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Intracellular proline accumulation in some algae exposed to copper and cadmium

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Abstract. The intracellular proline level in four species of algae: *Anacystis nidulans*, *Chlorella* sp., *Pediastrum duplex*, and *Nitzschia palea*, was enhanced when they were treated with deleterious concentrations of Cu^{2+} or Cd^{2+}. The maximum enhancement of proline level followed the order: *Chlorella* > *Pediastrum* > *Anacystis* >*Nitzschia* for either metal ion, but copper caused a greater accumulation of proline than did cadmium. Proline accumulation appears to be a general response of algal cells to copper and cadmium. The present study characterizes the time course of proline accumulation. In addition, experiments conducted with *Chlorella* sp. and *A. nidulans* showed that when incubated under illumination, metal-treated cells accumulated more proline than they did in the dark. The possible role of proline accumulation is discussed.

Keywords: *Anacystis*; Cadmium; *Chlorella*; Copper; Light dependence; *Nitzschia*; *Pediastrum*; Proline accumulation.

Introduction

Under stress conditions, cells tend to accumulate low-molecular-weight compounds intracellularly. Proline is such a compound, and accumulates in algal cell as a response to osmotic stresses such as drought and high levels of salinity and osmolarity (Liu and Hellebust, 1976; Schobert, 1977b). Freezing stress (Guy, 1990) and air pollution (Anbazhagan et al., 1988) have also been found to cause an increase in the intracellular proline levels of a variety of higher plants.

High concentrations of copper and cadmium inhibit the growth of algal cells. Wu and Lin (1987) reported that the amount of intracellular amino acid in *Coelastrum astroideum*, a green alga, changed in response to cadmium. At the time of a preliminary study (Chang, 1991) with *Chlorella* sp., which showed that the intracellular proline level was markedly enhanced when algal cells were treated with high concentrations of Cu^{2+} or Cd^{2+}, this interesting phenomenon had not been reported in the literature. Our present study, with various organisms, further characterizes this proline accumulation and attempts to ascertain its relationship to the heavy-metal tolerance of algae.

Materials and Methods

Algae for Study

We used four species of algae. Two green algae—*Chlorella* sp. (strain 2563, isolated from the estuary of the Tamsui River, Taipei, Taiwan) and *Pediastrum duplex* (strain 2060, a freshwater species isolated from the Tamsui River)—a diatom—*Nitzschia palea* (strain 2428, isolated from a freshwater pond in Taipei)—and a cyanobacterium—*Anacystis nidulans* (strain 1402-1, obtained from the Algensammlung Goettingen, Germany).

Cultivation of Cells

The different algal species were cultivated in various culture media. *Chlorella* sp. was cultivated in inorganic Aquil medium (Morel et al., 1979), containing 0.9% NaCl, in which they grew optimally. An inorganic medium, adopted from Kuhl (1962), was used for the cultivation of *P. duplex*. The culture media described by Jorgensen (1956) and Jüttner et al. (1983) were used for the culture of *N. palea* and *A. nidulans*, respectively. All cultures were kept at 27°C, with aeration (about 60 ml min^{-1}), illumination of about 400 $\mu E\ m^{-2}\ s^{-1}$, and a light:dark cycle of 14:10 h. Sulphate salts of copper and cadmium ($CuSO_4 \cdot 5H_2O$ and $CdSO_4$) were added to cultures at the exponential-growth phase.

Proline Determination

For quantitative analysis of intracellular proline, cells (about 10^{10}) were harvested by centrifugation (4,000 *g* for 10 min) and taken up with deionized water (1.5 ml). About one ml of glass pearl (0.1 mm diameter) was added to the cell suspension, which was then disrupted with a homogenizer (Glas Col, Terre Haute, Indiana, USA) for 15 min and centrifuged again (10,000 *g* for 15 min). The proline concentration in the supernatant liquid was determined according to the method of Bergman and Loxley (1970). The concentrations of proline are given as ng per cell or fmol per cell.

Quantitative Determinations of Chlorophylls and Proteins

The chlorophyll content of the cultures was used as the measure of cell mass, which is used for the calculation of cell growth rate. Chlorophyll was extracted from the cells with methanol (100%) at 60°C for 30 min in darkness. The absorbance of pigment was read at 665 nm with a spectrophotometer (Beckman DU-20, Irvine, USA). The concentration of chlorophyll *a* was determined using the coefficients and equations derived by Mackinney (1941). The equations given by Fogg (1975) were employed to estimate the growth rate of the cells. Cells were harvested for analysis of growth rate after a 24-h incubation with metal ions.

