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Bioaccumulation of Antimony by *Chlorella vulgaris* and the Association Mode of Antimony in the Cell

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The bioaccumulation and excretion of antimony by the freshwater alga *Chlorella vulgaris*, which had been isolated from an arsenic-polluted environment, are described. When this alga was cultured in a medium containing 50 μg cm^{-3} of antimony(III) for 14 days, it was found that *Chlorella vulgaris* bioaccumulated antimony at concentrations up to 12 000 μg Sb g^{-1} dry wt after six days' incubation. The antimony concentration in *Chlorella vulgaris* decreased from 2570 to 1610 μg Sb g^{-1} dry wt after the cells were transferred to an antimony-free medium. We found that the excreted antimony consists of 40% antimony(V) and 60% antimony(III). This means that the highly toxic antimony(III) was converted to the less toxic antimony (V) by the living organism.

Antimony accumulated in living *Chlorella vulgaris* cells was solvent-fractionated with chloroform/methanol (2:1), and the extract residue was fractionated with 1% sodium dodecyl sulfate (SDS). Gel-filtration chromatography of the solubilized part showed that antimony was combined with proteins whose molecular weight was around 4×10^4 in the antimony-accumulated living cells. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Antimony occurs widely in the environment in concentrations which are generally low but nevertheless significant owing to the high potential toxicity of this element.[1–5] Antimony trioxide (Sb_2O_3) has many uses, including flameproofing of textiles, paper and plastics, and as a paint pigment, ceramic pacifier, catalyst, mordant and glass decolorizer. When waste-containing fire retardant material is incinerated, antimony is emitted into the atmosphere.[6] As such products are increasingly used in the future, the emission of antimony into the atmosphere and waterways is likely to increase. Coprecipitation with ion [Fe(III)ion] is now the only method of removal of antimony from polluted water.[1] However, no method of disposing of the resulting precipitate has yet been generally accepted as safe.

Antimony, which is considered a nonessential element, is comparable in its toxicological behavior with arsenic and bismuth. Previously we have isolated (*Chlorella vulgaris*) from arsenic-polluted environments and found that this freshwater alga has a high ability to accumulate arsenic and to reduce its toxicity by oxidation.[7–10] It seems reasonable to suppose that this alga can also bioaccumulate and detoxify antimony, because this element is chemically like arsenic and is in the same Group of the Periodic Table. Only few attempts, however, have so far been made to determine the impact of antimony on living organisms. Therefore, we have started to study the possibility of bioaccumulation and excretion of antimony by *Chlorella vulgaris*, with the aim of finding an effective process of detoxifying antimony.

In this paper, the effects of antimony levels on the growth, bioaccumulation and excretion of antimony are discussed. We also report experi-

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mental results on the adsorption of antimony by the cell *in vitro*.

EXPERIMENTAL

General procedure of algal culture

The freshwater alga (*C. vulgaris*) isolated by the method described previously[9] was cultured and harvested under the conditions described in a previous paper.[10]

Determination of antimony concentration in algal cells

The total amount of antimony in the cells was determined following acid digestion. Algal cells were collected by centrifugation (4500 rpm, 10 min), and were washed twice or more with distilled water by repeating the centrifuge procedure. Dried cells were dissolved in concentrated nitric acid (10 cm^3) and heated at 80 °C to give a homogeneous clear solution. The resultant pale-yellow transparent solution was mixed with 0.1 M tartaric acid, diluted to an appropriate volume, and subjected to flameless atomic absorption analysis via a graphite furnace.

Determination of the valence of the antimony

The oxidation state valence of inorganic antimony in the aqueous phase was determined after extraction.[11] To the sample solution (10 cm^3), whose pH was adjusted to 0.5 by 10 M HCl, was added 0.1 M cupferron aqueous solution (5 cm^3) and 5 cm^3 of chloroform. After the mixture had been vigorously shaken for 3 min, the water phase was washed with 5 cm^3 of chloroform, and diluted to an appropriate volume. Then, the antimony concentration in the water phase was estimated by atomic absorption analysis. This represented antimony(V), since antimony(III) had been extracted into the chloroform as its chelate with cupferron. The concentration of antimony(III) was then found by subtracting this value from the total antimony concentration.

Solubilization of proteins in the residue of the solvent extraction

The wet living cells (1.5 g; 0.15 g on a dry weight basis) were homogenized with chloroform/methanol (2:1) using a Teflon homogenizer (Potter–Elvejhem type), the slurry was filtered under reduced pressure through double filter papers (No. 5C, Tokyo Filter Paper Co. Ltd), and the residue was washed with the mixed solvent until the filtrate became colorless. The residue was pulverized, mixed with 1% sodium dodecyl sulfate (SDS) (membrane-protein solubilizer: SPS-4, Nacali Tesque Co. Ltd) (15 cm^3, pH 8.6) and allowed to stand at 40 °C for 24 h. The suspension was centrifuged, and the supernatant was concentrated by a rotary evaporator at a reduced pressure.

