5 mg dm^{-3}; NH_4Cl, 0.5 g dm^{-3}. This is almost the same as the medium described by Kumazawa and Mitsui[16] but with a minor modification. The pH of the growth medium was adjusted to 8.6 by adding NaOH and by aeration of the medium. An adequate assortment of concentrations of phosphate and arsenate in media (chemical forms were KH_2PO_4 and KH_2AsO_4, respectively) was chosen as the occasion demanded. Such a variability in their composition can affect the basic characteristics of media such as pH, salinity, etc. Thus the pH in the culture media was monitored all through the experiment.

The light source was 40 W white fluorescent lamp and the light intensity on the surface of the media was about 2600 lx. The temperature of the media was kept at 26 ± 3°C. The cultures were aerated at a flow rate of about 660 cm^3 min^{-1} during the course of study.

The growth was determined as a change in dry weight which was measured based on a linear relationship between the dry weight and optical density at 620 nm of the culture. The correlation was over 0.998 throughout the experimental period.

Analysis and reagents

Phormidium sp., preliminarily starved of phosphate for at least one week, was collected, then washed twice with phosphate-free medium by centrifugation, and inoculated in the media of composition described above. Culturing vessels were three litre flasks set under white fluorescent

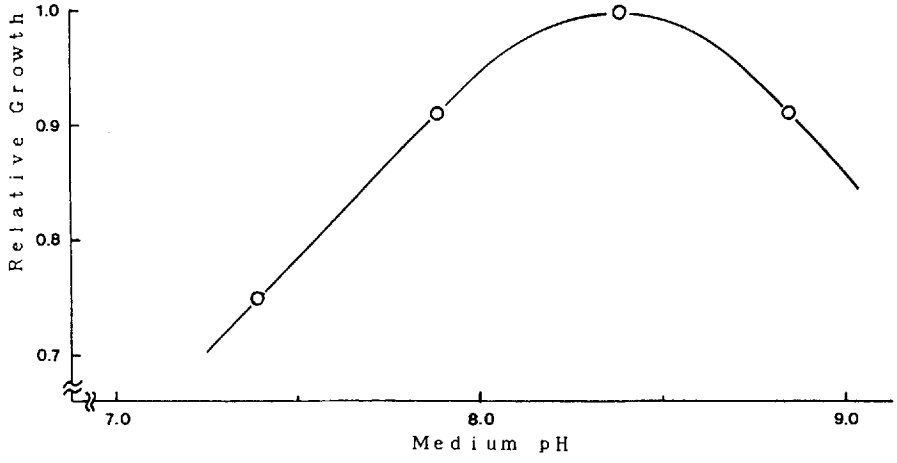

Figure 1 Growth dependence of *Phormidium* sp. on medium pH.

lamps. During growth, an aliquot of each culture was collected occasionally, its optical density (OD) measured at 620 nm and medium pH. The sample was then filtered on a GC50 glass-fiber filter (Advantec Toyo). The extraction of the inorganic arsenicals from the filtrate with toluene (according to Yasui *et al.*[17]) was succeeded by phosphate determination (according to Murphy and Riley[18]), in which phosphate was extracted as molybdenium blue with n-amyl alcohol and the optical density at 675 nm of the alcohol solution was used for phosphate determination. The inorganic arsenicals extracted were measured with flameless atomic absorption spectrophotometry. The analytical conditions and apparatus used for arsenicals were the same as those described before.[15] Carbohydrate content in a unit volume of the culture was also monitored with the method described by Dubois *et al.*[19] Reagents used in this study were all of analytical grade.

