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Cadmium-Binding Components in Soybean Plants[1]

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ABSTRACT

Soybean (*Glycine max* L.) plants exposed to ^{109}Cd readily absorb the element. Differential centrifugation of leaf, stem, and root homogenates followed by radioassay showed that Cd was associated primarily with the 105,000*g* supernatant. Separation of this fraction by gel chromatography and subsequent analysis by radioassays revealed that ^{109}Cd was bound to macromolecules of >50,000, 13,800, and 2,280 molecular weights. The >50,000 and 2,280 molecular weight fractions probably are nonspecific binding of Cd to normal cell components. The 13,800 molecular weight ^{109}Cd-bound component was found to be inducible by cadmium. It had a high ultraviolet absorbance at 254 nm and a low absorbance at 280 nm at pH 8.6.

Elemental analysis of many different plants growing in laboratories and in various parts of the world near mines and industrial areas have revealed that plants do not exclude toxic heavy metals, especially cadmium, during the uptake of essential minerals (4, 9, 13, 14, 23, 25). The plant-to-soil ratio for Cd was considerably higher than for Pb, Zn, or Cu (8). This suggests that the concentration of the Cd in plants is higher than it is in their environment. Cutler and Rains (7) suggested that one of the mechanisms involved in Cd accumulation involves an irreversible sequestering of Cd to binding sites, probably on cell constituents or macromolecules within the cell. Accumulation was greater in the roots than it was in the top of the plants (12). This accumulation at higher concentrations in various plant species was reported to be toxic (10, 11, 17). As low as 18 μM Cd in the nutrient solution inhibited pod fresh weight accumulation, nitrogenase activities, and photosynthetic rates in soybeans (11).

There is abundant literature pertaining to the binding of Cd in animal tissues (3, 5, 6, 15, 16, 18, 21, 22, 24) and in shellfish tissues (19). The most important finding was that 75 to 90% of the Cd that accumulated in the mammalian liver or kidney and shellfish tissues was bound by a cytoplasmic soluble protein of low mol wt. This protein was induced by Cd and was found to have an extremely high binding affinity for the element. This specific Cd-bound protein has been named "metallothionein" (15). Characteristically, it is deficient in aromatic amino acids and has high content of cysteine residues in the animal protein and of dicarboxylic residues in the shellfish protein. Recently, Rauser and Curvetto (20) have isolated a copper-containing metallothionein from *Agrostis gigantea*.

The mechanism whereby heavy metals are distributed and retained in plants is not fully understood. Inasmuch as environmental concentrations of Cd are increasing because of many industrial processes, we consider it important to isolate Cd-containing plant tissue fractions and to determine the nature of the Cd associating with these fractions. Soybean plants were chosen for this study because of their agricultural importance as a source of protein for man and animals.

MATERIALS AND METHODS

Experimental Growth Conditions. In a preliminary study, 20 soybean plants (*Glycine max* L.) were grown singly in aerated nutrient solution in 1,000-ml Erlenmeyer flasks for 35 d. The nutrient composition was: 2.5 mM $Ca(NO_3)_2$; 0.125 mM $MgSO_4$; 0.167 mM KH_2PO_4; 0.125 mM K_2SO_4, 5.9 μM ferric EDTA; 46.3 μM H_3BO_3; 0.91 μM $MnCl_2$; 0.81 μM $ZnCl_2$; 0.29 μM $CuCl_2$; and 0.10 μM $NaMoO_4$. The seedlings were grown at room temperature (19 to 26°C) in a room with a regulated cycle of 16 h of light and 8 h of dark at 220 lux. Fluorescent and incandescent lamps were used. The initial pH of the nutrient solution was 6.0. Twenty Erlenmeyer flasks covered with aluminum foil to keep the roots in darkness were used. On day 14, the nutrient solution was replaced with fresh solution containing the nutrients plus $CdCl_2$ at 0, 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} M, with four flasks at each level. The plants were grown for 35 d.