Gel-filtration chromatography of solubilized proteins

Solubilized proteins were fractionated according to their molecular weights by gel-filtration chromatography by use of Sephadex G-75 (Pharmacia LKB Biotechnology). The Sephadex G-75 column (1.8 cm i.d., 70 cm long) was preconditioned with an eluent solution of 0.1% SDS in 10 mM Bicine (pH 8.6). The clear aqueous protein solution was put on the column and eluted with the eluent at a flow rate of 0.5 $cm^3\ min^{-1}$. The eluates were collected by a fraction collector (220 drops, *ca* 4.5 cm^3 each) and the fractions were analyzed for protein and antimony. The protein concentration was determined from its UV absorbance at 254 nm. Total antimony was determined by flameless atomic absorption spectrophotometry.

RESULTS AND DISCUSSION

Effect of antimony(III) impact on the growth of *C. vulgaris*

The growth of *C. vulgaris* at various levels of antimony(III) as antimony potassium tartrate is plotted against culture time in Fig. 1. The growth was unaffected by antimony impact up to an antimony level of 100 $\mu g\ cm^{-3}$. Although cells did not survive at 5000 $\mu g\ cm^{-3}$, *C. vulgaris* can grow even at an antimony level of 4000 $\mu g\ cm^{-3}$. It should be noted that *Chlorella* grew faster at 10 $\mu g\ cm^{-3}$ than at 0 $\mu g\ cm^{-3}$. This growth rate enhancement reappeared in all the various experiments. This alga seems to prefer an antimony-containing medium to an antimony-poor medium even though antimony is considered to be a nonessential element.

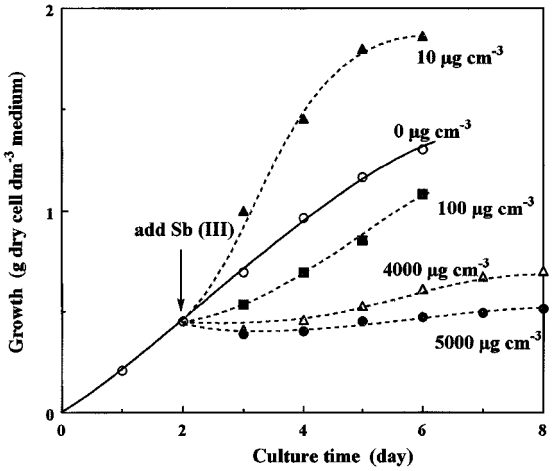

Figure 1 Growth curves of *C. vulgaris* in modified Detmer medium containing 0–5000 μg cm^{-3} of Sb(III).

Figure 2 shows antimony bioaccumulation at an antimony level of 50 μg cm^{-3}. The antimony concentration in the cell was highest at the log phase. We found *C. vulgaris* bioaccumulated antimony at concentrations up to 12 000 μg Sb/g dry cell after six days' incubation. Although the cell growth increased, the amount of accumulated antimony significantly decreased after seven days' incubation. This suggested that after it was accumulated by the *Chlorella*, antimony was rapidly excreted to the culture medium.

The following experiment was performed to determine whether the antimony accumulation is due to the life process of the cells. After algal cells had been collected by centrifugation and washed with distilled water, a part of the cells was freeze-dried to kill the cells. The dead cells and live cells were suspended in the medium containing 50 μg cm^{-3} of antimony(III) and incubated for five days. Then the cells were collected by centrifugation and the antimony concentration in the algal cells was determined by flameless atomic absorption spectroscopy. We found that antimony concentrations in the live cells and in the dead cells were 8080 μg Sb g^{-1} dry wt and 850 μg Sb g^{-1} dry wt, respectively. Hence most antimony in the cells was due to the life processes of *C. vulgaris*, and not to physical adsorption onto the cells.

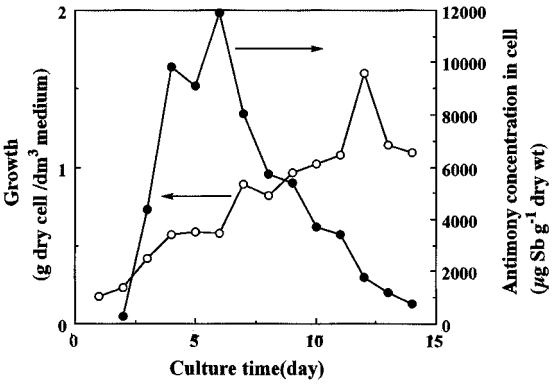

Figure 2 Bioaccumulation of antimony by *C. vulgaris* in modified Detmer medium containing 50 μg cm^{-3} of Sb(III).