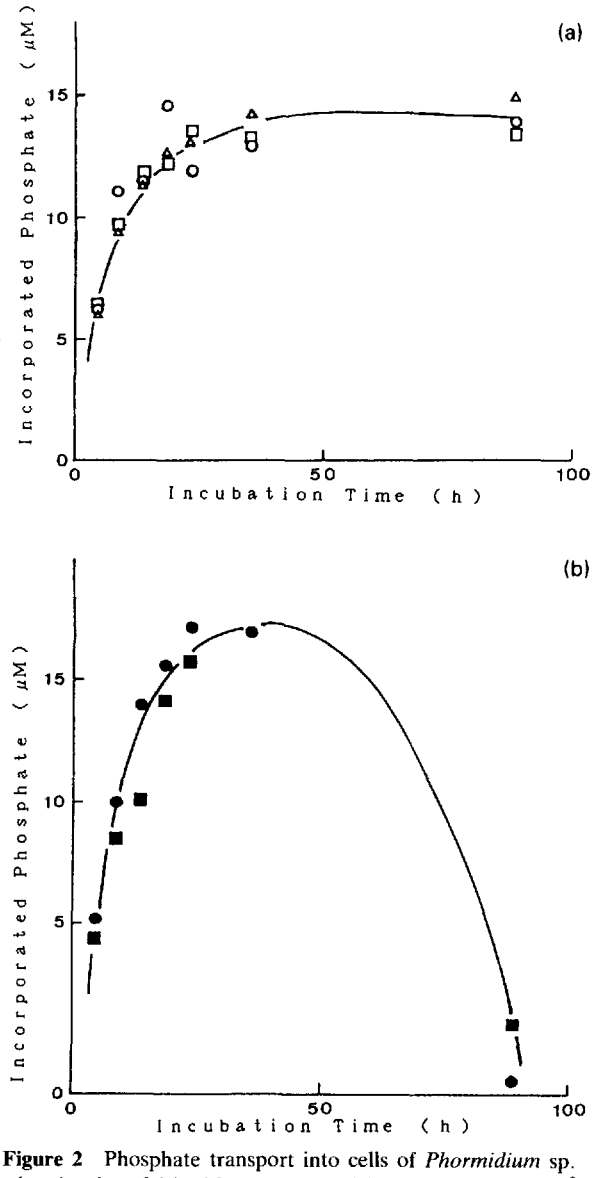

Figure 2 Phosphate transport into cells of *Phormidium* sp. when incubated (a) without arsenate (b) with 100 mg As dm^{-3} arsenate. Phophate concentrations: △, 30 μmol dm^{-3}; □, ●, 50 μmol dm^{-3}; ○, ■, 100 μmol dm^{-3}. The amount of phosphate incorporated was calculated based on the change in phosphate concentration in the medium.

RESULTS

pH and salinity of medium

Because of a strong buffer action of phosphate, the variation in medium composition could cause scattering in pH values, salinities and dissociation of salts in the media, which could possibly affect the growth or physiological characteristics of the strain. Such scattering may affect the experimental results. To avoid this, we needed to trace the pH changes in all the media throughout the experiment. The pH dependence of growth rate of the strain is shown in Fig. 1, showing that the optimum pH for the growth is around 8.4, but pH 8.6 was chosen in this study, because of a better stability of pH around 8.6. Thus, the scattering in pH values in media of different compositions was kept within 0.28 (data not shown) and thought to be sufficiently small to avoid confusion (see Fig. 1). The scattering in the salinity originated from the variety of composition was within 1.7% of total salinity; this was also thought to be sufficiently small.

Phosphate transport in phosphate-depleted cells and the effect of arsenate

Cells starved of phosphate for at least one week showed rapid incorporation of phosphate immediately after transfer to new media containing various concentrations of phosphate and arsenate, but the incorporation showed saturation within 40 h [Fig. 2(a) and (b)]. There was no effect due to phosphate concentrations, which were employed in the experiment on phosphate transport into cells, suggesting that 30 μmol dm^{-3} of phosphate was sufficient for the recovery of phosphate depletion in cells and the rate of incor-