For the detailed study, the above procedure was employed utilizing $CdCl_2$ at 0 and 2×10^{-10} M, in two and eight flasks, respectively. On day 21, 5 μCi ^{109}Cd was added to each flask containing the Cd solution. The nutrient solution level was maintained by adding distilled H_2O. Immediately after harvest on day 35, the leaves, stems, and roots were separated, rinsed with distilled H_2O, minced, weighed, and stored at −20°C until analyzed. The washings from the roots were transferred to the Erlenmeyer flasks containing the nutrient solutions for radioactivity count. A total of 84 plants were harvested. The ^{109}Cd uptake by the soybeans was obtained by subtracting the radioactivity of the nutrient solution on day 35 from that in the same solution at the time of addition of ^{109}Cd.

Tissue Preparation. Leaves, stems, and roots were homogenized, 20% w/v, in cold 0.25 M sucrose, by a high speed blender. The pellet fraction was removed by centrifugation at 105,000*g* for 90 min. The supernatant fractions were reduced in volume to 25% by lyophilization and saved for Cd determination and for column chromatography. The pellet from ultracentrifugation was twice washed with cold sucrose solution; washings were added to the supernatant fraction.

Sephadex G-75 Chromatography. Portions of the lyophilized supernatant fraction of both control and Cd-treated plant tissues were chromatographed at the beginning and at the end of the clean-up procedure (Fig. 1) on a 2.5- × 36-cm Sephadex G-75 column at 10°C, following the procedure described by Shaikh and Lucas (21). The flow rate was 30 ml/h, using 0.01 M Tris-HCl buffer (pH 8.6). Fractions of 5 ml were collected. The column was calibrated with proteins (20 mg each) of known mol wt, according to Andrews (1): ovalbumin, 45,000; chymotrypsinogen, 25,000; and ribonuclease A, 13,700. The column void volume was deter-

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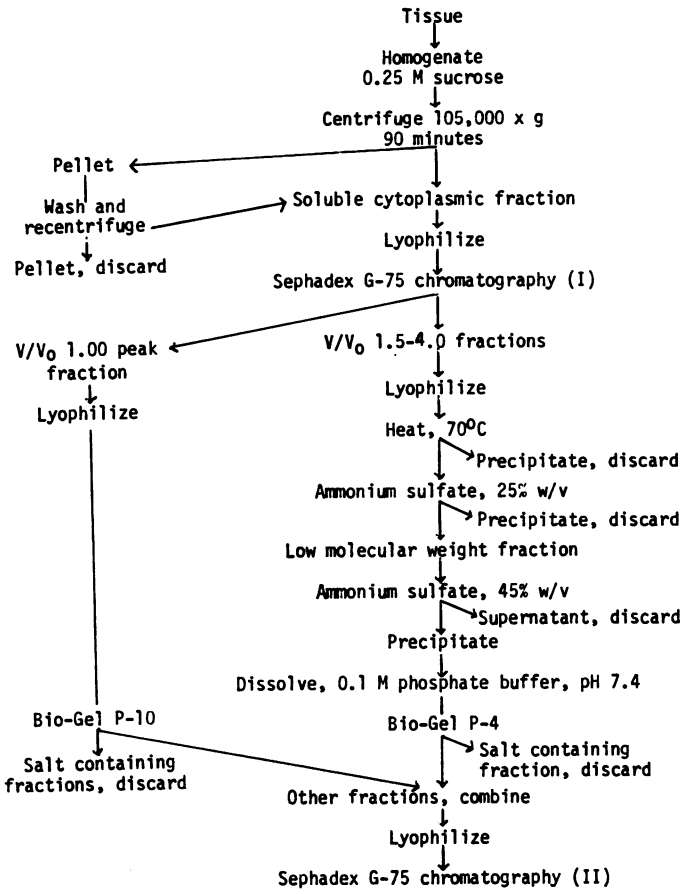

FIG. 1. Scheme for the isolation of plant Cd-binding components.

mined after every third run with Blue Dextran (2 million mol wt). The G-75 chromatography was done again at the end of the clean-up scheme to obtain V/Vo[2]-UV absorption curves for comparison purposes. After the first G-75 chromatography, the >50,000 mol wt fraction peak was pooled and reduced by lyophilization. Concentrated salts were removed through Bio-Gel P-10 chromatography by eluting with distilled H_2O. The other UV-absorbing fractions of lower mol wt (V/Vo 1.5–4.0) were pooled, partially lyophilized, and saved for $(NH_4)_2SO_4$ fractionation. The eluate from the Sephadex G-75 column was monitored at 254 nm and at 280 nm in separate runs.