Protein concentrations of cell extracts were determined with a modification of the Lowry method (Bollag and Edelstein, 1991), using bovine serum albumin (Merck, Darmstadt, Germany) as a standard.

Results

The species differed from each other in their tolerance to Cu^{2+} and Cd^{2+}. *Nitzschia palea* was the most sensitive to Cu^{2+} and Cd^{2+}, having the lowest EC_{50} value (the metal concentration that causes a 50% inhibition of the cell's growth rate) (Figure 1). *Chlorella* sp. was the most tolerant of either metal ion, having the highest EC_{50} value of the four species tested. *Pediastrum duplex* and *A. nidulans* exhibited similar, intermediate sensitivities to Cd^{2+} and also to Cu^{2+}. Cu^{2+} was more toxic than Cd^{2+} to all the tested species, excepting *N. palea*.

In the presence of deleterious concentrations of Cd^{2+} or Cu^{2+}, *Chlorella* sp. showed markedly enhanced intracellular proline contents (the proline contents are related to the concentration of metal; Cu^{2+} caused slightly greater proline accumulation than did Cd^{2+}), while the protein level of the cells was little affected. Figure 2 shows that it is approximately the EC_{50} that causes the maximum proline accumulation.

The four species tested had various maximum enhanced proline levels. The magnitude of enhanced proline follows the order: *Chlorella* sp.> *P. duplex* > *A. nidulans* > *N. palea*, independent of the metal species.

The levels of enhanced proline concentrations were compared with the cells' tolerance of the metals. A plot of enhanced proline levels versus the Cd^{2+} and Cu^{2+} EC_{50} (Figure 3) reveals that the more-tolerant species accumulate more proline intracellularly.

The time course of proline accumulation was further studied with *Chlorella* sp. When Cd^{2+} (160 μM) was added to cultures at the beginning of the illumination period, an intense increase in intracellular proline level was detected within the first hour (Figure 4). The proline level continued to increase slowly until 7th hour, followed by a slight decrease in proline level to the end of the illumination period. In the dark period, the proline level declined. Nontreated cells showed a similar course of fluctuation in proline level during incubation, but the amplitude of fluctuation was very low.

We compared the proline accumulation in *Chlorella* sp. incubated under illumination with that incubated in the dark. Two different concentrations of Cd^{2+} (10 and 160 μM) were added to cultures at the beginning of the LD cycle and the changes in intracellular proline levels were compared after 2 h. Figure 5 shows marked differences in proline level between illuminated and dark incubation conditions. Compared with non-treated cells, those treated with 10 μM Cd^{2+} did not exhibit a significant increase in

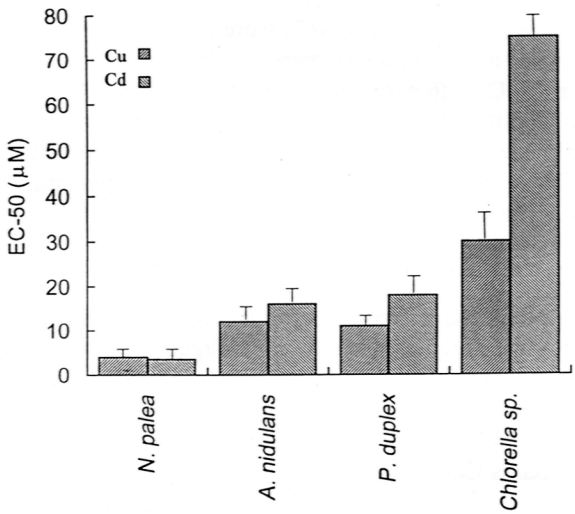

Figure 1. Medium inhibitory concentrations (EC_{50}) of copper and cadmium against the growth of *Nitzschia palea, Anacystis nidulans, Pediastrum duplex*, and *Chlorella* sp. Vertical bars indicate SE, n=4.

