

Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins

(glutathione/detoxification/homeostasis/cadmium)

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Communicated by H. A. Barker, September 15, 1986 (received for review May 28, 1986)

ABSTRACT Phytochelatins are a class of heavy-metal-binding peptides previously isolated from cell suspension cultures of several dicotyledonous and monocotyledonous plants. These peptides consist of repetitive γ -glutamylcysteine units with a carboxyl-terminal glycine and range from 5 to 17 amino acids in length. In the present paper we show that all plants tested synthesized phytochelatins upon exposure to heavy metal ions. No evidence for the occurrence of metallothionein-like proteins was found. All data so far obtained indicate that phytochelatins are involved in detoxification and homeostasis of heavy metals in plants and thus serve functions analogous to those of metallothioneins in animals and some fungi. Phytochelatins are induced by a wide range of metal anions and cations. Phytochelatin synthesis in suspension cultures was inhibited by buthionine sulfoximine, a specific inhibitor of γ -glutamylcysteine synthetase (EC 6.3.2.2). This finding and kinetic studies of phytochelatin induction point to a synthesis from glutathione or its precursor, γ -glutamylcysteine, in a sequential manner, thereby generating the set of homologous peptides.

Phytochelatins are small, cysteine-rich peptides capable of binding heavy metal ions via thiolate coordination. They primarily form a M_r 3600 complex with cadmium. Phytochelatins are assumed to be involved in the accumulation, detoxification, and metabolism of metal ions such as cadmium, zinc, copper, lead, and mercury in plant cells (1). The general structure of this set of peptides is $[\text{Glu}(\text{-Cys})]_n\text{-Gly}$ ($n = 2$ to 8). Two of these peptides ($n = 2$ and 3) have been observed in the yeast *Schizosaccharomyces pombe* (2), and we extended these findings to the same class of chelating compounds ($n = 2$ to 8) known to be present in higher plants (3).

The γ -glutamyl linkages present in these peptides imply that they are not synthesized via mRNA. In contrast, metallothioneins isolated from mammals, *Drosophila*, sea urchin, and the fungi *Neurospora* and *Saccharomyces* are primary gene products (4–8). They are low molecular weight, heavy-metal-binding proteins with high cysteine content, and their sequences have been strongly conserved during evolution, as evidenced by sequence homology between the metallothionein of *Neurospora* and that of humans (7). Their functions are not known definitively but are at least assigned to detoxification and homeostasis of heavy metal ions (9, 10).

In the present work, we investigated the ubiquity and possible functions of phytochelatins in higher plants, as well as the presumptive involvement of glutathione (1) in phytochelatin biosynthesis.

MATERIAL AND METHODS

Plants and Cell Cultures. Cell suspensions of *Rauvolfia serpentina*, *Agrostis tenuis*, and *Silene cucubalus* were

grown in Linsmaier and Skoog medium (11) on a gyratory shaker (100 rpm) at 23°C in continuous light (650 lux). Two-week-old seedlings of *Brassica oleracea* var. *capitata*, *Lycopersicon esculentum*, and *Zea mays* were cultivated in Hoagland medium (12) at 22°C with a daily light period of 12 hr. Plants of *Eichhornia crassipes* were maintained in Hoagland solution (12) diluted 1:10.

Gel Filtration. According to the method of Wagner (13), leaves of *B. oleracea* were extracted, and the extract was applied to a Sephadex G-50 (Pharmacia) column (2.5 × 51 cm) equilibrated and developed with 25 mM potassium phosphate buffer (pH 7.5). The Cd^{2+} concentration of the fractions (5.0-ml volume) was measured by atomic absorption spectrophotometry (Perkin-Elmer model 300, flame mode). In order to determine the apparent molecular weight, 0.4 mg of the M_r 3600 phytochelatin-Cd complex isolated from *R. serpentina* was subjected to gel filtration (Sephadex G-50 column, 1.5 × 60 cm, flow rate 12 ml/hr) at various ionic strengths [5 mM or 10 mM Tris Cl (pH 7.8) plus KCl]. At each ionic strength employed, the column was calibrated with the molecular weight standards bovine serum albumin (M_r 67,000), ovalbumin (M_r 44,000), soybean trypsin inhibitor (M_r 22,100), cytochrome *c* (M_r 12,300), insulin (M_r 6000), insulin chain B (M_r 3450), bacitracin (M_r 1450), and bradykinin (M_r 1060). Elution was monitored by measuring the UV absorbance at 250 nm and 280 nm.