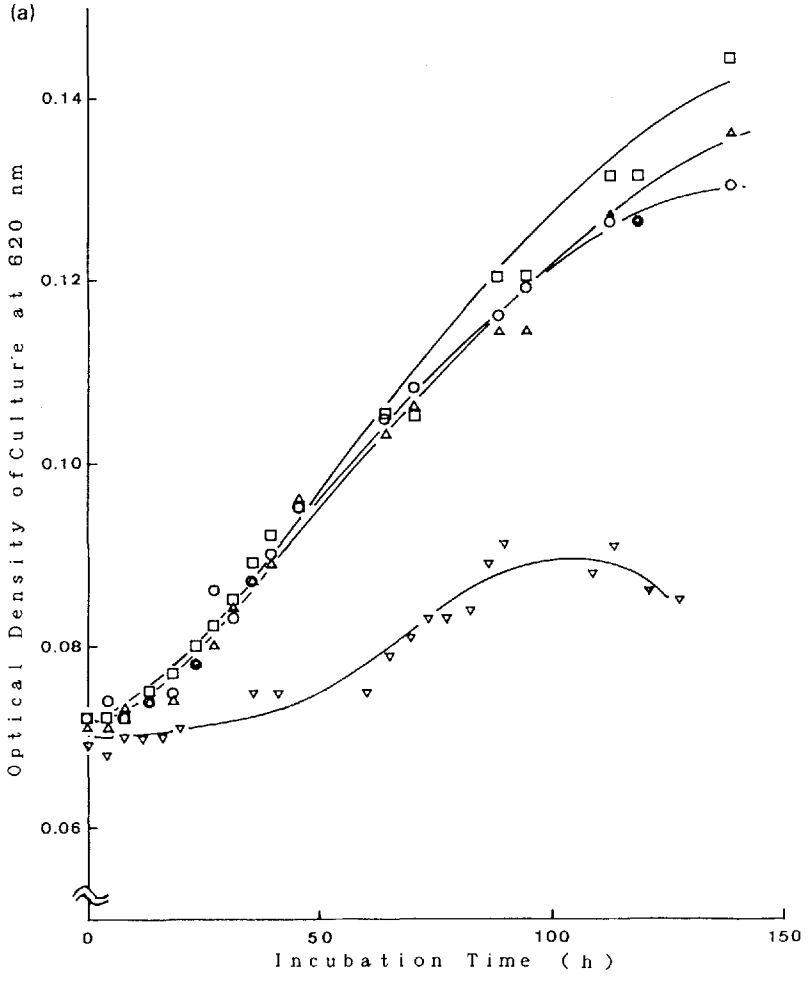

Figure 3 Growth curves of *Phormidium* sp. at various concentrations of phosphate when incubated (a) without arsenate and (b) with 100 mg As dm^{-3} arsenate. Phosphate concentrations: ▽, 0 μmol dm^{-3}; ○, 30 μmol dm^{-3}; □, 100 μmol dm^{-3}; △, 200 μmol dm^{-3}.

poration was already saturated at the concentration of phosphate. When incubated with arsenate and phosphate, cells showed a release of phosphate (once incorporated in growing cells) despite the cells still growing [see Fig. 3(a), (b), and Fig. 4(a), (b)], showing the inhibitory effect of arsenate on phosphate incorporation.

Growth of phosphate-depleted cells under various concentrations of phosphate and arsenate

Figure 3 shows the growth of cells when incubated in media containing various concentrations of phosphate. In the arsenate-free media [Fig. 3(a)], phosphate concentrations at 30 μmol dm^{-3} or more did not show any difference in their effect on growth, but the cells showed insufficient growth in the phosphate-free medium. In the media containing 100 mg As dm^{-3} of arsenate [Fig. 3(b)] on the other hand, growth inhibition was seen. Phosphate at concentrations 100 μmol dm^{-3} or more seemed to alleviate the inhibitory effect but 30 μmol dm^{-3} was insufficient for the alleviation. Arsenate at 100 mg As dm^{-3} sometimes gave zero or even a negative growth of cells when phosphate-free. However, saturation or breakdown in growth appeared earlier when cells were incubated with 100 mg As dm^{-3} arsenate than when they were arsenate-free [compare Fig. 3(a) and Fig. 3(b)].