Valence of antimony after excretion

Chlorella was cultured in a medium containing 100 μg cm^{-3} of antimony(III). After *C. vulgaris* had accumulated antimony, the cells were isolated and cultured with shaking in an antimony-free medium. The antimony concentration in *C. vulgaris* decreased from 2570 to 1610 μg Sb g^{-1} dry wt after the cells were transferred to an antimony-free medium. We found that the excreted antimony consists of 40% antitmony(V). This result means that the highly toxic antimony(III) was converted to the less toxic antimony(V) by the living organism. It is known that one of the methods of resistance to toxic metals for organisms is a change in an oxidation state, i.e. a more toxic form of metal is converted to a less toxic form such as mercury [Hg(II) to Hg(0)],[12] molybdenum [Mo(VI) to Mo(III)[13] and gold [Au(III) to Au(I) or Au(0)][14] by *Chlorella*. Such a biological transformation of antimony has not previously been shown to occur with living organisms, so our findings establish that it is possible.

Analysis of antimony-bound proteins in antimony-accumulated *C. vulgaris*

If this alga is to be used for removing antimony from polluted water, it is necessary to consider the fate of the antimony removed. It may be converted into a less toxic form, but this may be released into the environment when the cell dies. We have therefore started to study the chemical form of the antimony in the cells.

Live *C. vulgaris* was cultured for three days in a medium containing 50 μg cm^{-3} of antimony(III) by the general procedure, and the antimony-accumulated algal cells, which had accumulated antimony at a level of 710 μg Sb g^{-1} dry wt were harvested. The wet algal cells were fractionated with chloroform/

methanol (2:1) by the method described in the Experimental section.

Protein in the extract residue was separated by the solubilization technique, determined and fractionated on the basis of molecular weight by the method described in the Experimental section. In the same manner, antimony-free protein was separated from the extract residue of antimony-free *C. vulgaris* cells. Gel-filtration chromatograms of the solubilized proteins from antimony-bound and antimony-free residues are shown in Fig. 3. The peak positions of the proteins in Fig. 3 were determined by a UV method. Both chromatograms show two peaks. It was found, from a calibration curve obtained by the use of standard molecular weight proteins, that the lower peak corresponds to 4.7×10^4 Da. Antimony concentrations of each fraction were determined by flameless atomic absorption spectrometry and these data are shown in Fig. 3. We found that the existence of antimony was observed at the same position as the low-molecular fraction in Fig. 3(a), not in Fig. 3(b). From this it may be seen that the accumulated antimony co-elutes with the proteins of lower molecular weight. Further studies with undenatured proteins will be required to determine the form of the element, and whether it is protein-bound.

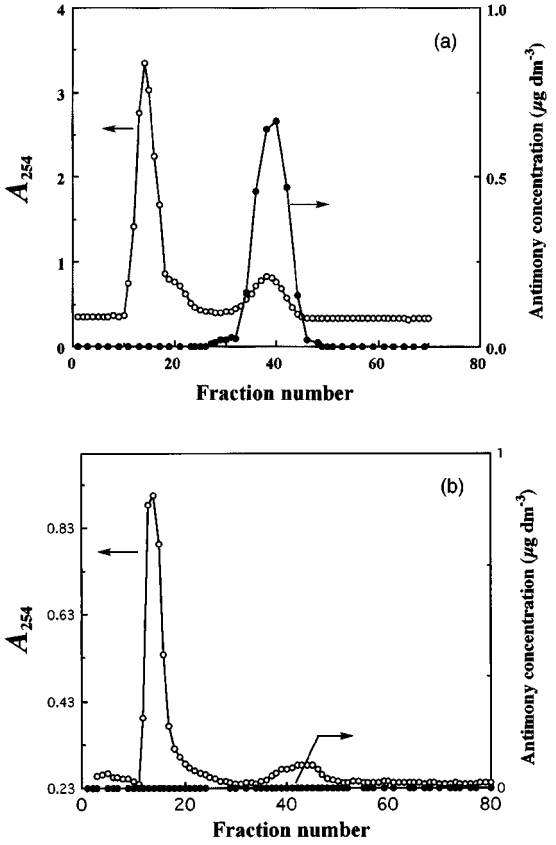

Figure 3 Gel-filtration chromatography on Sephadex G-75 of solubilized proteins separated from (a) antimony-accumulated and (b) antimony-free *C. vulgaris* cells.

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