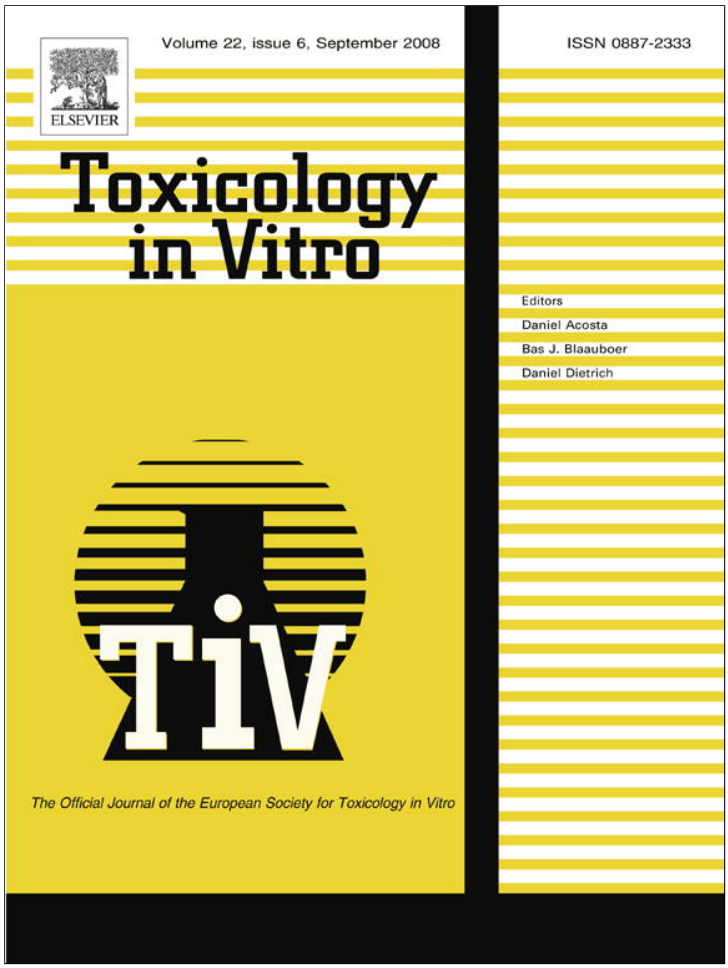

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Arsenic (V) induces a fluidization of algal cell and liposome membranes

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ABSTRACT

Arsenate is one of the most poisonous elements for living cells. When cells are exposed to arsenate, their life activities are immediately affected by various biochemical reactions, such as the binding of arsenic to membranes and the substitution of arsenic for phosphate or the choline head of phospholipids in the biological membranes. The effects of arsenate on the life activities of algae *Chlorella vulgaris* were investigated at various concentrations and exposure times. The results demonstrated that the living activities of algal cells (10^{10} cells/L) were seriously affected by arsenate at a concentration of more than 7.5 mg As/L within 24 h. Algal cells and the artificial membranes (liposomes) were exposed to arsenate to evaluate its effects on the membrane fluidization. In the presence of arsenate, the membranes were fluidized due to the binding and substitution of arsenate groups for phosphates or the choline head on the their membrane surface. This fluidization of the biological membranes was considered to enhance the transport of toxicants across the membrane of algal cells.

1. Introduction

Arsenic is one of the most significant global environmental toxicants. Humans can be exposed to arsenic through the intake of air, food, and water. Trace amounts of arsenic in drinking water can endanger human health, mainly because arsenic compounds in drinking water occur in inorganic forms which are toxic (Abernathy et al., 2003). Arsenic in natural waters mainly as inorganic arsenite and arsenate. Organic arsenicals also occur in natural, but their toxicities are much lower than inorganic arsenic (Chen et al., 1994; Li et al., 1995).

