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Inducible Cadmium Binding Complexes of Cabbage and Tobacco[1]

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ABSTRACT

Cadmium complexes with apparent molecular weights of 10,000 were observed in aqueous extracts of Cd-treated cabbage (*Brassica capitata* L., cv. red danish) and tobacco (hybrid of *Nicotiana glauca* and *N. langsdorffii*) plants. The amount of complex (as Cd) recovered was found to be dependent on the concentration of the metal in the growth medium and the total time of exposure of plants to the metal. Induction of the complex at moderate levels of ^{112}Cd exposure was monitored after labeling the complex with ^{109}Cd *in vitro*. The constitutive nature of the ligand of the complex in cabbage and tobacco leaves was suggested when control plant extracts were exposed to ^{109}Cd. Such extracts contained ^{109}Cd, which eluted from Sephadex G-50 in the region of Cd complex. Simultaneous labeling with ^{112}Cd and ^{35}S or ^{32}P indicated that the complex contained sulfur but probably not phosphorus. The amount of ^{35}S which eluted coincident with ^{112}Cd complex increased during complex induction. No evidence was found for the presence of 10,000 molecular weight Cd complex in stem exudates (vascular sap) of Cd-treated plants.

The results obtained are consistent with the presence in these tissues of a ligand which is both inducible and constitutive and binds Cd in mercaptide bonds. All of these properties, and others reported earlier, are characteristic of Cd-metallothionein formed in animals.

It is widely recognized that vegetable foods and (for those who smoke) tobacco smoke constitute two main sources of the toxic metal cadmium found in animals and man (11, 17). The presence of Cd in higher plants is explained by the fact that certain heavy metals, including Cd, are concentrated in plants during growth, often in tissues consumed by man, such as tubers, grains, leaves, and fruits (17).

Relatively little is known about the mechanisms of Cd (and other heavy metal) accumulation, tolerance, and toxicity in higher plants. The influence of soil chemistry on metal uptake, genus and species variation in uptake and accumulation, and the tissue distribution of accumulated Cd in plants have been extensively studied (2, 6), but little information is available concerning cell level effects of the metal and the chemical nature of Cd complexes formed by plants (1, 4, 10, 21, 22). The nature of plant-formed Cd complexes may be an important factor in human Cd toxicity. The chemical form of orally administered Cd has recently been shown to influence the body burden of the metal in mice (7).

In contrast to the relative lack of information on cell-level aspects of Cd in plants, much is known about the chemistry and biochemistry of this metal in animals. Proteins which bind the metal, called Cd-thioneins, appear to function in Cd sequestration and perhaps in trace metal homeostasis in animals (9, 15). Cadium-thioneins are inducible and constitutive proteins which have low mol wt (6,000 to 10,000) and high cysteine content (16). They lack aromatic amino acids and, therefore, have low A_{280} but show high A_{255}, because of the presence of Cd-mercaptide (cysteine) chromophores (19). Also, they are located in the cytosol of cells but are not normally found in body fluids (7, 8).

In an earlier report (21), we described the isolation and cytosol location of Cd complexes from several plant tissues. These complexes possessed some of the characteristics of animal Cd-thioneins. Recently, Bartolf *et al.* (1) isolated and partially purified a 10,000-dalton protein from the roots of Cd-treated tomato. Like Cd-thionein from animal sources, this protein exhibited high A_{250}, which was lost on titration of the complex from pH 7.8 to 2 and recovered when the pH was returned to 7.8. Several other reports have noted the presence of about 10,000 mol wt Cd complexes in higher plants (4, 10, 21, 22).

In this work, the inducible and constitutive nature of about 10,000 mol wt Cd complexes formed in cabbage and tobacco was studied. Very low mol wt complexes were also observed and are described.

MATERIALS AND METHODS

Cabbage (cv. red danish) and tobacco (hybrid of *Nicotiana glauca* and *N. langsdorffii*) were germinated in soil and transplanted as young seedlings into liquid culture. Nutrient solution contained (in mM concentration) 10 N; 6 K; 2 Mg; 5 Ca; 1 P; 2 S; and the trace elements (in μM concentration) 0.135 Mo, 0.315 Cu, 4.6 Mn, 0.085 Zn, and 23B. Iron (40 μg/ml) was added as Sequestrene 330 Fe (Geigy Agricultural Co.). The concentration of Cd in the culture solution was <1 pg/ml. Cultures were maintained at pH 5.5, 23°C, and ^{112}Cd, where added, was as $CdSO_4$.