Ammonium Sulfate Fractionation. Portions of the lower mol wt mixture described above (Fig. 1) were subjected to $(NH_4)_2SO_4$ fractionation, according to the method of Cherian (6), which includes heating of the mixture at 70°C for 1 min. This procedure has been used to isolate metallothionein from rat liver. The precipitates resulting from heating and from 25% w/v $(NH_4)_2SO_4$ treatment were discarded. The precipitate in the 25 to 100% saturation fraction was isolated by centrifugation. It was dissolved in phosphate buffer and then was filtered through Bio-Gel P-4 and eluted with distilled H_2O to remove ammonium salt. The fractions eluted from the P-10 and the P-4 columns were combined and lyophilized down to 25% in volume.

Liquid Scintillation Counting. Cadmium concentration of each fraction was determined by liquid scintillation counting of 1-ml aliquot in 15 ml of scintillation mixture. The scintillation solution consisted of 5.0 g 2,4-diphenyloxazole, 50.0 mg 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, 33.3 g Cab-o-sil (Packard Instrument Co.), 500 ml toluene, and 500 ml ethylene glycol monoethyl ether.

[2] Abbreviation: V/Vo, relative elution volume = (elution volume of fraction)/void volume.

RESULTS

Effect of Cadmium on the Growth of Soybean Plants. The vegetative growth of soybean plants in aerated nutrient solutions

Table I. *Effect of Cd on Growth of Intact Soybean Plants from Day 14 to Day 35*

Cadmium Treatment	Fresh Weight	Inhibition
	g ± SE	*%*
None	10.00 ± 1.06	0
10^{-12} M	6.90 ± 0.68	31
10^{-10} M	3.53 ± 0.36	65
10^{-8} M	1.12 ± 0.09	89
10^{-6} M		100

Table II. *Distribution of ^{109}Cd in Soybean Plants Grown in Nutrient Solution Supplemented with ^{109}Cd*

Plant Organ[a]	Total Fresh Weight of Organ per Plant	Total ^{109}Cd Content	^{109}Cd Concentration
	g ± SE	*cpm ± SE*	*cpm/g fresh wt*
Leaf	4.60 ± 0.55	273,000 ± 2,900	50,000
Stem	4.45 ± 0.43	710,000 ± 12,000	160,000
Root	5.33 ± 0.75	3,052,000 ± 41,000	573,000
Total		4,035,000	

[a] Homogenate, 20% w/v.

Table III. *Differential Centrifugation of Plant Tissue Homogenates*

Sample	^{109}Cd Concentration	^{109}Cd in 105,000 *g* Supernatant
	cpm/g fresh wt	*%*
Leaf homogenate	59,301	
Leaf supernatant	45,500	76.7
Stem homogenate	159,576	
Stem supernatant	92,552	58.0
Root homogenate	572,561	
Root supernatant	301,167	52.6

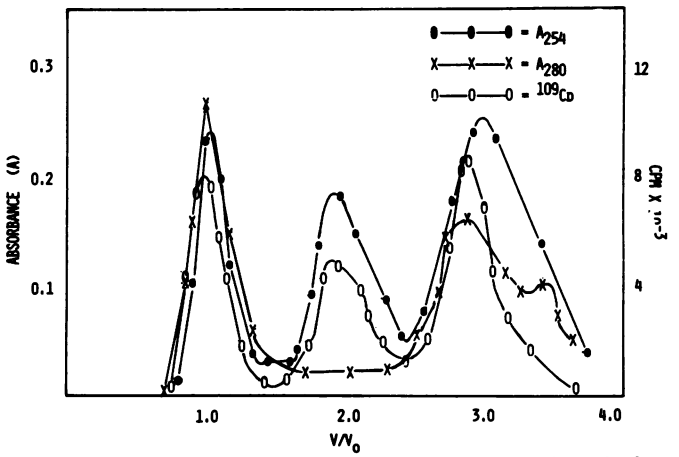

FIG. 2. Sephadex G-75 column chromatography of soybean leaf supernatant.