In living cells, arsenic exists in the states of +5, +3, 0, and −3. It can form alloys with metals and covalent bonds with carbon, hydrogen, oxygen, and sulfur (Ferguson and Gavis, 1972). Because its biochemical properties are similar to those of phosphate, arsenate can replace phosphate in energy transfer phosphorylation reactions, resulting in the formation of adenosine diphosphate (ADP)-arsenate instead of adenosine triphosphate (ATP) (Gresser, 1981). However, the arsenate concentration required for the formation of ADP-arsenate is reported to be as high as 0.8 mM (Moore et al., 1983). Arsenic is well known for its ability to induce the production of superoxide (Barchowsky et al., 1999; Lynn et al., 2000). If excess superoxide is produced in the pancreatic cells, an impairment of insulin secretion is expected (Tseng, 2004). With respect to biological membranes, there are some reports indicating that arsenic compounds affect the structure and functions of cell membranes, especially those of human erythocytes (Zhang et al., 2000; Winski et al., 1997; Winski and Carter, 1998).

The final purpose of the current study was to examine the effects of low concentrations of arsenate at different exposure times on the life activities of algal cells and on liposome, the "artificial" membrane system discovered more than 40 years ago by Bangham et al. (1965) that has become a versatile tool in biology, biochemistry and medicine. The cell membrane is a diffusion barrier which protects the cell interior. Therefore, the change of its structure and functions results in the disturbance inside or outside membrane. In the present study, to more deeply understand the molecular mechanisms of the interaction between arsenic and biomembrane, we exposed the cell membranes of algae and artificial membranes (liposomes) to arsenate. By using the fluorescence microscope, spectrofluorometer and the reverse-phase high performance liquid chromatography, as well as the atomic absorption spectrometry analyzing bound arsenic on the membrane, we initially determine the interaction between arsenate and biological membranes. The results showed that arsenate changed the characteristics of the membrane by substituting phosphate or the choline head of

Abbreviations: As, arsenic; As (V), arsenate; As (III), arsenite; POPC, 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine; Calcein, 3,3-bis [*N*,*N*-bis(carboxymethyl)-aminomethyl] fluorescein.

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phospholipid molecules on the biomembrane. Fluidization of the membrane or increase of the membrane fluidity, permitting the toxicants to move into and/or be released from the cells, was also investigated under arsenic toxic stress. Based on the results, the toxic effect of arsenate on the artificial and cell membrane was finally discussed.

2. Materials and methods

2.1. Materials

Arsenate solution with a concentration of 60% was purchased from Wako Pure Chemicals (Osaka, Japan). $FeCl_3$ from Sigma–Aldrich was used for the comparative experiments to estimate the toxic strength of arsenic (V) and iron (III). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and was used for liposome preparation. Calcein 3,3-bis [*N*,*N*-bis(carboxymethyl)-aminomethyl] fluorescein, purity 98%) was obtained from Wako (Osaka, Japan).

Algal cells of *C. vulgaris*, purchased from Wako Pure Chemicals, were used after a purification procedure in which the cells were centrifuged at 800g for 5 min and the supernatant was removed. The other reagents were of analytical grade.

2.2. Liposome preparation

POPC was used to produce liposomes. In brief, the phospholipid was dissolved in chloroform, the solvent was evaporated under vacuum, and the resulting dry thin film was dried for 2 h under vacuum at room temperature. The lipid film was hydrated by dispersion in 50 mM phosphate buffer (pH 7.3) to form multilamellar vesicles. The multilamellar vesicle suspension was frozen in dry ice-ethanol (–80 °C) and was dispersed above the phase transition temperature for five cycles. The resulting suspension was passed through two stacked polycarbonate filters of 100 nm pore size by using an extrusion device.

For successful separation of all membrane lipids, algal cells were ground to fine powder in the presence of liquid nitrogen. While cells were still frozen, methanol–chloroform–water (2:1:0.8, v/v) (Bligh and Dyer, 1959) was added in a ratio of one part tissue to three parts solvent. The extraction was continued until the sample completely lost its color. After filtration, the cleared extract was transferred into a separation funnel. A 5 ml volume of chloroform and water was added for complete separation of the two phases. The chloroformic fraction was collected and evaporated to dryness using a vacuum evaporator at 40 °C. The dry residue was then dissolved in 0.2–0.5 ml chloroform. The above lipids were analyzed to determine the membrane-bound arsenic.

2.3. Toxic effect of arsenate on algal cell membranes

Pure cells were incubated with different arsenate doses to evaluate the effects of arsenic toxicity on living cells. Algal cells of *C. vulgaris* with a density of 10^{10} cells/L were incubated in Proteos medium, a modified form of Bristol medium (Nichols, 1973), with various concentrations of arsenate (H_3AsO_4) under neon lights with an intensity of 3000 lux at 30 °C.