To prepare extracts, deribbed leaves and other tissues were frozen at −70°C, crushed, and homogenized (VirTis homogenizer) in 25 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 2 mM DTT (about 1 ml/g fresh tissue). Homogenates were filtered through Miracloth, and the filtrate was centrifuged at 27,000*g* for 10 min at 23°C. The supernatant was shaken with an equal volume of $CHCl_3$:BuOH (10:1, v/v), after which the mixture was centrifuged at 5,000*g* for 5 min at 23°C to define the phases (extraction and centrifugation were carried out in 125-ml Corex bottles [Beckman]). The aqueous phase was removed and lyophilized. Dried extracts were suspended in H_2O and centrifuged at 10,000*g* for 5 min in preparation for gel filtration chromatography. The supernatants (5 ml) were applied to columns (2.54 × 61 cm) containing Sephadex G-50 fine. Elution (44 ml/h with 5.9-ml fractions collected) was with 25 mM KH_2PO_4/K_2HPO_4 (pH 7.4) at 10°C. Columns were calibrated with Cyt *c*, RNAse A, myoglobin, and chymotrypsinogen, having mol wt of 12.4×10^3, 13.7×10^3, 17.8×10^3, and 25×10^3, respectively.

In certain experiments, a solution containing ^{109}Cd in homogenization medium was added to the aqueous phase of the $CHCl_3$:BuOH extract to effect ^{109}Cd binding to Cd-containing complexes. The mixture was held at 23°C for 15 to 30 min before being applied to Sephadex G-50. Isotopic Cd was determined by gamma

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scintillation spectroscopy, and ^{112}Cd and Zn, by flame atomic absorption spectroscopy. For the latter, column fractions were made 3.7% with HCl (w/w) and directly analyzed. Homogenate-insoluble materials were washed and extracted for 1 h at 40°C with 19% HCl (w/w), and the extracts were diluted to 3.7% HCl (w/w) for analysis. In some cases, extracted insolubles were oxidized using a ternary acid mixture (12).

RESULTS

Elution profiles (Sephadex G-50) obtained with extracts of cabbage and tobacco leaves grown for 26 d in the presence of 11 μg/ml Cd are shown in Figures 1 and 2. About 50% and 10% of the Cd eluting from G-50 occurred as a complex (mol wt of about 10,000) for cabbage and tobacco, respectively. Both profiles were examined for the presence of zinc, A_{280} (protein), and A_{255} (Cd-mercaptide chromophore). The cabbage (Fig. 1) and tobacco (Fig. 2) profiles both showed 2 to 3 times as much A_{255} as A_{280} in the region of the 10,000 mol wt complex (A_{255} shown only for tabacco). Neither profile showed zinc to be present in this region (zinc profile shown only for cabbage). In cabbage, only one peak of ^{112}Cd occurred in the region of elution of very low mol wt compounds (<2,000 mol wt) and free metal, whereas, in tobacco, two peaks occurred—one coincident with high A_{255}. The similar elution profile for tobacco reported earlier (21) was incorrectly labeled as having been obtained with plants grown in 60 μg/ml Cd. The actual level of Cd during growth in that instance and in the experiment described by Figure 2 was 11 μg/ml.

In our first attempt to test the inducibility of the 10,000 mol wt complexes observed, three cabbage plants were grown for 26 d in 7 L of medium containing 6.5, 13, 19.5, and 32.5 μg/ml ^{112}Cd (Fig. 3). A gradual depletion of Cd from the nutrient solution (approximately linear with time—data not shown) was observed over this period. Also, a Cd-enriched precipitate was observed after several weeks in cultures containing >7 μg/ml Cd. The soluble Cd was measured at the beginning and end of each experiment, and values are presented in Figures 3 and 4. Analysis of extracts made after

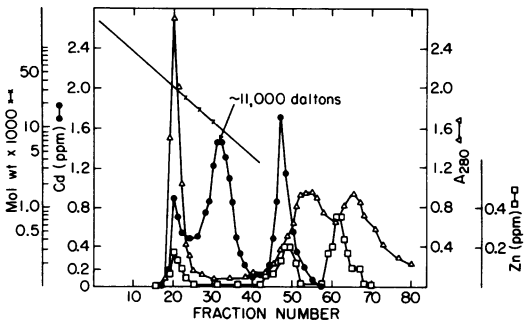

FIG. 1. Sephadex G-50 elution profile of a leaf extract from cabbage plants grown in the presence of 11 μg/ml Cd.

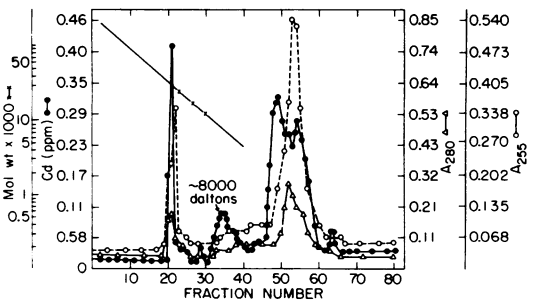

FIG. 2. Sephadex G-50 elution profile of a leaf extract from tobacco plants grown in the presence of 11 μg/ml Cd.