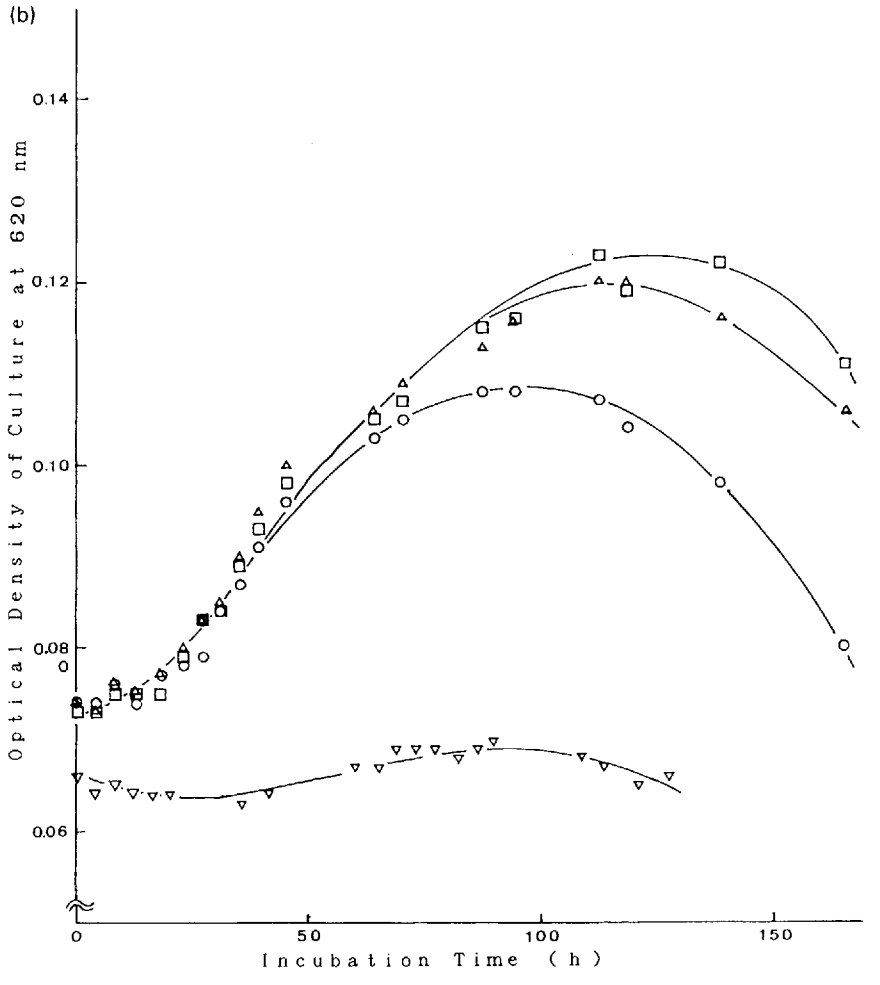

Figure 3b

Figure 4 shows the growth of cells when incubated in media containing various concentrations of arsenate. When incubated in phosphate-free media, 30 mg As dm^{-3} of arsenate was sufficient for growth inhibition [Fig. 4(a)]. There seemed to be a concentration effect of arsenate for growth inhibition, though it was small. The growth inhibition at 150 mg As dm^{-3} of arsenate seemed slightly stronger than that at 30 mg As dm^{-3}. In the media containing 100 μmol dm^{-3} phosphate [Fig. 4(b)], cells incubated with arsenate at concentrations up to 150 mg As dm^{-3} apparently showed good growth but an earlier saturation again appeared, suggesting arsenate inhibition and its alleviation to some extent by phosphate.

Effects of arsenate and phosphate on accumulation of carbohydrate and other cell components

In Fig. 5, changes in content of carbohydrate and other cell components in unit volume of culture are shown. A slight increase in carbohydrate was seen only when cells were incubated in a medium containing no phosphate and arsenate, but the increase was suppressed by addition of phosphate above 30 μmol dm^{-3} or arsenate above 30 mg As dm^{-3}. On the other hand, other cell components (which means the subtraction of carbohydrate from the dry weight in unit volume of culture) showed almost no change in media containing lower concentrations of arsenate

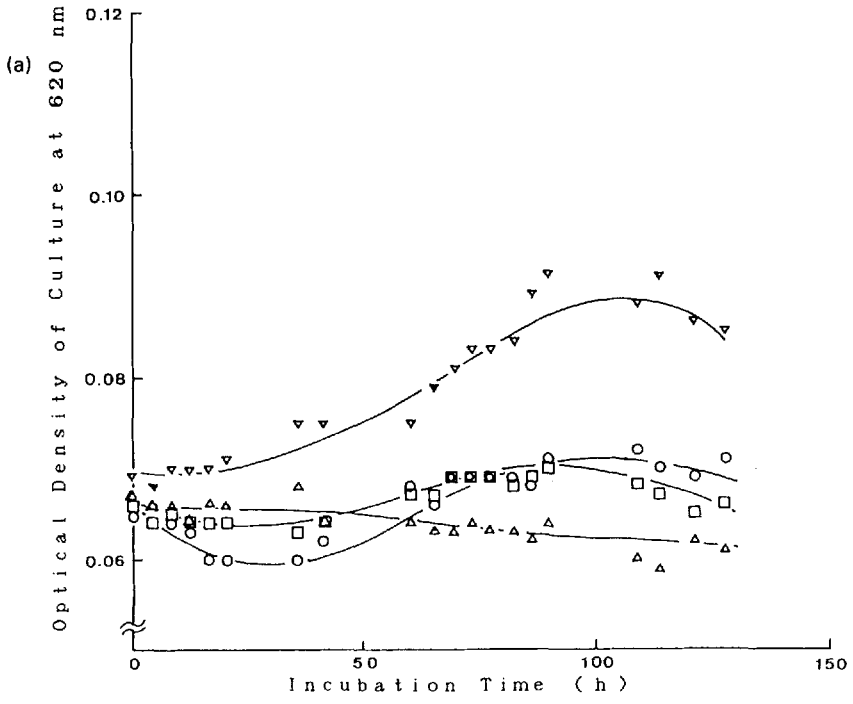

Figure 4 Growth curves of *Phormidium* sp. at various concentrations of arsenate when incubated (a) without phosphate and (b) with 100 μmol dm^{-3} phosphate. Arsenate concentrations: ▽, 0 mg dm^{-3}; ○, 30 mg dm^{-3}; □, 100 mg dm^{-3}; △, 150 mg dm^{-3}.

(50 mg As dm^{-3} or less), or decreased gradually in media containing higher concentrations of arsenate (100 or 150 mg As dm^{-3}) when incubated without phosphate. Addition of phosphate at a concentration above 30 μmol dm^{-3} gave an increase in the other components regardless of arsenate concentration, but a saturation in increase or a decrease appeared earlier when incubated in media containing higher concentrations of arsenate than in the media containing no or lower concentration. These results are summarized in Fig. 5 as typical cases.