The cells were incubated for 24 h, and the effects of the arsenate concentration on the living cells were investigated by measuring fluorescence intensity with a synchronous scan as an index of the number of living cells (Liu et al., 2005). At the same time, the optical density at 650 nm of media solution was measured in combination with counting the number of cells in a Quadrate chamber.

The time-dependence of the toxicity of arsenate on cell viability was then examined. Algal cells were incubated with different concentrations of arsenate for 12, 24, and 48 h, and cell viability was determined by measuring the fluorescence intensity.

2.4. Effect of arsenate on model cell membranes (liposomes)

In order to clarify the mechanism of the cellular toxicity of arsenic, the structure of the compound formed between arsenic and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) liposome was examined. POPC (5 mM) in 50 mM phosphate buffer was incubated at 30 °C for 12 or 48 h with the following molar ratios of arsenate to POPC: 1/300, 1/200, and 1/100. The pH value was kept at 7.3. POPC without arsenate was also incubated as a control. The change in the binding structure between POPC and arsenic according to the incubation time was investigated by running reverse-phase high performance liquid chromatography (RP-HPLC) to detect arsenic bound to POPC liposomes in the incubated solutions.

2.5. Fluidization of membranes of POPC liposomes and algal cells under arsenic toxicity

Fluidization increases the permeability of membranes, permitting the toxicants to move into and/or be released from the cells. In this study, we investigated changes in permeabilization by arsenate dosage in both algal cells and POPC liposomes using two parallel experiments.

Cell membrane permeability under a toxic condition induced by arsenate was examined by means of the accumulation of calcein, a fluorescent probe, in algal cells of *C. vulgaris*. Algal cells were incubated in Proteos medium with MES buffer containing 7.5 mg As/L of arsenate and without arsenate as a control. Calcein (0.05 mM) was added to the medium as a fluorescent probe. The concentration of calcein in the media solution, which was analyzed periodically during the incubation, was calculated to estimate the transport of calcein into the cells.

A series of experiments was conducted to examine the fluidization of POPC liposomes that had been treated with arsenate. Calcein was entrapped in POPC liposomes, which had a diameter of 100 nm prior to incubation. Arsenate (50 μM as a final concentration) was added to liposome solution (5 mM of POPC) at 30 °C. The change in the concentration of calcein, which was released from the liposomes by fluidization of the membrane structure into the solution, was measured according to exposure time. Triton X-100 at a concentration of 1% was added to the solution to release all the calcein entrapped in liposome membranes (Felix et al., 2002).

2.6. Analytical methods

A Shimadzu reverse-phase HPLC (RP-HPLC) system equipped with an FCV-10AL pump and DGU-$20A_3$ degasser was used, as were a SPD-10A UV–vis detector and LC-10AD liquid chromatograph. Elution profiles were monitored at 254 nm on the UV detector. The mobile phase of acetonitrile/water (65/35 v/v) with a flow rate of 1 mL/min was applied at 30 °C. An Inertsil ODS-SP column (0.46 cm × 2.5 cm) was used throughout this study. POPC-bound arsenic lipids were detected by an RP-HPLC.

Free arsenic and bound arsenic was detected by hydride generation atomic absorption spectroscopy (HG-AAS). A Varian SpectrAA 220 Atomic Absorption instrument (with software SpectrAA 220 ver. 10) connected with Varian Vapor Generation Accessory, VGA-77, was used. An heated oven containing a Varian quartz hydride absorption cell was connected to the hydride system and placed in the optical path of the instrument. With HG-AAS, the minimum concentration of arsenic for the possible detection is 1 ppb.

In the exposure experiments, the concentration of calcein in the supernatant was determined after centrifugation at 2200g by a fluorescent spectrophotometer (JASCO FP 6500; Jasco, Tokyo, Japan) at wavelengths of 495 nm and 515 nm for excitation and emission, respectively.

2.7. Statistical Analysis

Results are expressed as the mean ± standard deviation (SD). All experiments were performed at least in triplicate. The data distribution was analyzed, and statistical differences were evaluated using the Student *t*-test. A *P* value of <0.05 was considered to be significant.