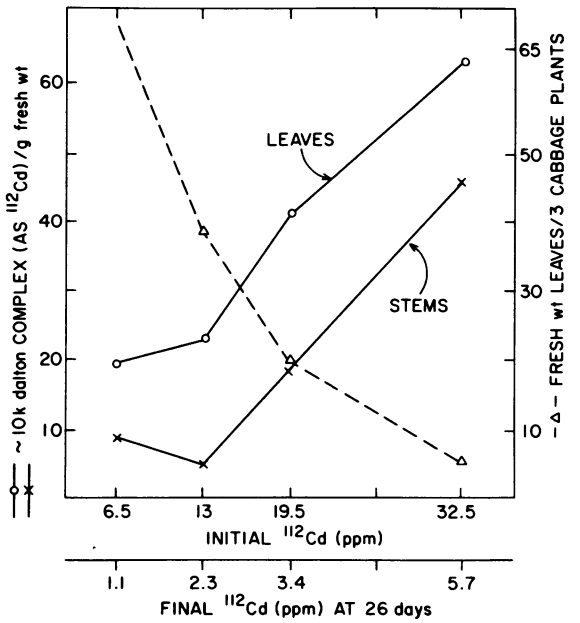

FIG. 3. Induction of an approximately 10,000 mol wt Cd complex in leaves and stems of cabbage. Depletion of Cd occurred during the course of the experiment. See "Results." Each sample was separated on Sephadex G-50, and μg ^{112}Cd occurring in the region of 10,000 mol wt were determined.

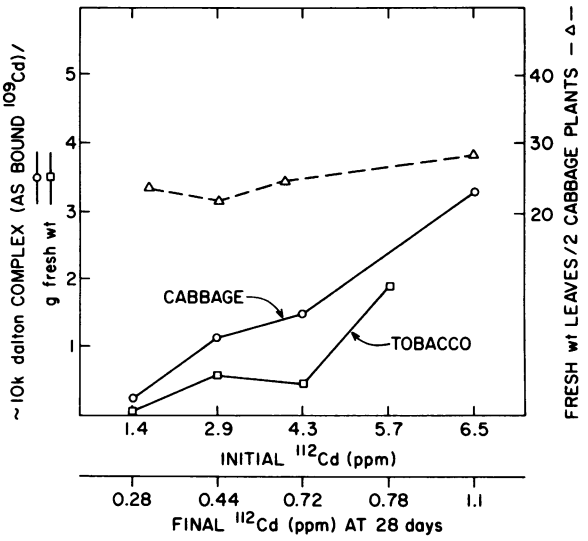

FIG. 4. Induction of an approximately 10,000 mol wt Cd complex in leaves of cabbage and tobacco measured after ^{109}Cd binding. As in the experiment described in Figure 3, depletion of Cd occurred during the experimental period. Each sample was separated on Sephadex G-50, and ^{109}Cd (cpm) occurring in the region of 10,000 mol wt was determined.

26 d showed that the amount of complex (as ^{112}Cd) in leaves and stems was higher where higher levels of Cd had been supplied during growth (Fig. 3). Substantial growth inhibition occurred at >6.5 μg Cd per ml (initial Cd concentration).

To avoid the large growth-inhibiting effects of high Cd levels, similar experiments were performed using 1.4 to 6.5 μg/ml Cd (initial concentration). Levels of complex observed in G-50 profiles from the 1.4- and 2.9-μg/ml Cd experiments were too low for accurate determination of complex as ^{112}Cd due to dilution during fractionation. To improve the sensitivity of detecting low levels of complex, the experiment was repeated, and, after $CHCl_3$:BuOH extraction, ^{112}Cd of complex was labeled by binding of ^{109}Cd.

Complex (as ^{109}Cd) was found to be higher in leaves of cabbage and tobacco where levels of Cd supplied were higher (Fig. 4). Also, more complex (as ^{112}Cd) was recovered from leaves of plants supplied 6.5 μg/ml than from those supplied 4.3 μg/ml (data not shown). Each extract was incubated with the same amount of ^{109}Cd for the same period of time during ^{109}Cd binding. This was found to be important, since the extent of exchange was somewhat time-dependent. If ^{109}Cd was added to homogenates before $CHCl_3$: BuOH extraction, about 20% of the label was recovered, with insoluble materials formed during extraction. No ^{109}Cd was found in the organic phase. No loss of complex (as ^{109}Cd or ^{112}Cd) occurred during extraction. In steady-state exposure experiments described below (constant 7 μg/ml exposure), the same qualitative pattern was observed for complex monitored as ^{109}Cd and ^{112}Cd. Introducing isotopic Cd by *in vitro* binding was, therefore, found to be an effective means of monitoring low levels of complex.