Assay for Phytochelatin. Unless otherwise stated, plant material was exposed to heavy metal ions, added to the nutrient solution, for 3 days. Differentiated tissues were frozen in liquid nitrogen prior to homogenization by mortar and pestle. These tissues and sucked-dry cell suspension cultures (0.40 g fresh weight) were suspended in a solution (0.4 ml) containing 1 M NaOH and 1 mg of NaBH_4 per ml. After sonication and centrifugation (5 min, 11,000 × *g*) of the sample, the supernatant (0.5 ml) was acidified with 3.6 M HCl (100 μl). Precipitated material was sedimented again, and the cleared extract was separated by HPLC as described (1), followed by an additional post-column derivatization with 75 μM 5,5'-dithiobis(2-nitrobenzoic acid) [Ellman's reagent (14)], which is specific for sulfhydryl groups, in 50 mM potassium phosphate buffer (pH 7.6) (flow rate 2 ml/min, detection at 410 nm). Induction of sulfhydryl-containing compounds was tested by comparing chromatograms of extracts from heavy-metal-treated and untreated plant material. As a control, a second HPLC separation of the extract was performed without application of Ellman's reagent. Glutathione synthesis was inhibited by L-buthionine (*S,R*)-sulfoximine (Sigma).

Isolation and Sequence Determination. The phytochelatin-Cd complex was isolated, and the individual peptides were purified by HPLC as described (1). Amino acid sequences of phytochelatins were established by consecutive enzymatic and chemical degradation steps (γ -glutamyl transpeptidase, Edman degradation) and by partial hydrolysis and

subsequent analysis of the resulting fragments by two-dimensional thin-layer chromatography (1).

RESULTS

Phytochelatin Are Ubiquitous in Higher Plants. Several authors have reported (13, 15–21) that metallothionein-like proteins of $M_r \approx 10,000$ occur in differentiated higher plants. We reinvestigated those plants to determine whether the heavy-metal-binding factors are, in fact, phytochelatin.

B. oleracea (red cabbage) plants were grown and exposed to Cd^{2+} exactly as described by Wagner (13). Fig. 1 shows the elution profiles obtained from gel filtration of cell-free leaf extracts. As a control, Cd^{2+} was added to the homogenate from untreated plants (Fig. 1A). Comparison of the Cd^{2+} distribution in the chromatograms of the control (Fig. 1A) and Cd^{2+} -treated cabbage (Fig. 1B) revealed that the heavy metal exposure resulted in an induction of Cd^{2+} -binding material (Fig. 1B, fractions 20–30). Approximately 60% of total Cd^{2+} was associated with this peak, as compared to noninducible binding of 12% and 29%, respectively, associated with high molecular weight (M_r 20,000, fractions 14–17) and low molecular weight (M_r 1000, fractions 35–50) material of unidentified character. The fractions of the inducible Cd^{2+} -binding complex correspond to the M_r 10,000, metallothionein-like material reported earlier (13). These fractions (nos. 20–30) were pooled and subjected to HPLC, yielding peaks corresponding to phytochelatin with two to six γ -glutamylcysteine units (Fig. 2A). The major species was isolated and sequenced and unequivocally gave the primary structure $[\text{Glu}(\text{-Cys})]_3\text{-Gly}$. Consequently, the inducible Cd^{2+} -binding complex from red cabbage is in fact phytochelatin. The observed molecular weight, 10,000, can be explained by the drastic dependence of the apparent molec-