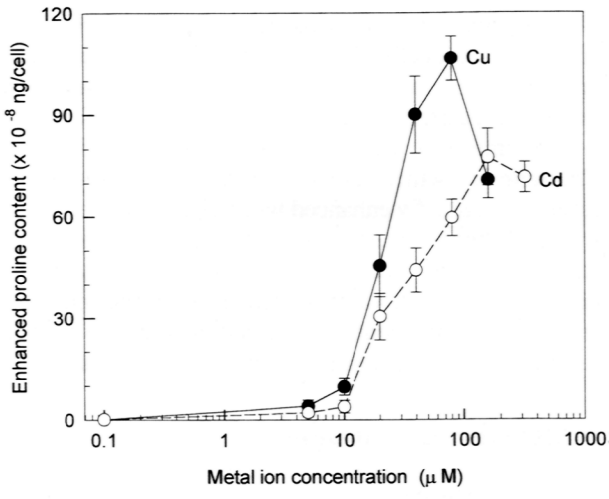

Figure 2. Enhancement in intracellular proline contents caused by treatment with various concentrations of copper and cadmium in *Chlorella* sp. Vertical bars indicate SE, n=3.

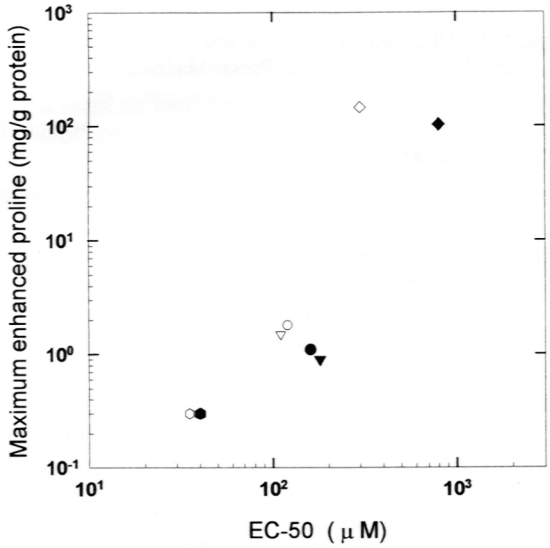

Figure 3. Relation of the medium inhibitory concentrations (EC_{50}) of copper (unfilled) and cadmium (filled) to the maximum enhanced intracellular proline levels in *A. nidulans* (triangular), *Chlorella* sp. (tetragonal), *N. palea* (hexagonal), and *P. duplex* (circular).

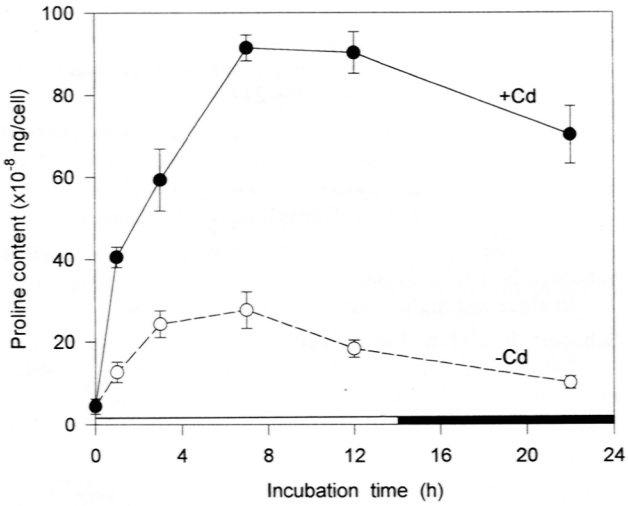

Figure 4. Courses of the change in intracellular proline content in *Chlorella* sp. cells caused by treatment with 160 μM $CdSO_4$ (+Cd), in comparison with nontreated (-Cd) cells. The treatment was conducted at the beginning of a light:dark growth cycle of 14:10 h. Vertical bars indicate SE, n=3.

proline level when incubated in the dark, while a marked increase in proline level was observed in those incubated under illumination. The difference in the enhancement of proline levels under illuminated and dark incubation was more pronounced with 160 μM Cd^{2+}.

When *A. nidulans* was treated with 20 μM Cu^{2+} and incubated under illumination, the level of intracellular proline increased from 0.006±0.002 to 0.021±0.003 fmol per cell after 2 h. The proline level increased to 0.011±0.003 fmol per cell, when the Cu^{2+}-treated cells were incubated in darkness, suggesting that the enhanced proline levels are related to the illumination.