Depletion of Cd from the growth medium occurred during the course of all the experiments described thus far. Depletion is thought to be due to adsorption and uptake by plants and precipitation of Cd salts from solution. Facilities for testing various concentrations of Cd while maintaining a constant level of exposure were not practical. However, induction of complex under conditions of constant exposure to 7 μg/ml Cd was tested to examine time-related effects of steady-state exposure. This level of Cd was chosen so that complex could be monitored as both ^{109}Cd (after ^{109}Cd binding *in vitro*) and ^{112}Cd. Twelve cabbage seedlings were grown in 33 L of nutrient solution containing 7 μg/ml Cd. Over the course of these experiments, levels of Cd and Ca (the latter a monitor of nutrient—assayed by atomic adsorption spectrophotometry) were found to remain constant. Two plants were sampled after 8, 14, 21, 29, 39, and 44 d of exposure. Recovery of Cd in complex and in <2,000 mol wt materials (free-metal region of G-50 profile) from leaves and roots is shown in Figure 5. Complex accumulated slowly in leaves until about 14 d, after which it increased more rapidly (Fig. 5A). Metal associated with <2,000 mol wt materials also increased slowly for about 14 d, after which it continued to increase for the duration of the experiment. In roots (Fig. 5B), a similar lag in the formation of complex and Cd associated with <2,000 mol wt materials was seen, but, unlike that in leaves, the 10,000 mol wt complex of roots appeared to decrease after reaching a maximum at about 20 d. Only 39- and 44-d treated plants showed overt signs of stress—slight chlorosis and increased anthocyanin production. Fresh weight of leaves and roots continued to increase over the 44-d period, but some inhibition of growth was observed. In separate control experiments, plants grown for 44 d under the same conditions, but, in the absence of Cd, they weighed about 1.7 times as much as plants grown in the presence of 7 μg/ml Cd.

The nature of the Cd associated with <2,000 mol wt materials observed in these experiments has not been determined. However, in other experiments, cabbage and tobacco plants were pulse-labeled with ^{109}Cd (1 μg/ml Cd) and, subsequently, were maintained in Cd-free nutrient solution for 10 d. To determine whether the metal associated with the <2,000 mol wt materials observed consisted of free or complexed Cd, extracts and <2,000 mol wt components of leaves of these plants were examined by paper electrophoresis. Electrophoresis was for 1 h at 400 volts on Whatman 1 paper using 0.05 M NaOH/acetic acid (pH 5.56) as buffer. In this system, most standard ^{109}Cd moves to the anode (R_F 0.3), but about 20% remains at the origin—presumably bound to exchange sites on the paper. The 10,000 mol wt Cd complex of cabbage and tobacco remains at the origin under these conditions of electrophoresis. Most of the label in leaf extracts of pulse-labeled cabbage and tobacco remained at the origin. About 10 to 20% moved to the cathode (R_F 0.04 to 0.5), and about 1% moved to the anode (R_F 0.3). Most of the metal associated with <2,000 mol wt materials from both tissues moved to the cathode (R_F 0.04 to 0.5). The migration of about 10% of the label was like that of free Cd. Similar results were found for fractions from leaves of pulse-labeled pea. These observations suggest that the Cd associated with <2,000 mol wt materials observed in pulse-labeled (1 μg/ml Cd) tissues is primarily present as complexed Cd and not as free metal.

The possibility that the ligand of the 10,000 mol wt complex from cabbage and tobacco leaves might be constitutive was tested by adding ^{109}Cd to extracts of leaves of plants grown in the absence of Cd. Gel filtration chromatography of ^{109}Cd-treated extracts revealed the presence of label resembling complex (Fig. 6). The $A_{255/280}$ in the 10,000 mol wt regions were 1.05 and 1 for cabbage and tobacco, respectively. These results, which were observed in controls of several experiments, suggest the constitutive nature of the ligand.

Extractable (19% HCl [w/w]) Cd associated with insoluble materials from leaves of plants supplied 6.5 μg/ml Cd was only about 3-fold that recovered from plants supplied 1.4 μg/ml Cd (Table I). In contrast, Cd recovered as 10,000 mol wt complex from 6.5 μg/ml Cd-supplied plants was 40-fold that observed in 1.4 μg/ml Cd-supplied plants. The ratio of Cd of 10,000 mol wt complex to that extractable from insolubles (Table I) reflects the

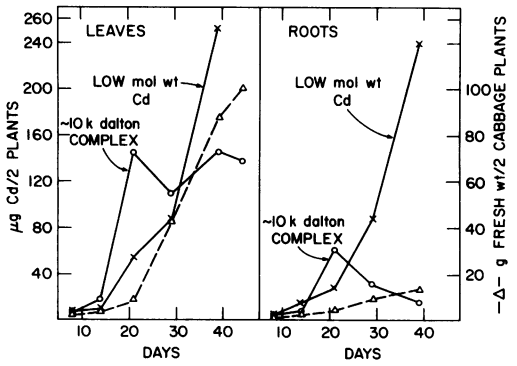

FIG. 5. Time course of occurrence of approximately 10,000 mol wt and <2,000 mol wt Cd in leaves and roots of cabbage supplied constant 7 μg/ml Cd.