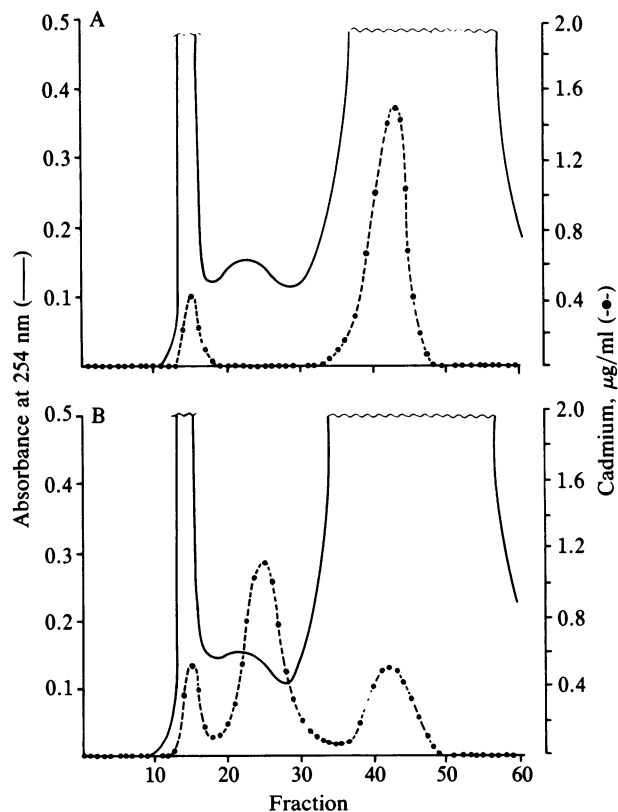


FIG. 1. Elution profiles of crude extracts from leaves of *B. oleracea* grown in the absence (A) or presence (B) of $90 \mu\text{M}$ $\text{Cd}(\text{NO}_3)_2$. Prior to Sephadex G-50 gel filtration, $\text{Cd}(\text{NO}_3)_2$ was added to homogenates of untreated plants (A).

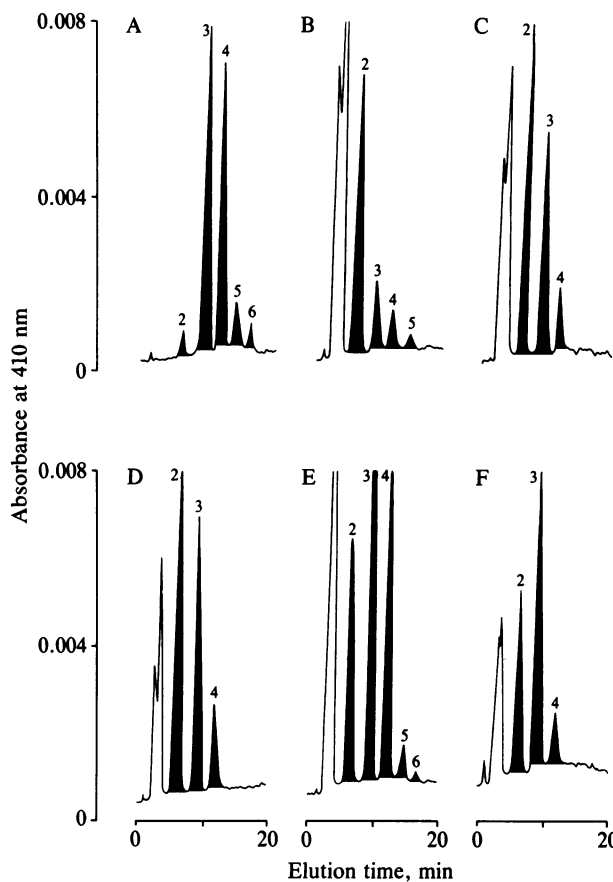


FIG. 2. Chromatogram of sulfhydryl-containing material from Cd^{2+} -exposed plants. Purified phytochelatin isolated from leaves of *B. oleracea* (A) was analyzed by HPLC, as well as crude extracts from cell suspension cultures of *S. cucubalus* (B) and *A. tenuis* (C) and from roots of *Z. mays* (D), *L. esculentum* (E), and *E. crassipes* (F). For A and E, plants were treated for 3 weeks with $90 \mu\text{M}$ $\text{Cd}(\text{NO}_3)_2$, according to Wagner (13), whereas the other tissues were exposed for 3 days to $20 \mu\text{M}$ $\text{Cd}(\text{NO}_3)_2$. The peaks corresponding to the individual phytochelatin are shaded and are identified by index number (n , the number of γ -glutamylcysteine units). The unshaded double peak corresponds to cysteine, which was eluted at a lower retention time, and glutathione. Samples from plants not exposed to Cd^{2+} contained either no phytochelatin or only negligible quantities ($A_{410} < 0.0005$ in this assay).