3. Results and discussion

3.1. Toxic effect of arsenate on algal cell membranes

The toxic effect of arsenate on the algal cells was first investigated. Fig. 1a shows the color change of the algal cell suspension induced by 24 h of incubation of algal cells with arsenate. Fig. 1b indicates the spectra of fluorescence intensity after 24 h of incubation with various concentrations of arsenate. Increases in the arsenate concentration resulted in decreases in fluorescence intensity, which implied the death of algal cells. Arsenate attached to the cell membrane to produce reactive oxidative species, which easily oxidize lipids and membrane proteins and which could result in cell destruction (Delnomdedieu et al., 1995). Although there are many previous reports on the binding of metal ions on algae, few efforts have been made to characterize the complex of metal ions and algae. The photodegradation of estrogens and photoproduction of hydroxyl radicals in algae solutions with (or without) Fe (III) were observed in previous work (Liu et al., 2003, 2004). Therefore, experiments were conducted with arsenic (V) and iron (III) to compare their toxicities (Fig. 1c). The above results with arsenate and iron (as a control experiment) indicated that arsenate was more toxic than iron under the same experimental conditions. By destructing the cell membrane (images obtained from the fluorescence microscope), arsenate induced cell death, which appears after only a short incubation time and increases drastically in experimental period (24 h).

Fig. 2 shows the cell viability after 24 h of incubation with various concentrations of arsenate. The viability of cells decreased sharply as the arsenate concentrations increased. Under a fluorescence microscope, cell aggregation, in which the cells were still alive, was observed at arsenate concentrations between 30 mg As/L and 45 mg As/L, while a cell disruption process, indicating cell death, was observed at arsenate concentrations of 60 and 75 mg As/L.

According to our experimental results, even a low concentration of arsenate adversely affected the viability of algal cells. A 75 mg As/L of arsenate induced cell destruction and 45 mg As/L of arsenate induced cell aggregation after 24 h of incubation. This acute toxicity can be explained by membrane fluidization, which enhanced the permeability of the membrane, leading to high accumulation of arsenate in the cells.

The viability of algae was also examined by incubating algal cells with different concentrations of arsenate for a time-dependence of exposure. The results shown in Fig. 2 indicate that the viability of algae was affected not only by the arsenate concentration but also by the incubation time under toxic conditions. An experiment was conducted to determine the site on which arsenate was adsorbed. After incubation of alga with arsenic, the lipid component of the algal membrane was extracted and was analyzed by an atomic absorption spectrometry device to determine the percentage of the total arsenic that had accumulated on the membrane. Fig. 2b shows that the algal membrane adsorbed an increasing amount of arsenic along with the increasing arsenic concentration in the culture solution. The membrane is the main place where adsorption reactions of nutrients occur both passively