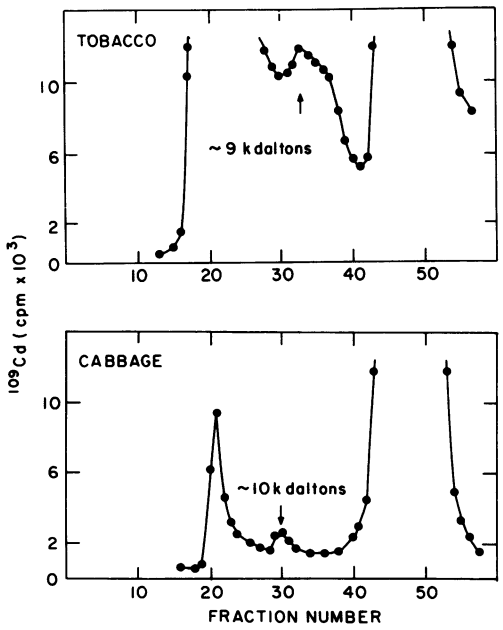

FIG. 6. Apparent constitutive nature of the approximately 10,000 mol wt complex ligand in cabbage and tobacco. Extracts of plants grown in the absence of Cd were labeled with ^{109}Cd to allow detection of ligand after metal binding.

Table I. *Homogenate Insoluble and 10,000 Molecular Weight Complex Cd Recovered from Cabbage and Tobacco Leaves after 28-Day Exposure to Cd*

	Exposure[a]		Extractable Cd of Insolubles	10,000 Mol Wt Complex Cd	10,000 Mol Wt Complex Cd / Extractable Cd of Insolubles
	Initial	Final			
	μg/ml		*μg ^{112}Cd*	*μg ^{112} Cd*	*ratio*
Cabbage					
	1.4	0.28	4.7	4[b]	0.82[b]
	2.9	0.44	8.8	8[b]	0.85[b]
	4.3	0.72	12.7	86	6.8
	6.5	1.1	13.4	174	13.0
Tobacco					
	4.3	0.72	24.3	9.6	4.0
	5.7	0.78	21.3	211	9.9

[a] Depletion of Cd from the growth solution occurred during the course of the experiment; see "Results."

[b] Estimates.

apparent saturation of what are probably cell-wall exchange sites under conditions which induce formation of 10,000 mol wt complex. Oxidation of samples of insoluble materials (after extraction with 19% HCl [w/w]) indicated that 90% or more of the bound Cd was released during acid extraction.

Animal Cd-metallothionein binds the metal in mercaptide bonds with cysteine. Cousins *et al.* (9) have demonstrated the induction of thionein in rats by feeding ^{35}S and ^{112}Cd. Similar experiments were performed here. Two cabbage seedlings which had been grown for 20 d in nutrient solution containing 7 μg/ml Cd were exposed to 0.85 mCi of carrier-free (^{35}S) Na_2SO_4 in 100 ml of nutrient solution containing 7 μg/ml Cd and lacking trace elements. Similarly, two seedlings were incubated with 1.3 mCi of carrier-free (^{32}P) H_3PO_4 in nutrient solution containing 7 μg/ml Cd and lacking PO_4. Nearly all of the ^{32}P and 75% of the ^{35}S were taken up before the plants were returned to complete nutrient solution containing 7 μg/ml Cd. Leaves from one plant of each experiment were homogenized at 4 and 9 d after labeling. A composite elution profile of the 4-d extracts is shown in Figure 7. A peak of ^{35}S was observed coincident with complex (^{112}Cd), while little ^{32}P was observed in this region. The small amount of ^{32}P in the region was not precisely coincident with complex. The ratio of ^{35}S in the region of the 10,000 mol wt complex to that in the region of <2,000 mol wt materials for the 4-d sample was 0.012. That for the 9-d sample was 0.035. For ^{32}P, the ratios were 0.009 and 0.01, respectively. Coincidence of ^{35}S and ^{112}Cd in the 10,000 mol wt region of the profile of a root extract was also observed (data not shown). Complex (as ^{112}Cd) increased by about 5% between 4 and 9 d after labeling in both the ^{35}S and ^{32}P experiments. These results are consistent with the induction of a sulfur-containing ligand under the conditions tested.