Effect of phosphate on arsenate incorporation

The change in arsenate concentration in media was too small to be detected in any experiment. The cells incubated in media containing 100 mg As dm^{-3} arsenate and various concentrations of phosphate were collected after the experiment (10 days later) and arsenic accumulation was determined. After sufficient rinsing with distilled water with centrifuging, the collected cells were dried, pulverized and preweighed, and then immersed in 9 N HCl for the extraction of arsenicals (according to Yasui *et al.*[17]) for 1 day. The arsenic in the extract was determined by atomic absorption spectrophotometry. The accumulated amount of arsenic in cells was plotted against the phosphate concentration in media (Fig. 6). Inhibition of arsenic incorporation by phosphate was seen but 400 μmol dm^{-3} of phosphate was not enough for complete elimination of arsenate entry.

Alleviation of inhibitory effect of incorporated arsenate by phosphate

The cells preliminarily incubated in media containing 100 mg As dm^{-3} of arsenate but no phosphate for 162 h were collected, rinsed with fresh medium containing no phosphate and arsenate, and then transferred to a medium containing 100 μmol dm^{-3} of phosphate but no arsenate (Fig. 7). The cells showed a favorable growth after the transfer.

DISCUSSION

Some workers have published excellent studies on arsenic accumulation or tolerance in microalgae

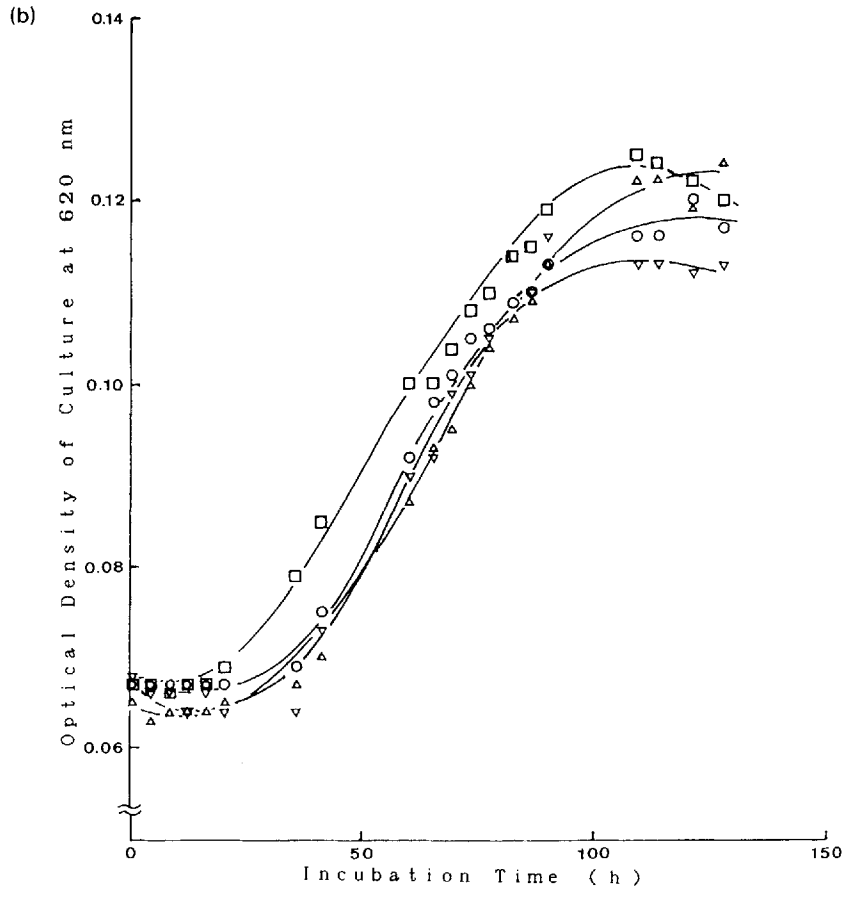

Figure 4b

(for both marine and fresh water), focusing their interest mainly on the environmental or biological problems of arsenic compounds.[2,3,7–10] In such studies, the chemical analogy between arsenate and phosphate has been one of the most difficult problems to be solved.[2,3,7,9,10] As is well known, arsenate or phosphate are polyvalent anions that act as strong buffers in solutions. Chemical characteristics of solutions containing such anions are very complicated and this makes research in organisms complicated. In solutions containing many components (like seawater), such species are in quasi-equilibrium under given chemical and physical conditions. A deviation in concentrations of components from equilibrium causes shifts in the parameters characterizing the equilibrium (such as pH, electrochemical potential, dissociation of salts, etc.), towards a new equilibrium state, and this should affect the incorporation of polyvalent anions like carbonate, phosphate or arsenate in microalgae. Therefore, in this study, we paid much attention to make the characteristices of media uniquely defined. As already mentioned (Results), the maximum deviation in total salinity was within 1.7% and that in pH was within 0.28, and both were thought to be sufficiently small for the purpose of the study.