ular weight of phytochelatin upon ionic strength during gel filtration (Fig. 3). At high ionic strength ($>0.3 \text{ M}$), the Cd^{2+} complex isolated from *R. serpentina* has an apparent molec-

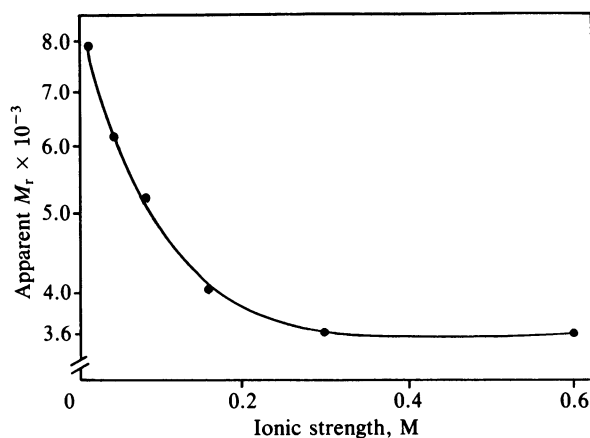


FIG. 3. Dependence on the ionic strength of the apparent molecular weight of phytochelatin, as determined by gel filtration.

Table 1. Induction of phytochelatin by heavy metal ions

Salt		Phytochelatin,* μmol/g [†]			Total γ-glutamyl- cysteine in phytochelatin, μmol/g [†]
Formula	Conc., μM	n = 2	n = 3	n = 4	
Cd(NO ₃) ₂	100	1.27	2.91	2.30	20.5
Pb(NO ₃) ₂	1000	1.78	2.28	0.25	11.4
ZnSO ₄	1000	1.51	1.68	0.12	8.5
SbCl ₃	200	0.94	1.72	0.37	8.5
AgNO ₃	50	1.07	1.90	0.08	8.2
Ni(NO ₃) ₂	100	1.53	0.82	0.07	5.8
Hg(NO ₃) ₂	10	1.28	0.55	0.02	4.3
Na ₂ HAsO ₄	20	1.40	0.34	0	3.8
CuSO ₄	50	0.88	0.41	0.04	3.1
SnSO ₄	100	0.86	0.33	0.03	2.8
Na ₂ SeO ₃	100	0.75	0.22	0.07	2.4
AuCl	50	0.71	0.17	0.03	2.0
Bi(NO ₃) ₃	100	0.69	0.18	0	1.9
TeCl ₄	10	0.58	0.13	0.05	1.8
WCl ₆	100	0.42	0.09	0	1.1
None	—	0	0	0	0

R. serpentina cell suspension cultures were exposed to the indicated salts for 3 days. A zinc- and copper-free medium (11) was used in all experiments. No phytochelatin was detected in cells exposed to 2 mM Ca(NO₃)₂, Al(NO₃)₃, FeSO₄, MgSO₄, MnCl₂, or NaCl; to 0.1 mM NaMoO₄, CsCl, or Cr(NO₃)₃; to 0.05 mM UO(NO₃)₂; or to 0.02 mM VOSO₄.

*Individual species with *n* γ-glutamylcysteine units per molecule.

[†]Dry weight of cells.

ular weight of 3600, but this value increases to 8000 at low ionic strength. A similar dependence has been noted for metallothioneins (22). We conclude that the molecular weight of the native metal-containing phytochelatin complex is 2000–4000 (23–26), rather than the ≈10,000 observed at low ionic strength (13, 15–21).