Cadmium-metallothioneins are generally absent from body fluids of animals. It was, therefore, of interest to determine whether the 10,000 mol wt complex observed in cytosol (21) and leaf extracts of plants was present in the vascular sap of Cd-contaminated plants. After removal of leaves of plants used for the induction experiments described in Figures 3 and 4, sap exuding from the cut surfaces of stems was collected in some cases—the first 50 μl of sap from each stem was discarded. The concentration of Cd in exudates collected is shown in Table II. The exudate of plants supplied 19.5 μg/ml Cd (Fig. 3) was applied to Sephadex G-50 after addition of ^{109}Cd to effect ^{109}Cd binding in complexes which might be present. No evidence was found for the presence

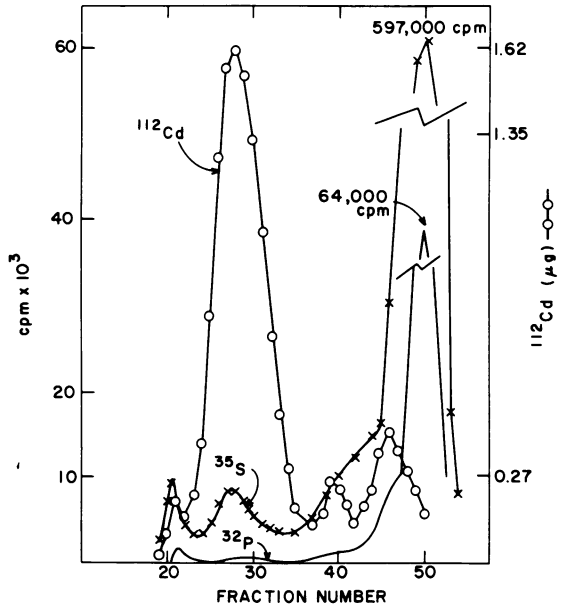

FIG. 7. Composite of elution profiles obtained for extracts of plants supplied ^{112}Cd and ^{35}S and plants supplied ^{112}Cd and ^{32}P.

Table II. *Cd Recovered from Stem Exudates of Cd-Treated Cabbage*

Exposure of plants was for 26 d under conditions in which depletion of Cd from the growth medium occurred.

Initial Exposure	Cd in Stem Exudate
μg/ml	
Control	ND[a]
0.1	0.05
0.5	0.2
2.9	1.15–2.05
6.5	0.9–1.7
13.0	7.7–7.3

[a] Not detected.

of 10,000 mol wt Cd complex in this exudate. All the Cd eluted in the region of <2,000 mol wt compounds and free metal.

DISCUSSION

In this study, the production of Cd complexes in plants was examined as a function of the level and time of exposure of the plants to the metal. The main focus was on the formation of complexes having an apparent mol wt of about 10,000. As noted in the introduction, complexes similar to those described here have been observed by us and others in a variety of tissues. However, as noted previously, we have not observed them in all tissues examined (21).

A 10,000 mol wt Cd complex was easily shown to be a major component of the total soluble metal recovered from leaves (Fig. 1), stems, and roots of cabbage plants grown in liquid culture in the presence of 11 μg/ml Cd. Less of the soluble Cd of tobacco leaf extracts obtained from plants grown under the same conditions was present in a complex having a similar mol wt (Fig. 2). In both cases, about 3 times as much A_{255} as A_{280} was found to be coincident with 10,000 mol wt complex, a result which is consistent with metal-ligand interaction through mercaptide bonds like that found in Cd-metallothionein (16). No zinc was found coincident with 10,000 mol wt complex. In animals, metal from Zn metallothionein is displaced by Cd (3, 14).

In this study, we found that extraction of crude homogenates with $CHCl_3$:BuOH (to remove high mol wt compounds of low

polarity) removed substantial material without loss of 10,000 mol wt complex or metal associated with <2,000 mol wt materials. Extraction reduced the color and viscosity of homogenates. Ethanol:$CHCl_3$ extraction has been used to clarify kidney homogenates without loss of Cd-metallothionein (14). Attempts to purify 10,000 mol wt complex from homogenates by metal chelate affinity chromatography were unsuccessful. Both Cd- and Zn-charged columns were prepared and tested using the conditions described by Porath *et al.* (18). Transferin was bound to and released from the Zn-loaded column essentially as described by Porath, but this column did not bind substantial 10,000 mol wt complex of extracts or that recovered from G-50. Free Cd was tightly bound and only eluted with EDTA.