Some workers report a competition between phosphate and arsenate for entry into cells or into metabolic pathways of some microalgae,[2,3,7,10] but others do not.[9] For example, Thiel reported a noncompetitive inhibition of phosphate transport into cells of *Anabaena variabilis* by arsenate,[10] but Budd and Craig reported that phosphate transport in *Synechococcus leopoliensis* is highly phosphate-specific and not inhibited by arsenate.[9] In *Phormidium* sp. in our laboratory, a competition between phosphate and arsenate, which is apparently different from that reported by Thiel in *Anabaena*, was seen. The incorporation rate of

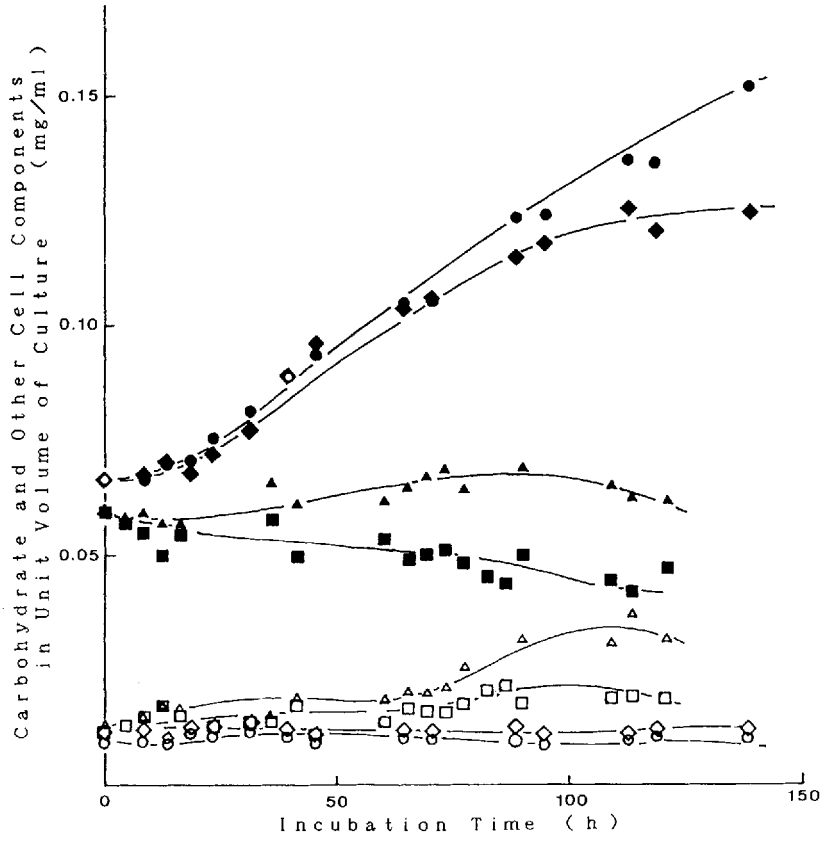

Figure 5 Effects of arsenate and phosphate on production of carbohydrate and other components in cells. Open symbols (△, ○ etc.) and closed ones (▲, ● etc.) indicate the amounts of carbohydrate and other cell components in a unit volume of culture, respectively. Concentrations of arsenate and phosphate are as follows: △, ▲, 0 mg As dm^{-3}, phosphate 0 μmol dm^{-3}; □, ■, 150 mg As dm^{-3}, phosphate 0 μmol dm^{-3}; ◇, ◆, 100 mg As dm^{-3}, phosphate 100 μmol dm^{-3}; ○, ●, 0 mg As dm^{-3}, phosphate 100 μmol dm^{-3}.

phosphate in phosphate-depleted cells was high immediately after the transfer into new media but rapidly decreased with time when incubated in media without arsenate, similarly to *Anabaena* as reported by Thiel [Fig. 2(a)]. The phosphate concentration in media did not affect the incorporation rate of phosphate regardless of the arsenate concentration, suggesting that the rate was completely saturated at 30 μmol dm^{-3} of phosphate. However, a release of phosphate, once incorporated into cells, was seen only when cells were incubated with arsenate [Fig. 2(b)]. On the other hand, arsenate incorporation in cells was clearly inhibited by phosphate, showing that transport mechanism is rather specific to phosphate (Fig. 6), but 400 μmol dm^{-3} of phosphate was not enough for complete elimination of arsenate entry in cells. These observations suggest that there are two types of competition between arsenate and phosphate. The competition for entry into cells favors phosphate; the competition for metabolism within the cells could be responsible for the release of phosphate once incorporated in cells.