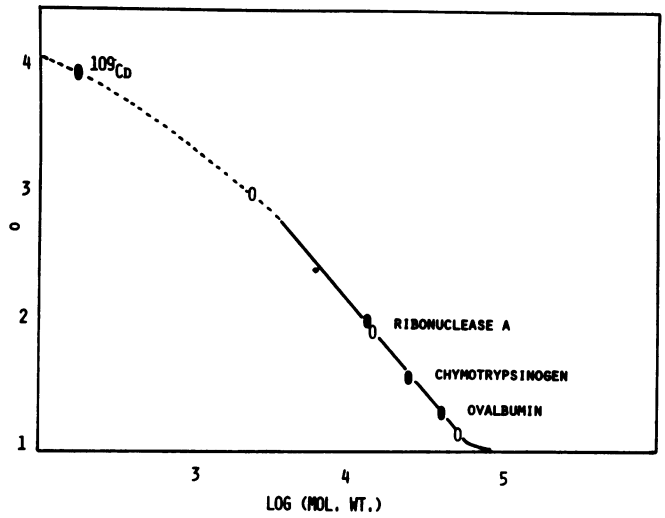

FIG. 3. Calibration of mol wt *versus* relative elution volume in Sephadex G-75 column chromatography of Cd-containing complexes (○) and mol wt standards (●).

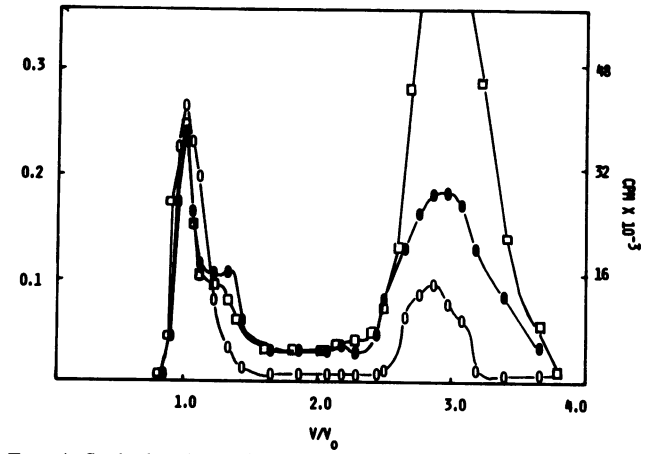

FIG. 4. Sephadex G-75 chromatography of supernatant of untreated soybean leaves (□, A_{254}) and of a similar supernatant dialyzed before chromatography against 10^{-10} M $CdCl_2$ containing 1,000 cpm/ml ^{109} Cd (●, A_{254}; ○, ^{109}Cd cpm).

at the various Cd levels is shown in Table I. Growth was inhibited at all Cd levels from day 14, when $CdCl_2$ was added, to day 35. The Cd level that produced 50% inhibition (I_{50}) was estimated to be 10^{-11} M. Cadmium at 10^{-6} M killed all plants. We also observed that the influence of the Cd poisoning caused chlorosis. There was growth of lateral buds and inhibition of root elongation.

Uptake and Distribution of Cadmium. Fourteen d after ^{109}Cd was added to the nutrient solution (2×10^{-10} M $CdCl_2$), the average uptake of the radioisotope was 4.5×10^6 cpm per plant. This uptake was computed from the initial and final ^{109}Cd counts in the nutrient solution. The homogenates of the plant contained an average of 89.4% of the computed uptake. Of this plant count, 6.8% was found in the leaves, 17.6% in the stems, and 75.6% in the roots (Table II). Differential centrifugation of leaf, stem, and root tissue homogenates of 35-d soybeans showed that ^{109}Cd was primarily in the 105,000*g* supernatant fraction (Table III).