Results of experiments performed to study induction of the 10,000 mol wt complex indicated that its formation responded to both the amount of metal present during exposure (Figs. 3 and 4) and the time of exposure of plants (Fig. 5). In some of these experiments, high, growth-inhibiting levels of Cd were used so that complex could be clearly detected and quantitated from gel filtration profiles. However, evidence was also obtained for concentration-dependent induction where moderate levels of Cd, which did not greatly reduce growth (fresh weight), were tested, and ^{109}Cd binding was utilized to enhance the detection of 10,000 mol wt complex (Fig. 4).

The relationship between the amount of 10,000 mol wt Cd complex formed in cabbage and the time of exposure of plants to constant 7 μg/ml Cd was examined to study the effect of constant exposure to a single concentration of metal and the changes in Cd species formed over an extended exposure period. In leaves and roots (Fig. 5), a lag of about 14 d was observed before the rate of complex formation began to increase rapidly. The finding that metal associated with <2,000 mol wt materials showed a similar lag suggests that, under the conditions tested, the metal reaches soluble compartments within the plant and is complexed slowly even though uptake into the cell-wall spaces of the root epidermis and cortex may be rapid. Roots of exposed plants were thoroughly washed with H_2O (at 23°C for 30 min) prior to homogenization to remove external and free space metal. Two other aspects of results described in Figure 5 are of interest. First, in leaves, 10,000 mol wt complex rapidly increased between 14 and 21 d, and then it remained constant. This may reflect a balance between synthesis and turnover of complex resulting from toxicity. Total soluble metal (sum of complex and <2,000 mol wt Cd) continued to increase during the experiment. Second, the level of complex in roots appeared to decline after 21 d. This may also reflect turnover, or perhaps transport, out of the root. Experiments similar to these, but using constant exposure to lower levels of ^{109}Cd (≤1 μg/ml Cd), are needed to clarify these results. The concentration of available Cd in relatively uncontaminated soils is thought to be about 0.3 μg/g, while soils close to pollution point sources may contain 100 times as much available Cd (20). The relationship between available Cd and bioavailability has been discussed by Bingham (2).

Both ^{112}Cd and ^{109}Cd (the latter added to extracts) were monitored in the 7 μg/ml, constant-exposure experiments to provide evidence that ^{109}Cd binding could be used to monitor complex both qualitatively and quantitatively. The ratios of the 10,000 mol wt to <2,000 mol wt-associated ^{109}Cd observed were compared with those of ^{112}Cd observed in roots and leaves at all the time samples described in Figure 5. Ratios computed from ^{109}Cd data were 1.2 ± 0.16 times those obtained from ^{112}Cd data. Thus, isotope binding appears to be a reliable monitor of Cd in 10,000 mol wt and <2,000 mol wt materials.

A substantial amount of Cd was found in association with <2,000 mol wt materials recovered from cabbage plants exposed to both high and moderate Cd levels (initial concentrations, 6.5 to 32.5 μg/ml and 1.4 to 7.5 μg/ml, respectively). In these experiments, the <2,000 mol wt fraction contained 10 to 40% of the Cd eluting from G-50. Similarly, Cd has been observed in this fraction from plants pulse-labeled with ^{109}Cd—as 1 μg/ml Cd (21). Also, in the present study, cabbage and tobacco plants were pulse-labeled with ^{109}Cd (1 μg/ml Cd) to prepare extracts, 10,000 mol wt complex, and <2,000 mol wt materials for analysis by paper electrophoresis. Results of these efforts suggest that Cd associated with <2,000 mol wt materials is present as complexed Cd and not as free metal (see "Results" for details). Further study is required to determine if Cd present in the <2,000 mol wt fraction of cabbage grown continuously in the presence of high and low concentrations of the metal exists as free or complexed Cd.

Cd was extracted from insoluble materials recovered after homogenization of tissues supplied constant 7 μg/ml Cd. In leaves, Cd recovered in this fraction increased only 1.7 times between 8 and 39 d, while 10,000 mol wt complex and <2,000 mol wt material-associated Cd increased 24 and 42 times, respectively. Corresponding values for roots were 0.2, 4.2, and 100, respectively. Also, Cd associated with insoluble material (HCl extractable Cd) of cabbage plants supplied 1.4 and 6.5 μg/ml Cd differed by a factor of 3 while that recovered as 10,000 mol wt complex Cd differed by a factor of 40 (Table I). These results suggest rapid saturation with Cd of insoluble-material binding sites under the conditions of exposure studied. In earlier experiments (21), protoplasts isolated from pulse-labeled plants (^{109}Cd as 1 μg/ml Cd) contained less than 5% of the label in the leaves from which they were prepared. The bulk of the remaining label was recovered from protoplast isolation medium as an 80% ethanol precipitate, suggesting that most of the Cd in pulse-labeled leaves was associated with cell-wall polysaccharides, which were partially degraded and released during protoplast preparation. Results obtained from these low-level exposure, pulse-labeling studies and those reported here from high- and moderate-level, continuous-exposure experiments suggest that cell-wall polysaccharides are a first, but saturable, site for Cd binding in these plants.