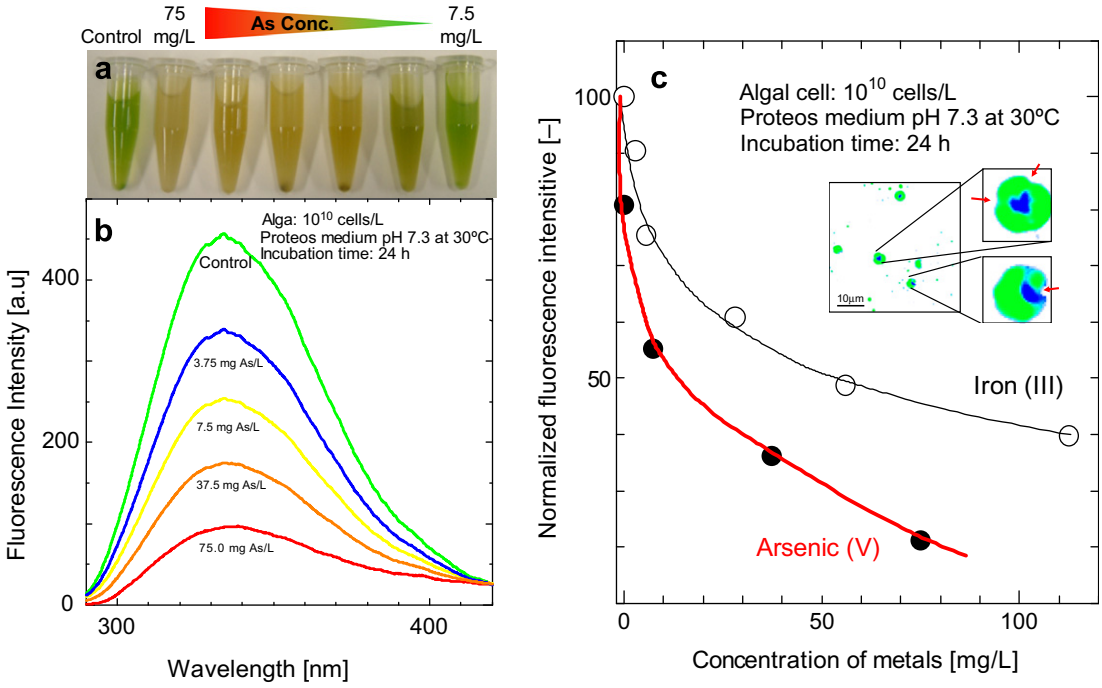

Fig. 1. Effects of arsenic (V) on the synchronous-scan fluorescence intensity of 10^{10} cells/L *C. vulgaris* solution with As (V). Control (without As) and with 3.75, 7.5, 37.5, and 75.0 mg As/L of arsenic (V) and with Fe (III) as a comparison. Algal cells were incubated with arsenic at 30 °C for 24 h. (a) The color change of algal solution at different concentrations of arsenic. (b) The fluorescent intensity of algal solution. (c) The effect of toxicants on algal culture and disruption of cell membrane under toxic stress of arsenate.

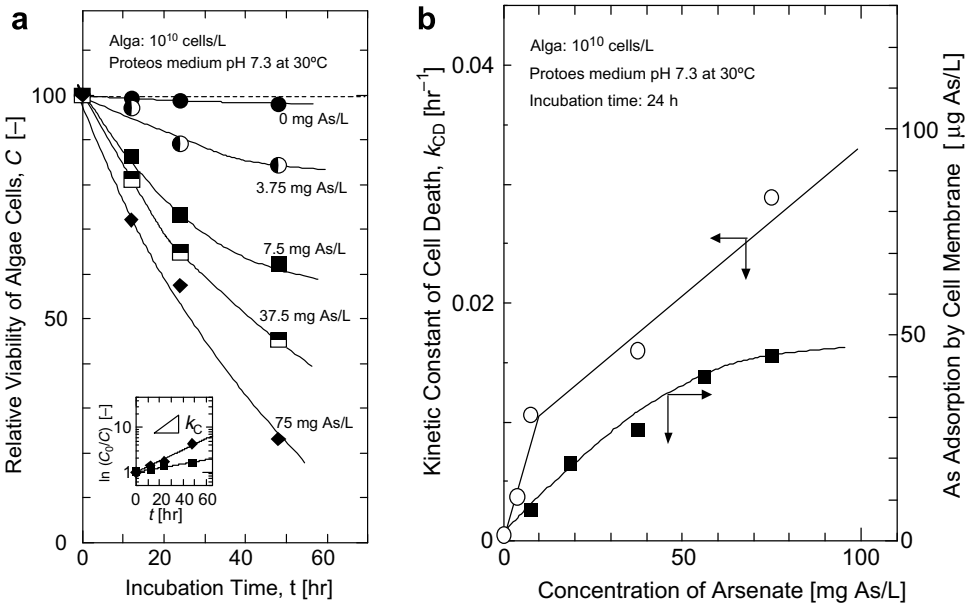

Fig. 2. (a) Cell viability as a function of time and arsenic concentration. (b) Kinetic constant of cell death (○) and arsenic adsorption on the cell membrane (■). After incubation of cells with arsenic, the cell membranes were extracted (Section 2) to determine the arsenic amount on biomembranes.

and actively. Remarkably, it is not the toxicant itself which induces cell death, but rather the cell membrane, following its adsorption of arsenic. Thus, an increase in the arsenic concentration resulted in an increase in cell death (Fig. 2a). However, in some cases, the membrane plays an important role in removal of toxicants from the environment. One of the essential removal mechanisms is the binding of arsenic on the membrane. This mechanism is an indispensable part of the interactions between toxicants and the cell membrane. To demonstrate this phenomenon, an artificial membrane model (liposome) was used in the following experiments for the reaction with arsenate.