Isolation and Characterization of ^{109}Cadmium-Bound Biomolecules. Plant tissue was homogenized and fractionated as described in Figure 1. Further clean-up steps after Sephadex G-75 chromatography I were done to remove a large amount of nonradioactive low mol wt UV-absorbing substances. The combined radioactive fractions of the ^{109}Cd plant leaves, stems, and roots after Bio-Gel P-10 and P-4 chromatography were resolved into UV-absorbing fractions by Sephadex G-75 gel chromatography II with an exclusion limit of 50,000 mol wt (Fig. 2). Chromatographs of stem and root supernatants were similar (data not shown). Radioassay of the eluted fractions showed activity in the regions which had ratios of elution volume to void volume (V/Vo) of 1.00, 1.92, and 2.92, with a predominance in 2.92. The mol wt of these peak regions were estimated at >50,000, 13,800, and 2,200 on the calibrated column (Fig. 3). Prolonged exposure to Cd apparently caused a small, but significant, increase in the concentrations of 254 nm UV-absorbing substances in the V/Vo region between 1.6 and 2.4. No UV absorption was observed in this region in the control plant extract (Fig. 4) and in the control plant extract dialyzed against 10^{-10} M $CdCl_2$ plus ^{109}Cd at pH 6.2 (Fig. 4) utilizing Spectrapor membranes with mol wt cutoff at 6,000. Excess Cd was removed by redialysis against 0.01 M Tris-HCl buffer (pH 8.6). Chromatography of radiocadmium ($^{109}CdCl_2$) gave a single radiopeak which appeared at V/Vo 3.9.

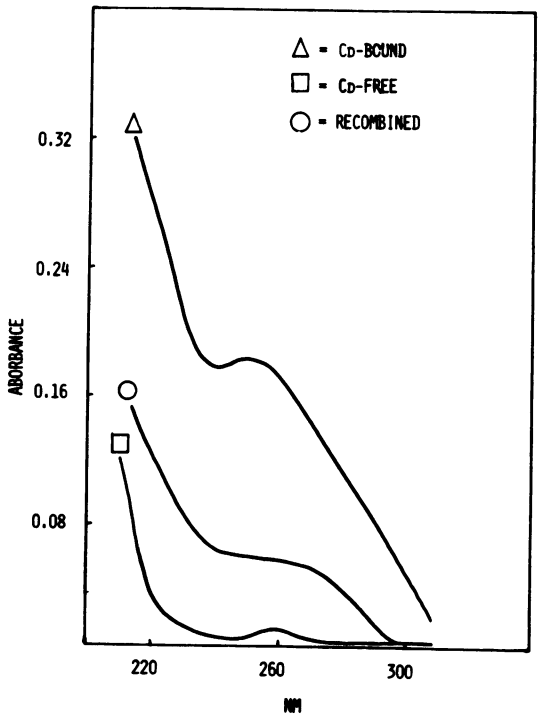

FIG. 5. Absorption spectra of V/Vo 1.92 fraction.

Treatment of 2 ml of Sephadex G-75 II V/Vo 1.00 fraction with 1 mg of pronase in 0.02 M $CaCl_2$ for 16 h and rechromatography resulted in 92% reduction in ^{109}Cd content (count dropped from 4,835 cpm to 405 cpm). Pronase had no effect on other radioactive fractions. Another 2 ml of each sample which had only $CaCl_2$ added to it were used as a control. The UV absorbance profile of pronase-treated fractions was omitted from this study, because of absorption interference by the pronase itself.

Figure 5 shows the spectral characteristics of the V/Vo 1.92 material before and after acid treatment. The chromatography of V/Vo 1.92 fraction through a 1.5- × 30-cm Sephadex G-25 column equilibrated with 0.01 M HCl resulted in a reduced UV absorbance of the Cd-binding component. Subsequent recombination of this fraction and the free ^{109}Cd-containing fraction partially restored the absorbance at pH 8.6.

DISCUSSION

This study has shown that, in the leaf, stem, and root tissues of soybean plants, Cd was associated primarily with the soluble fraction of the cells. A very large amount of nonradioactive low mol wt substances were present in the soluble fraction. Fraction-

ation with $(NH_4)_2SO_4$ and by heating, dialysis, and molecular-exclusion chromatography removed most of the substances, but it has resulted in some loss of Cd-containing fractions of relatively low mol wt.