Thioneins of humans and animals are thought to be constitutive components whose synthesis is induced by administration of inducer metals. A portion of the Cd-thionein, which is observed after high levels of Cd have been administered to animals, may derive from endogenous Zn-thionein, whose metal has been displaced by Cd. In this study, control plants were shown to contain a small amount of a 10,000 mol wt complex when ^{109}Cd was added to extracts of these plants. Similar results were found with extracts which were heated at 80°C for 2 min prior to addition of ^{109}Cd, so that it is unlikely that complex formation occurs in extracts on addition of ^{109}Cd. Further study is needed to establish that the apparently constitutive component is identical to inducible complex.

The induction of the thionein ligand in animals has been demonstrated in several laboratories after simultaneous administration of ^{112}Cd and ^{35}S or [^{35}S]cysteine (9, 13). Similar experiments were performed here with ^{112}Cd and ^{35}S. Parallel experiments with ^{112}Cd and ^{32}P were performed to rule out the possibility that the ligand of the 10,000 mol wt complex contains phosphate. Both polyphosphates and nucleic acids (23) have been suggested as ligands for heavy metal binding in plants and algae. Results indicated that the ligand contains sulfur but probably not phosphate (Fig. 7). The amount of ^{35}S which was found to be coincident with 10,000 mol wt complex ^{112}Cd after gel filtration increased with time after labeling, as did complex ^{112}Cd. These results suggest the presence of sulfur in the ligand. Dabin *et al.* (10) observed that both ^{109}Cd and ^{35}S of soluble extracts of rice roots (from plants supplied 0.01 μg/ml Cd, ^{109}Cd, and [^{35}S]cysteine) were eluted in the void volume from Sephadex G-25. The exact size of these ≥5,000 mol wt components was not determined.

Cadmium-metallothionein in animals is thought to be localized in the cytosol of cells of various tissues but to be absent from body

fluids. A low mol wt form of the metal has been found in biliary excretions of Cd-treated rats and is thought to be the species of the metal excreted to the feces (8). Here, we examined exudates collected from cut stems of Cd-contaminated plants and observed no 10,000 mol wt Cd in these exudates. Only low mol wt Cd species were detected, even though the concentration of Cd in exudates was similar to that initially added to the growth medium (Table II). Therefore, like Cd-metallothionein, the 10,000 mol wt complex observed in cabbage appears to be found only intracellularly. Previously, we examined the subcellular location of Cd in several plant tissues, including tobacco leaves (pulse-labeled plants), and found Cd to be present in the cytosol fraction but absent from vacuoles and chloroplasts (21). Attempts to prepare vacuoles from ^{109}Cd pulse-labeled cabbage leaves produced contaminated vacuole preparations which, nevertheless, showed no evidence of enrichment with Cd (data not shown). Experiments are in progress to determine whether long-term exposure of plants to low and high levels of Cd results in vacuolar accumulation of the metal.

Characterization of the 10,000 mol wt complex is in progress and will be described in detail in another report. Briefly, attempts at purification of cabbage complex by ion exchange chromatography have not been successful. Conditions similar to those used by Bartloff *et al.* (1) to apply and elute G-50 separated complex from both DEAE cellulose and DEAE Sephadex do not allow elution of the complex from the resin. However, similar to the finding of Bartloff *et al.*, gel electrophoresis on pH 8.8 gels results in migration of the complex with the dye front. The 10,000 mol wt complex observed here is heat-stable and relatively protease-insensitive and shows A_{250}, which is reversibly lost on titration from pH 7.8 to 2. Mercury, but not Zn, causes a similar loss of A_{250} (G. Wagner, unpublished). All of these chemical properties are characteristic of animal Cd-thionein. It is noted that a 10,000 mol wt complex similar to those described here has been found in pea seeds. In these experiments, 3-d-old pea seedlings (cv. Alaska) growing in the nutrient solution described in "Materials and Methods" were pulsed with ^{109}Cd in H_2O for 10 h and, subsequently, allowed to grow to maturity in nutrient solution lacking Cd. Mature seeds contained a higher level of Cd (most as 10,000 mol wt complex) than did pods or adjacent leaves or stems, suggesting remobilization of metal from old leaves to seed (data not shown). Remobilization of nickel (a metal which, like Cd, is readily taken up by plants) has been demonstrated in soybean (5).

In summary, from the results of this study and those presented in an earlier report, the ligand of the approximately 10,000 mol wt Cd complexes observed in various plants appears to be constitutive and inducible and to contain sulfur. The complex appears to be restricted to intracellular space, probably the cytosol. All of these properties, as well as the chemical characteristics mentioned above, are like those of animal Cd-thionein.

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