We reexamined the heavy metal metabolism in *S. cucubalus* (19) and *A. tenuis* (23) cell cultures, as well as in plants of *L. esculentum* (26) and *Z. mays*[‡]. Our results showed that phytochelatin was the only inducible heavy-metal-binding substance (Fig. 2 B–E). From Cd²⁺-exposed water hyacinths (*E. crassipes*), Fujita (24) isolated a compound whose properties suggested a similarity to the Cd²⁺-binding peptides from *Schizosaccharomyces pombe* (2). This assumption is now confirmed (Fig. 2F). These data imply that the heavy-metal-chelating principle in higher plants as well as in plant cell cultures is phytochelatin. The previously reported amino acid compositions of metal-binding "proteins" (13, 15, 19, 23) are similar but not identical with those of phytochelatin, presumably due to impurities.

Induction of Phytochelatin by Various Metals. The induction of phytochelatin formation by several heavy metal ions was reported previously (1). Table 1 summarizes the results obtained with a wider range of metal salts, following exposure of *R. serpentina* cell suspension cultures to high but nonlethal concentrations. The metal salts employed contained <0.001% other metals as impurities. No induction of phytochelatin was seen with Al³⁺, Ca²⁺, Co²⁺, Cr²⁺, Cs⁺, K⁺, Mg²⁺, Mn²⁺, MoO₄²⁻, Na⁺, or Va²⁺. Induction was observed with Ni²⁺ and the anions AsO₄³⁻ and SeO₃²⁻ (Table 1), not known to induce metallothionein synthesis in humans and animals (9). In general, the ions most active in inducing heavy-metal-binding molecules are Cd²⁺, Zn²⁺, Pb²⁺, Ag⁺, and Sb³⁺. Phytochelatin synthesis in cabbage leaves is also triggered by zinc salts, which is contrary to previously

reported experiments with this plant (13). We did not detect induction of the metal-binding peptides after heat shock or cold acclimation under conditions that induce synthesis of specific proteins (27, 28).

The most potent inducer is Cd²⁺, resulting in the formation of phytochelatin containing up to 11 γ-glutamylcysteine residues. The largest phytochelatin peptide so far detected by HPLC has a molecular weight of 2630 in the metal-free form. Metal analysis of purified phytochelatin–Cd complex isolated from *R. serpentina* gave 14.4% Cd, 0.038% Zn, and 0.04% Cu. The cells had been exposed for 5 days to 200 μM Cd(NO₃)₂ in addition to the regular amount of Zn²⁺ (37 μM) and Cu²⁺ (0.1 μM) present in the medium (11).

Involvement of Glutathione in Phytochelatin Biosynthesis. Kinetic studies were conducted to determine the rate of phytochelatin formation in plant cells after heavy metal exposure. We attempted to elucidate the expected role of glutathione [Glu(-Cys-Gly)] in phytochelatin formation (1), first by monitoring the level of both glutathione and phytochelatin during induction, and second by specific inhibition of glutathione biosynthesis prior to heavy metal exposure.

For study of the time course of phytochelatin synthesis, cells of *R. serpentina* were inoculated in Zn²⁺- and Cu²⁺-free medium, and after 3 days 200 μM Cd(NO₃)₂ was added to the vigorously growing culture. Immediately after Cd²⁺ administration, only phytochelatin containing two γ-glutamylcysteine units was synthesized (Fig. 4). Concomitant with the synthesis of this peptide, a decrease in the cellular glutathione pool was observed. In fact, incorporation of γ-glutamylcysteine residues into phytochelatin almost equaled the level of glutathione consumption for the first 3 hr after induction. *In vivo*, pulse-chase experiments using ³⁵S-labeled glutathione were in perfect agreement with this observation (data not shown). This decrease in glutathione was not observed in control cells to which Cd²⁺ was added after homogenization. While [Glu(-Cys)]₂-Gly was formed rapidly, the other phytochelatin peptides appeared only after