One of the Cd-component complexes, V/Vo 1.92, isolated from the Cd-treated plant tissues by Sephadex-gel chromatography, was absent from this V/Vo region of the control leaf preparation dialyzed against $CdCl_2$. This indicates that the Cd treatment induced the formation of this Cd-component complex in soybean plants. This complex has the characteristic *A* at 254 nm. The removal of Cd from V/Vo 1.92 fraction at pH 2.0 reduced 254 nm *A*. This absorbance was partially restored after Cd was recombined with the above fraction. Pronase treatment of this fraction of the Cd-treated plant leaves showed the complex to be pronase-resistant. This, however, does not rule out that the Cd-bound component is a protein. Based on the resistance to proteolysis of the Cd-binding protein, metallothionein, found in animals (22), Cd may have a stabilizing effect on the structure of the proteins.

The above characteristics are similar to those described for "metallothionein" in animals and molluscs. Cd-binding protein isolated from a variety of animals, including man (3, 16, 18), is cysteine-rich and aromatic amino acid-deficient. The UV absorption at 250 nm was shown to be dependent on the Cd-mercaptide bond, and it is generally believed that the absence of aromatic acids in the protein structure causes the lack of absorption at 280 nm (16, 18). Its mol wt is about 10,000 daltons (V/Vo = 2.11 to 2.14). Previous studies have shown that, during administration of Cd to rats, the element induced the formation of the protein and was sequestered by it. Cadmium was found to stimulate the incorporation of [^{14}C]cystine into the protein in the rat liver (22). Also, actinomycin D and cycloheximide were found to prevent the induction of the protein. These two findings suggest that Cd stimulates the *de novo* synthesis of the protein, probably through an event that involves formation of a specific mRNA (5). The inducible protein from various animals showed minor differences in amino acid composition. Studies conducted on molluscs exposed to Cd have also shown the presence of a low mol wt Cd-binding protein with chromatographic properties similar to mammalian metallothioneins (19). However, the mollusc protein, which is dominated by dicarboxylic amino acids, was found to be different from that in mammals, which is rich in sulfhydryl-containing amino acids. The metallocomponent in *Agrostis* has 1.9 to 2.4 cysteinyl residues per Cu (20). This metallothionein has not been characterized with respect to UV absorbance. A Cd-binding protein of 10,000 daltons was isolated from the roots of Cd-treated tomato (2). This bound protein was partially characterized; it has a UV-absorbance spectrum characteristic of metallothionein, and its synthesis appeared to have been induced by the treatment of the plants with Cd.

The physiological significance of the Cd-bound plant component is not known. There is some evidence that metallothionein participates in Cd-detoxification in animals (16). Many plants growing on heavy metal-rich soils are known to develop a specific tolerance against these same heavy metals in their environment. Cutler and Rains (7) explained that the binding of Cd may serve to protect the cells from toxic effects. However, further work using highly heavy-metal-tolerant plants is needed to determine whether tolerance (or an increase in the critical threshold level of toxicity for Cd) can be achieved through the formation of heavy-metal-binding components. Cd below the critical threshold level would first induce a biological component and then be sequestered by it, and the element above the threshold level would have a toxic effect on the plant. It is not known if these Cd-binding components are identical with Rauser and Curvetto's (20) Cu-binding metallothionein isolated from Cu-tolerant grass.

The significance of Cd associating with the plant components in the V/Vo 1.00 and 2.92 fractions is obscure. These two fractions probably bind nonspecifically to normal cell components, because ^{109}Cd was found in these fractions after *in vitro* binding of the metal by control supernatant. They are not usually found in animals. Up to 99% of total Cd in the soluble fraction of the liver of rats treated with ^{109}Cd was found bound to metallothionein (15). The Cd-bound biomolecules in V/Vo 1.0, but not those in V/Vo 2.92 fraction, were found to be highly susceptible to pronase. Cadmium in the V/Vo 2.92 fraction is a bound Cd, because it emerged from the column before the free ^{109} Cd, which emerged at V/Vo 3.9.

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