3.2. Effect of arsenate on model cell membranes (liposomes)

The cell membrane can be considered a "frontier" facing the attack of toxicants (Zhang et al., 2000). In order to better understand the interactions between the membrane and toxicant, experiments were carried out using POPC liposomes and arsenate. The results indicated that the liposomes were fluidized and disrupted by arsenate, which was regarded as evidence that arsenic bound to the liposomes and affected them directly. However, the chemical binding with POPC molecules might occur after they are loosely attached to liposomes. The relatively high level of membrane-bound arsenic at the beginning of the total process implies a rapid binding of arsenate from the bulk solution to the membrane. The release after rapid binding might also result from the transfer of arsenic from kinetically favored binding sites in the membrane to more thermodynamically stable ones in both the membrane and cytosol (Winski and Barber, 1995). In a recent report about As (III), it was suggested that the arsenite anion may form a hydrogen bond directly with the PO_4^- group of dimyristoylphosphatidylethanolamine (DMPE) molecules in competition with hydrating water molecules as well as amino groups. Reduction of the effective PE–PE head group interaction should leave the phosphorus group free, and hence its mobility should increase as well as the interfacial area of lipid. Thus, there is a direct insertion of arsenic into the head group (Suwalsky et al., 2007).

Fig. 3a and b show the results of RP-HPLC analysis for the POPC solutions after 12 h and 48 h incubation, respectively. In Fig. 3a, the first peak (P1) was detected at 2.7 min, which represented the POPC, while the second peak (P2) shifted to 4.0 min. This shift indicates the change in the binding structure of arsenic to the POPC molecule. One of the possibilities is that arsenate molecules bound to liposomes by a hydrogen bond or electrostatic force, and then were chemically entrapped in liposomes by the substitution of arsenate for the phosphate group or choline group of POPC molecules, as described above, and this process was accelerated by 48 h incubation (Fig. 3b). It has recently been reported that a new series of cationic phosphonolipids characterized by a cationic charge with a phosphorus or arsenic atom is being developed (Stekar et al., 1995 and Guenin et al., 2000). The transfection activities of new cationic As-phosphonolipids were studied *in vitro* in different cell lines (HeLa, CFT1, and K562) and *in vivo* using a luciferase reporter gene. It was also demonstrated that cation substitution on the polar domain of cationic phosphonolipids (N replaced by P or As) resulted in a significant increase in transfection activities in both the *in vitro* and *in vivo* assays, as well as a decrease in cellular toxicity (Floch et al., 2000).

In a previous report, arsenate was shown to replace phosphate in many biochemical reactions, because two compounds have similar structures and properties (Dixon, 1997). For example, arsenate reacts *in vitro* with glucose and gluconate (Gresser, 1981 and Lagunas, 1980) to form glucose-6-arsenate and 6-arsenogluconate, respectively. Glucose-6-arsenate is a substrate for glucose-6-phosphate dehydrogenase and can inhibit hexokinase, as does glucose-6-phosphate (Lagunas, 1980). Arsenate can also replace phosphate in the sodium pump and the anion exchange transport system of human red blood cells (Kenney and Kaplan, 1988). However, the concentration of arsenate required for such reactions is high and not physiologically relevant. Furthermore, these effects may happen only in acute intoxication and may not be effective in subjects chronically exposed to low-dose arsenic.

When binding, arsenate interacts with phospholipid by a hydrogen bond or electrostatic force only. In the case of substitution, arsenate replaces the phosphate or choline groups of POPC molecules. Consequently, arsenate was able to change some of the characteristics of both the artificial and algal membranes in the present study.