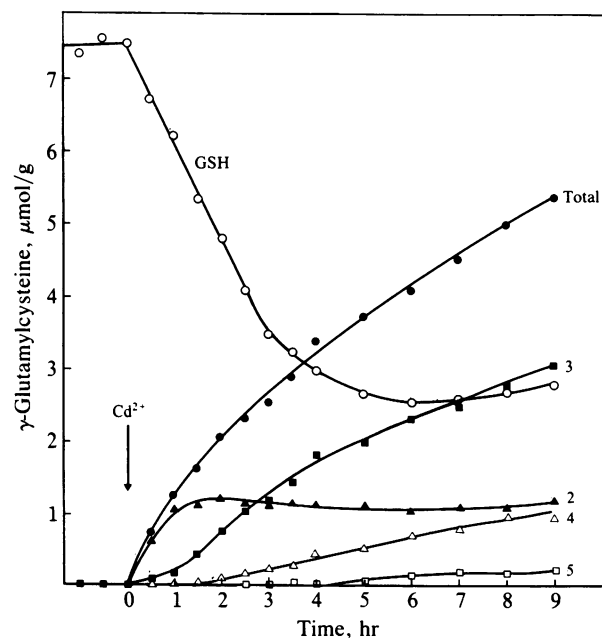


FIG. 4. Time course of phytochelatin induction and glutathione consumption after administration of 200 μM Cd(NO₃)₂ to *R. serpentina* cell suspension culture. Quantities of glutathione (GSH, ○), total phytochelatin (●), and individual phytochelatin with *n* (no. of γ-glutamylcysteine units per molecule) = 2 (▲), 3 (■), 4 (△), or 5 (□) are expressed as μmol of γ-glutamylcysteine per g (dry weight) of cells.

[‡]Bernhard, W. R. & Kägi, J. H. R. (1985) Second Meeting on Metallothionein, Aug. 21–24, 1985, Zürich (abstr.).

a significant lag period. A similar result was gained with the fission yeast *S. pombe* (3). These findings suggest that the heavy-metal-binding peptides are synthesized by sequential addition of γ -glutamylcysteine residues to glutathione.

The participation of glutathione in phytochelatin synthesis is substantiated by the drastic reduction of phytochelatin formation following inhibition of the biosynthesis of glutathione (Fig. 5). Buthionine sulfoximine is a specific and potent inhibitor of γ -glutamylcysteine synthetase [L-glutamate:L-cysteine γ -ligase (ADP-forming), EC 6.3.2.2] (29). It strongly diminished phytochelatin synthesis regardless of the Ag^+ concentration (10–100 μM) employed to induce the peptides. Phytochelatin formation was reduced 62% and 94%, respectively, in cells treated with 0.2 mM and 2 mM buthionine sulfoximine, as compared to control cells at maximal phytochelatin induction. Buthionine sulfoximine did not appear to be toxic to cells not exposed to heavy metal ions: the dry mass of the cells was 13.4 g/liter for the control and 13.9 and 13.1 g/liter for the cells treated with 0.2 mM and 2 mM buthionine sulfoximine, respectively. However, a significant growth retardation of buthionine sulfoximine-treated cells (0.2 mM and 2 mM) was registered after a 3-day exposure to 35 μM AgNO_3 , accounting for 21% and 67%, respectively, as compared to the control. These data suggest a biosynthesis of phytochelatins via glutathione and/or its biosynthetic precursor γ -glutamylcysteine.

Apparent Function of Phytochelatin. In order to study the possible role of phytochelatin in detoxifying metal ions, *R. serpentina* was exposed to 50 μM Cu^{2+} ions, and the effect on cell growth and phytochelatin induction was recorded. As shown in Figs. 4 and 6, the basal level of phytochelatin expression was zero or close to zero in plant cells grown in medium without the metal ions Zn^{2+} and Cu^{2+} . Upon heavy metal addition, the synthesis of phytochelatin was triggered. Coincident with phytochelatin induction, cell growth ceased for about 10 hr, presumably due to the inhibition of primary metabolism by toxic ion concentrations (30). Two days after