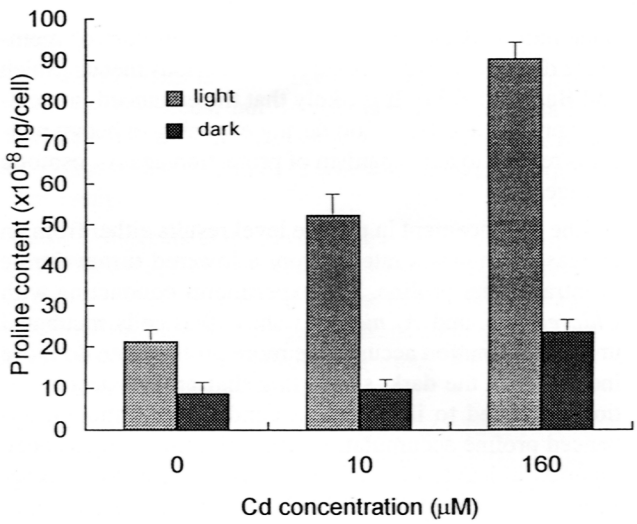

Figure 5. Comparison of the enhanced proline levels in *Chlorella* sp. cells incubated under illumination with that of those incubated in the dark, after treatment with two different concentrations of cadmium. Vertical bars indicate SE, n=3.

Discussion

The intracellular proline level was enhanced in all of the species studied when they were treated with copper or cadmium; the species differed in the magnitude of enhancement. A plot of proline level versus the cell susceptibility to metal ions shows that the tolerant species accumulate more proline than do the sensitive ones. We suggest that the enhancement of proline levels can be used as an indicator of the tolerance of a species to copper and cadmium.

Algae have a variety of mechanisms of metal-stress tolerance. These include reduced accumulation of the metals (Bariaud et al., 1985; Collard and Matagne, 1994), metal sequestration in intranuclear complexes (Silverberg et al., 1976) or in cytoplasmic inclusions (Qureshi and Stokes, 1985), and an excretion mechanism based on a complexing or precipitation of metals (Butler et al., 1980). Our previous study (Wu et al. 1995) with *A. nidulans* indicated that the enhancement in proline level is the most pronounced change in the intracellular amino acid pool during copper treatment. In addition, an experiment conducted with a supply of exogenous proline indicated that proline can markedly lower the toxicity of copper to *A. nidulans*. These data suggest that the enhancement of the proline level during metal treatment is related to the cells' tolerance of metals.

Proline is not regarded as a primary determinant conferring stress tolerance to salt or cold in higher plants (Duncan and Widholm, 1991). In algae and higher plants, intracellular proline has been regarded as an osmoprotectant that is accumulated in relation to the level of intracellular potassium ions (Schobert, 1977a). Our previous study (Wu et al., 1995) shows that a supply of exogenous proline can give rise to a marked lowering in

potassium leakage from algal cells (a symptom of membrane damage during exposure to deleterious metals [Shieh and Barber, 1973]). It is likely that the enhanced intracellular proline accumulation during exposing to heavy metals is related to a mechanism of protection against osmotic change.

The enhancement in proline level results either from an increased synthesis rate or from a lowered turnover rate of intracellular proline. The experiments conducting with *Chlorella* sp. and *A. nidulans* show that cells incubated under illumination accumulate more proline than do those incubated in the dark, suggesting that proline accumulation is related to illumination conditions. Although enhanced proline accumulation under illumination has been reported in leaves of wheat and barley treated with exogenous abscisic acid (Pesci, 1992), it is still unclear whether the influence of illumination is related to photosynthesis or to some other light-induced processes that are involved in the metabolism of proline.

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銅和鎘促使藻細胞堆積脯氨酸

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本研究以四種藻類：安那藻 (*Anacystis nidulans*)、菱形矽藻 (*Nitzschia palea*)、星盤藻 (*Pediastrum duplex*) 及小球藻 (*Chlorella* sp.) 爲材料，研究藻細胞在銅和鎘處理下之反應。結果發現，在高濃度銅和鎘之下，細胞內會堆積脯氨酸，其堆積量與此四藻種對銅和鎘之敏感度成負相關，脯氨酸之堆積量似可作爲細胞對銅和鎘之敏感度指標。在添加鎘後，細胞內脯氨酸之堆積，會持續增加至第七小時，然後維持穩定量。在照光下，經銅或鎘處理之藻細胞其脯氨酸堆積量比在黑暗中爲高，顯示脯氨酸之堆積受光照之促進，其原因尚待進一步研究。

關鍵詞：脯氨酸堆積；銅；鎘；安那藻；菱形藻；星盤藻；小球藻；侍光性。