The competition had some effect on the growth of cells as shown in Figs 3 and 4. When arsenate-free, phosphate at concentrations above 30 μmol dm^{-3} gave almost the same growth, again suggesting that phosphate demand was completely satisfied at these concentrations [Fig. 3(a)]. In Fig. 3(a), growth was also seen in a phosphate-free medium, though it was insufficient. This should be attributed to nutrition in the renewed medium and phosphate slightly carried over within cells. When incubated with 100 mg As dm^{-3} arsenate, on the contrary, growth was inhibited at phosphate concentrations of 50 μmol dm^{-3} or less, but not at 100 μmol dm^{-3}

or more. This suggests phosphate above 50 μmol dm^{-3} can overcome the inhibition by arsenate incorporated within cells. However, growth in 100 mg As dm^{-3} arsenate seemed to reach saturation more rapidly than that in arsenate-free media. These observations are consistent with the results from Figs 2 and 6. Growth inhibition by arsenate was more clearly seen in Fig. 4(a). Arsenate at concentrations of 30 mg As dm^{-3} or more completely inhibited growth when phosphate-free, and even a negative growth was seen at higher concentrations (100 or 150 mg As dm^{-3}) of arsenate. It was shown that inhibition such as in Fig. 4(a) can be at least partially alleviated by the addition of 100 μmol dm^{-2} phosphate [Fig. 4(b)].

An inhibitory effect of arsenate incorporated in cells was seen in the carbohydrate accumulation metabolism and its turnover to other cell components (Fig. 5). When incubated in media containing no phosphate and arsenate, carbohydrate content in cells showed a slight increase, but other components of cells remained unchanged, showing that the turnover of carbohydrate demands phosphate. This was confirmed by the unchanged carbohydrate content and the increase in other components of the cells when incubated with phosphate but no arsenate. Thus the insufficient growth seen in Fig. 3(a) can be attributed only to the increase in carbohydrate. In such a case, a small amount of phosphate carried over in the cells could be utilized exclusively for photosynthesis and subsequent synthesis of carbohydrate but it was not enough for the turnover of carbohydrate to other components. On the contrary, when incubated only with arsenate, the content of other components remained unchanged at lower concentrations of arsenate, or decreased gradually at higher concentrations, though the carbohydrate content showed no or very low increase at any arsenate concentration. This shows that arsenate incorporated in cells inhibits not only carbohydrate synthesis but its turnover to other cell components. Phosphate at 100 μmol dm^{-3} showed an alleviation of arsenate inhibition of carbohydrate and other syntheses to some extent, but the content of other components saturated and began to decrease more rapidly when incubated with arsenate than without it (Fig. 5).