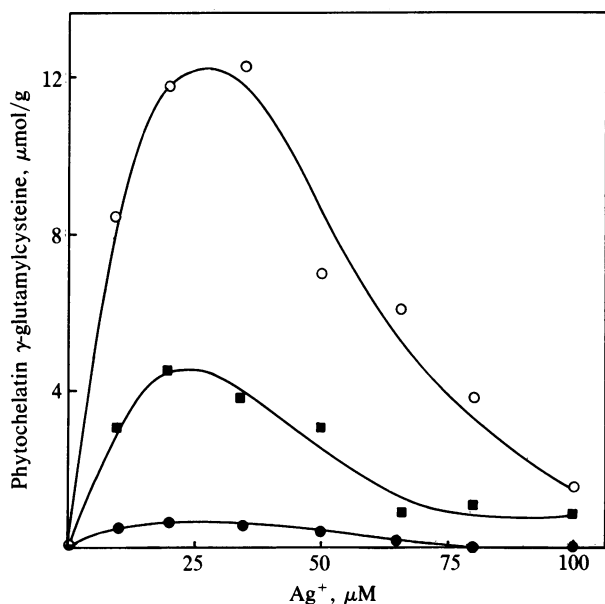


FIG. 5. Inhibition of phytochelatin synthesis by buthionine sulfoximine. *R. serpentina* cells were exposed to AgNO_3 6 hr after addition of buthionine sulfoximine, a specific inhibitor of the first enzyme of glutathione biosynthesis. After 3 days, the level of phytochelatin (expressed as μmol of γ -glutamylcysteine per g of dry weight) was determined in control cells (\circ) without inhibitor and in cells incubated in medium containing 0.2 mM (\blacksquare) or 2 mM (\bullet) inhibitor. Growth of cells not exposed to Ag^+ was not affected by buthionine sulfoximine.

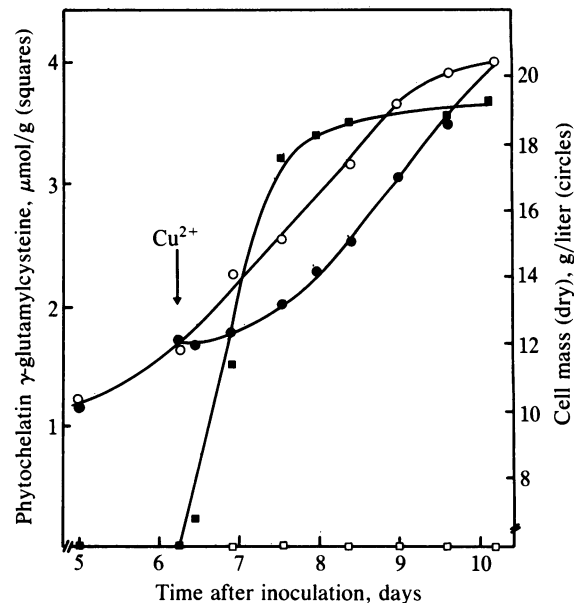


FIG. 6. Growth retardation and phytochelatin synthesis of *R. serpentina* cells during heavy metal stress. After administration of 50 μM CuSO_4 , phytochelatin was formed (\blacksquare); in nontreated cells, no phytochelatin was identified (\square). Growth of Cu^{2+} -treated cells (\bullet) was inhibited compared to the control (\circ).

Cu^{2+} administration, the intracellular phytochelatin concentration reached a constant level, and $\approx 80\%$ of the Cu^{2+} ions had been removed from the culture medium. At that time, cell growth fully resumed (3 g of dry weight per liter per day).

Phytochelatin biosynthesis is induced by the minimal amounts of Zn^{2+} and Cu^{2+} (37 μM and 0.1 μM , respectively) present in the growth medium (11) for cell cultures. Under these conditions the phytochelatin content of *R. serpentina* reached its highest level 3 days after cell transfer into fresh medium, the distribution of phytochelatins with two, three, and four γ -glutamylcysteine units being 0.35, 0.04, and 0.01 $\mu\text{mol/g}$ of dry weight, respectively. After 4 days more, the phytochelatin concentration had dropped to $\approx 8\%$ of maximal induction in the stationary phase (meanwhile the cells had proliferated nearly 3-fold).

DISCUSSION

The data presented here and in a previous report (1) demonstrate the absence of inducible metallothioneins or similar proteins in differentiated plants, as well as in plant cell suspension cultures. Instead, the principal pathway by which plants sequester heavy metals involves the phytochelatins. They are found in plants ranging from the phylogenetically primitive algae to the highly advanced order *Orchidales* (unpublished results). Phytochelatins have been observed in sizes varying from 2 to 11 γ -glutamylcysteine units. The molecular weight of the Cd-phytochelatin complex is dependent on the ionic strength, as is the situation in metallothioneins.