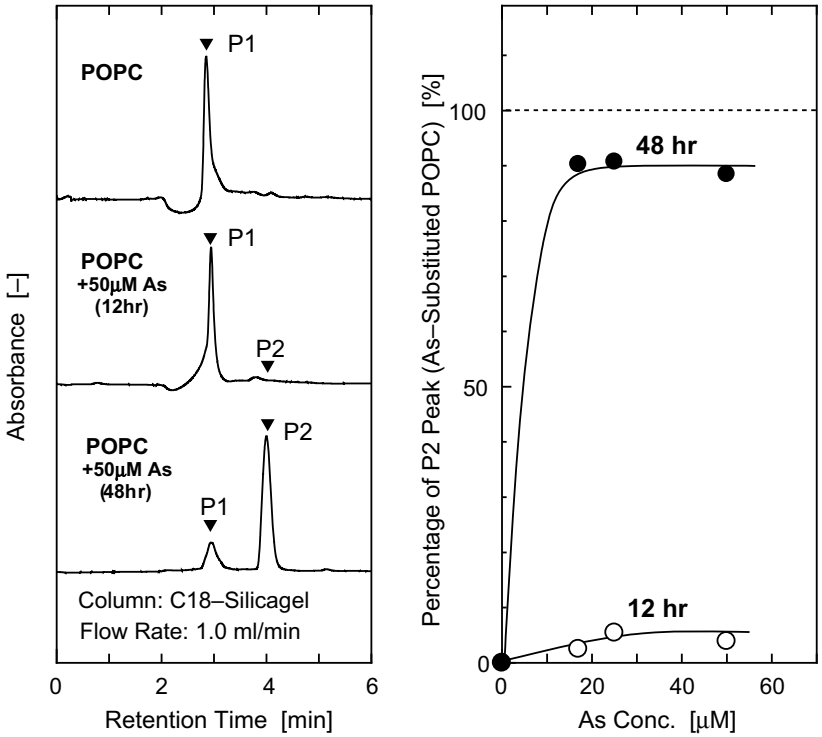

Fig. 3. RP-HPLC analysis of arsenic-substituted POPC. POPC 5 mM was incubated with arsenate 50 μM at 30 °C in phosphate buffer (pH 7.3). POPC-bound arsenic lipids were detected by an RP-HPLC. Elution profiles were monitored at 254 nm on the UV detector. The mobile phase of acetonitrile/water (65/35 v/v) with a flow rate of 1 mL/min was applied at 30 °C. An Inertsil ODS-SP column (0.46 cm × 2.5 cm) was used throughout this study. P1 represents POPC. P2 represents a product of the reaction between POPC and arsenic.

3.3. Fluidization of membranes of POPC liposomes and algal cells by arsenic toxicity: a mechanism for cell death by arsenic toxicity at low arsenate concentration

Fig. 4 shows the release of calcein from the POPC liposomes after exposure to 50 μM arsenate. Fifty percent of the total amount of calcein was released in the first 1 h of incubation, and the remainder was continuously released over the remainder of the 24 h experimental period. On the other hand, no calcein release was observed in the control experiment without arsenate. This is possibly because of the membrane fluidization of the POPC liposomes under arsenic toxicity. When liposome membranes become

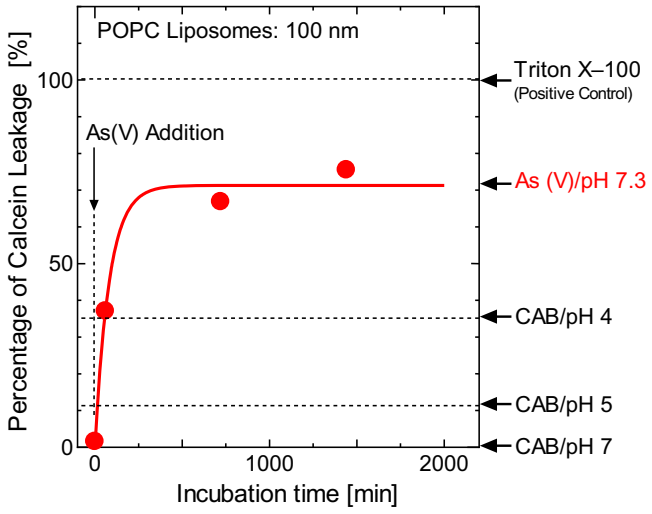

Fig. 4. Arsenate induced the fluidization of POPC liposome resulting in the release of calcein from the inside to the outside of the liposome membranes. Calcein was captured inside the liposomes. Free calcein was washed by phosphate buffer, and then arsenate was added to the liposome solution. The release of calcein indicated that arsenate induced membrane fluidization. Previously reported results on the effect of bovine carbonic anhydrase (CAB) and triton X-100 on membrane fluidization were plotted (Kuboi et al., 2004) for purposes of comparison.