The cells preliminarily incubated in a medium

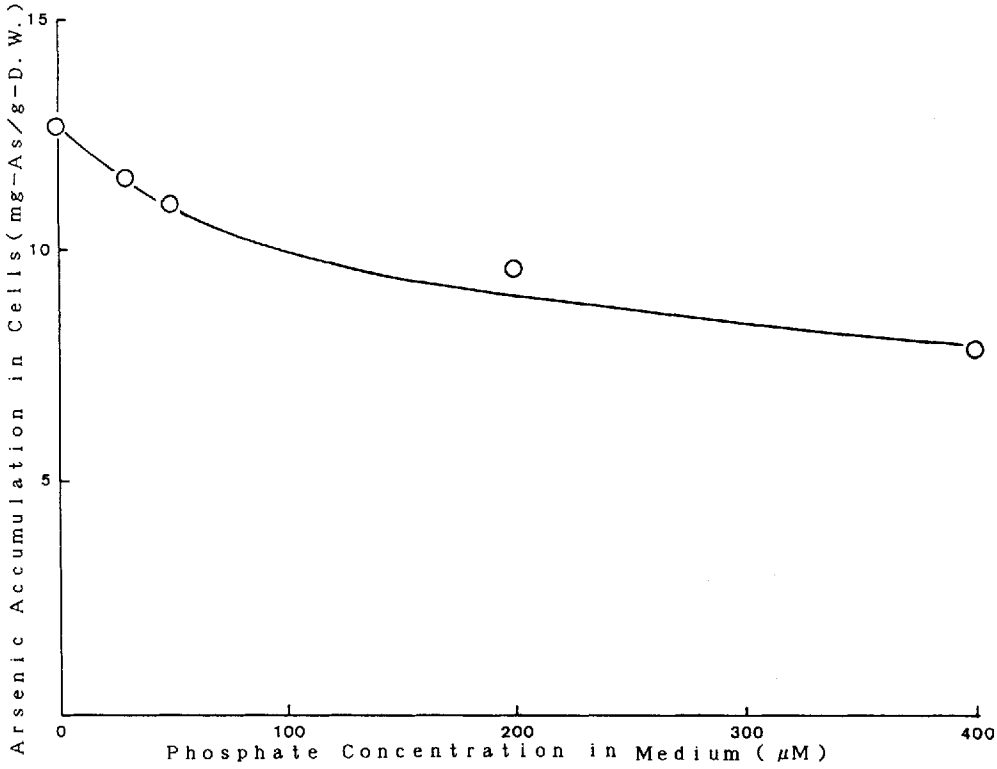

Figure 6 Effect of phosphate on arsenate accumulation in cells. Arsenate accumulation in cells incubated with 100 mg As dm^{-3} arsenate for 10 days was plotted against the phosphate concentration in the media.

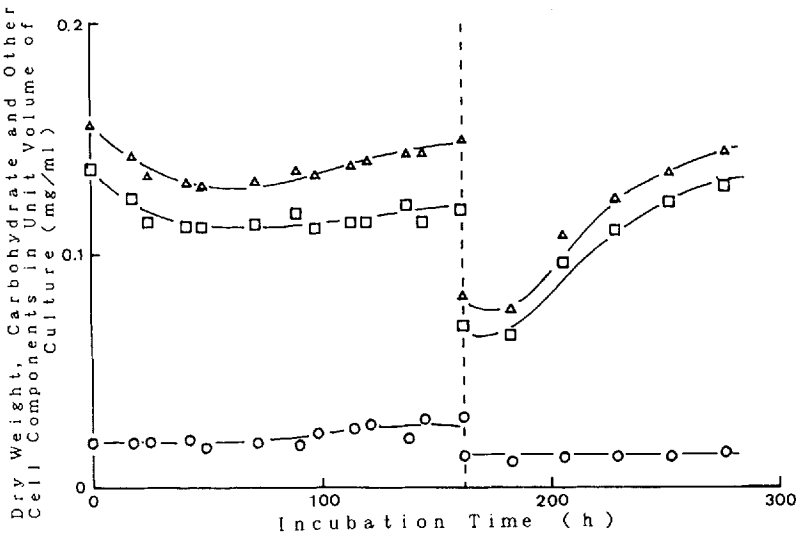

Figure 7 Alleviation of inhibitory effect of arsenate by phosphate. Cells preliminarily incubated for 162 h in a medium containing 100 mg As dm^{-3} arsenate but no phosphate were transferred to a new medium containing 100 μmol dm^{-3} of phosphate. △, Dry weight of cells in a unit volume of culture; ○, carbohydrate in cells in a unit volume of culture; □, other cell components in cells in a unit volume of culture.

containing 100 mg As dm^{-3} arsenate but no phosphate for 162 h were transferred into a new medium with 100 μmol dm^{-3} phosphate but no arsenate to see whether arsenate within cells inhibits the phosphate transport or biosyntheses. The cells showed favorable growth after transfer, suggesting that arsenate incorporated in cells cannot completely inhibit either phosphate transport into cells or the entry of phosphate into metabolic pathways. This is contrary to the case for *Synechococcus* reported by Thiel.[10] Competition between anions in the cells should be the same as that for cells incubated in media containing both anions.

CONCLUSION

Arsenate inhibition of metabolism in *Phormidium* sp. was clear only when incubated without phosphate, and 30 mg As dm^{-3} of arsenate was sufficient for growth inhibition. When incubated with 100 μmol dm^{-3} phosphate, on the contrary, the inhibition of growth, carbohydrate synthesis and turnover of carbohydrate to other cell components was alleviated to some extent, but phosphate at 30 μmol dm^{-3} or less was not enough for the alleviation. Arsenate incorporation into cells was inhibited by phosphate but even 400 μmol dm^{-3} of phosphate was not enough for the complete elimination of the entry of arsenate. On the contrary, arsenate in media showed no direct inhibition of phosphate transport in cells, but arsenate incorporated in cells seemed to force the cells to release phosphate. And, in addition, incorporated arsenate showed inhibition of carbohydrate synthesis and of turnover of carbohydrate to other cell components. In phosphate-depleted cells, phosphate seemed to be utilized exclusively for photosynthesis and subsequent carbohydrate synthesis, suggesting that arsenate inhibition is mainly of phosphorylation, in which arsenate should be a poisonous substitute for phosphate. Inhibition of phosphorylation should result in a lack of ATP and consequently other substances, including phosphate, nucleic acids, etc. Nucleic acids are indispensable to cell division and shortage of nucleic acids can be an important reason for growth inhibition.

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