The phytochelatin response of plants to metal salts reflects the reactivity of the ions with essential groups (e.g., sulfhydryl groups), which in turn primarily determine the toxicity of heavy metals (31). In addition to Cd^{2+} , Cu^{2+} , Hg^{2+} , Pb^{2+} , and Zn^{2+} , the cations Ag^+ , Au^+ , Bi^{3+} , Sb^{3+} , Sn^{2+} , and Ni^{2+} , and the anions AsO_4^{3-} and SeO_3^{2-} also induce phytochelatin biosynthesis. In contrast, alkali and alkaline-earth ions, as well as some other metabolically essential elements, do not induce phytochelatin synthesis.

The heavy metal ions Cd^{2+} , Zn^{2+} , Pb^{2+} , and Hg^{2+} were found intracellularly almost exclusively chelated with

phytochelatin (1). Thus, a detoxification function of phytochelatin has been postulated. This hypothesis is now supported by the following findings. First, exposure of growing plant cells to a sublethal concentration of CuSO_4 resulted in a sudden arrest of growth coinciding with a rapid synthesis of Cu^{2+} -binding phytochelatin. When Cu^{2+} uptake was almost complete, phytochelatin biosynthesis decreased and cell growth resumed. Second, the toxicity of Ag^+ for plant cells was enhanced, as monitored by growth retardation, when phytochelatin biosynthesis was reduced by application of as little as 0.2 mM buthionine sulfoximine. This specific inhibitor of γ -glutamylcysteine synthetase (31) did not influence the growth of non- Ag^+ -treated cells even when present at a 10-fold higher concentration.

Finally, it has previously been shown that Cd^{2+} -resistant cell lines of *Datura innoxia* (32) and *Lycopersicon esculentum* (26) and a Cu^{2+} -tolerant *Silene cucubalus* (19) produce higher levels of heavy-metal-binding molecules than nontolerant ones. In the case of *Lycopersicon* and *Silene* (Fig. 2) these low molecular weight peptides were inducible and have been shown here to be phytochelatins. Since there is no rigorous selectivity for the uptake of ions in plants, these organisms had to develop a general system for inactivation of toxic ions. The phytochelatin peptides serve this function.

Phytochelatins are already induced at physiological heavy metal concentrations. Cell cultures revealed oscillating levels of heavy-metal-binding peptides with maxima several days after transfer into fresh medium and minima at stationary phase. Therefore, phytochelatins may also be involved, as a degradable storage form, in the homeostasis of Zn^{2+} , Cu^{2+} , Ni^{2+} , and possibly other heavy metal ions necessary for plant growth and full development.

The time course of phytochelatin induction and the reduction of phytochelatin formation following inhibition of glutathione synthesis suggested that the phytochelatins are synthesized from glutathione or its precursor, γ -glutamylcysteine. This is supported by the recent observation that homoglutathione [Glu(-Cys- β Ala)]-containing plant species of the order *Fabales* synthesize phytochelatins in which the carboxyl-terminal glycine is replaced by β -alanine (33). Thus, the class of peptides could be enzymatically produced by stepwise condensation of γ -glutamylcysteine moieties to glutathione itself and to the growing phytochelatin chain. The mode of formation will need to be clarified by the identification and characterization of the enzyme(s) involved in phytochelatin biosynthesis.

S-conjugates of glutathione are well-known intermediates in the biotransformation of some drugs in eukaryotic cells (34), and glutathione is apparently involved in the excretion of Cd^{2+} in the bile (35). Presumably, phytochelatins chelate heavy metal ions by a rather similar mechanism of cytoplasmic detoxification. This mechanism is realized in plants and in certain fungi. In *Saccharomyces cerevisiae* and *Neurospora* (where no phytochelatins were identified) and in animals the structurally different metallothioneins have evolved. Thus, a fundamental evolutionary divergence in heavy metal sequestration has occurred between animals and plants.

We thank Ms. S. Kunz for excellent technical assistance and Prof. A. Meister for suggesting the application of buthionine sulfoximine. This study was supported by Bundesminister für Forschung und Technologie (Bonn, F.R.G.) and Fonds der Chemischen Industrie.

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