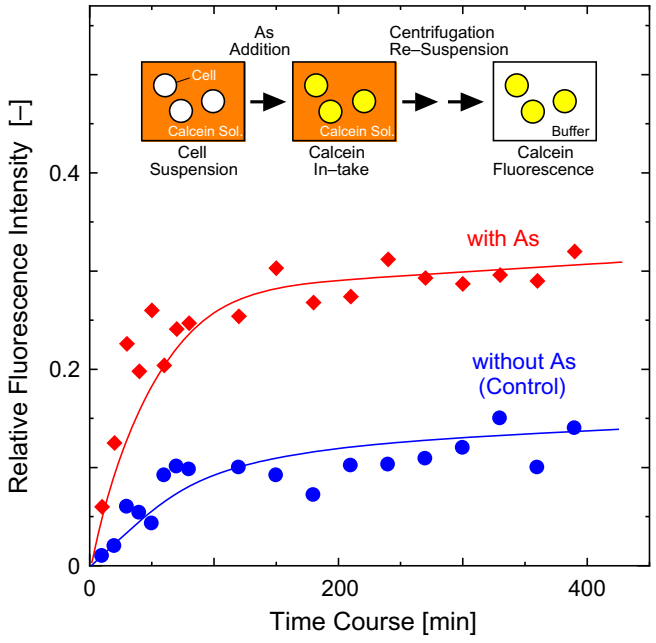

Fig. 5. Calcein accumulation in algal cells due to fluidization of the algal membrane. The cells were incubated in a Proteos medium with (♦) and without arsenic (●), and the fluorescent probe. Calcein adsorption by algal cells under arsenic toxic stress was analyzed periodically during the reaction.

fluid or are disrupted, entrapped calcein is released into the environment, resulting in increased fluorescence intensity. The results show that arsenate induced membrane fluidization. A previous study was conducted to compare the toxicity of arsenate with that of other substances such as bovine carbonic anhydrase and triton X-100 (Kuboi et al., 2004). Like this previous study, our present results underscore that arsenate is one of the most toxic elements.

To examine whether arsenate induces biomembrane fluidization, experiments were conducted with the exposure of algae to arsenate and calcein. The results are shown in Fig. 5. The permeability of fluorescent probe (calcein) into cells initially occurred quickly. The process of calcein adsorption into cells reached a state of balance even though the concentration of calcein outside the cells was still higher than that inside. The accumulation of calcein in the algal cells was dependent on the incubation time. The enhanced accumulation of calcein by increasing exposure to arsenate was considered to have been induced by fluidization of the cells, leading to an increase in membrane permeability.

In inducing cell death, arsenate initially fluidizes the membrane and then destroys it. The adsorption of arsenate by the cell membranes through passive or active mechanisms also can lead to generation of reactive oxygen species (Yamanaka et al., 1989, 1990) that oxidize lipids, proteins and genes (Delnomdedieu et al., 1995). Cell death is the inevitable consequence. The information obtained in the present study is necessary for application of algae to the treatment of toxicants (heavy metals).

4. Conclusion

The present study demonstrates that low concentrations of arsenate adversely affected algal cells. Arsenate with more than 7.5 mg As/L induced cell aggregation and death after 24 h. This acute toxicity induced the fluidization of the membrane, which in turn enhanced the membrane permeability and led to the accumulation of arsenic and subsequent cell death. Arsenate fluidized liposomes as well as cell membranes. At high concentrations of arsenate, the liposomes or cell membranes were destroyed, resulting in liposome disruption or cell death, respectively. When arsenate bound to the POPC liposomes, binding sites occurred at the phosphate head of POPC or choline of POPC liposomes. Arsenic (V) was also substituted for the phosphate or choline group of POPC molecules as well. According to the view point of previous report (Floch et al., 2000), we initially suggest that arsenate can replace the choline group more easily than the phosphate group of POPC molecules of the biomembranes. Thus, we considered that arsenic toxicity mainly affects cell membrane and model membrane. However, in some cases, the membranes play an important role in the removal of toxicants, because the removal activities of toxicants happen mainly on the membranes. The current research is a promising model for future applications to the treatment of arsenic contamination.

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