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Computer-Simulated Evaluation of Possible Mechanisms for Quenching Heavy Metal Ion Activity in Plant Vacuoles

I. Cadmium

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ABSTRACT

Various mechanisms have been suggested for the quenching of Cd ion activity in plant vacuoles. These include solution complexation with organic acids and sulfhydryl-containing peptides and precipitation as sulfides. Because direct experimental support for these mechanisms is lacking and difficult to obtain, we have used a computer model to evaluate the quenching role of possible organic and inorganic ligands of tobacco cultured cells exposed to Cd. Results of this thermodynamic evaluation, which assumes that a chemical equilibrium state is met in the vacuole, support the conclusion that sulfhydryl-containing peptides and certain organic acids may form soluble Cd complexes. Although complexation of malate and oxalate with Cd is predicted to be less significant, citrate in the concentration range encountered in the tobacco cultured cell vacuoles has high potential for forming soluble complexes with Cd over the entire possible vacuolar pH range, especially 4.3 to 7.0, even in the presence of low levels of Cd-binding peptides. In addition, results show that inorganic chloride, sulfide (if present), and phosphate may also act to sequester Cd ion activity in the vacuole by forming soluble Cd-Cl and insoluble CdS and Cd-phosphate.

Understanding the fate of Cd in plants is of interest because of concerns of Cd transfer from plants to animals and man. Recent studies suggest that heavy metals accumulated in the higher plant are mainly compartmentalized in the vacuole (5, 7, 9, 27). Various mechanisms have been proposed to account for the accumulation of these potentially toxic heavy metal ions in the plant vacuole (18, 30). In general, these mechanisms include formation of soluble metal-organic acid complexes (4, 9, 14) or metal-phytate (25, 26), formation of metal-peptide or metal-peptide-sulfide complexes (9, 18, 27), or precipitation of metal-sulfides (2, 21, 25).

Support for a particular mechanism of accumulation/sequestration of an ion is gained if the compartment of accumulation/sequestration is verified and if speciation of the ion in that compartment is determined. Both direct and indirect approaches (10) used to determine compartmentation of ions, including heavy metals, can only provide qualitative and quantitative estimates of vacuole contents (28). They cannot identify the species of ion complexes occurring in vacuoles. Therefore, mechanisms proposed on the basis of compartmentation analysis alone are not sufficient to argue the validity of a mechanism of accumulation/sequestration. However, it is possible with computer assistance to simulate the ion species distribution in the plant vacuole and thus to evaluate a proposed mechanism.

Computer calculations have been used to model the chemistry of xylem sap of soybean and tomato (29). Here, we use data obtained previously regarding sap composition of vacuoles from Cd-treated tobacco (*Nicotiana tabacum*) cultured cells and the GEOCHEM-PC computer model (16, 23) to predict ion species of these vacuoles *in vivo*. The prediction of ion speciation in vacuoles of Zn-treated tobacco cultured cells has been considered elsewhere.

MATERIALS AND METHODS

The study was carried out using a computer model, GEOCHEM-PC (16, 23), to simulate the chemical thermodynamic state of the vacuole based principally on chemical data of vacuole content of tobacco suspension cells (9). The GEOCHEM-PC computer model uses an iterative procedure to solve simultaneously all equations describing dissolved and solid species. Its features have been described in detail elsewhere (16). In general, thermodynamic simulations using the GEOCHEM-PC model are made by utilizing the equation for estimating effective ionic strength and the Davies and Helgeson equations (23) for calculating activity coefficients of dissolved species.

The estimated chemical composition of vacuoles under a specific set of experimental conditions in this system is given in Table I. Because complexation reactions are expected to be fast relative to movement of ligands into and out of the vacuole, we assume near chemical equilibrium conditions within the vacuole when cells are disrupted to examine their contents. The selected stability constants for various possible Cd-ligand species assumed to be present in tobacco cell vacuoles are shown in Tables II and III, and the thermodynamic protonation constants for various ligands are listed in Table IV. Concentrations of organic acids and ions were estimated from extracts of Cd-treated cultured cell assuming that 75% of packed cell volume was vacuolar sap and that major ion and organic acid species occur in the vacuole *in vivo* (9). The last assumption (for acids and nutrient ions) is based on earlier studies in which efflux and pulse-chase analyses (15) were used and (for acids, nutrient ions and Cd) on more recent

Table I. *Computer Input Data Describing the Estimated Composition of Tobacco (Nicotiana tabacum) Vacuoles*

Data were obtained for suspension cultured cells exposed to 45 μM Cd for 4 h (see ref. 9).

Metal Ion	Concentration	Ligand Ion	Concentration
	−log C(M)		*−log C(M)*
K^+	1.35	NO_3^-	2.57
Mg^{2+}	2.80	Cl^-	2.11
Ca^{2+}	3.00	SO_4^{2-}	4.00
Cd^{2+}	4.57	PO_4^{3-}	2.96
Zn^{2+}	4.30	$Malate^{2-}$	1.78
		$Citrate^{3-}$	2.25
		$Oxalate^{2-}$	3.34

studies in which direct vacuole/extravacuole analyses were used (9, 10, 27, 28).

Because the vacuolar sap pH varies from plant to plant and is thought to vary with growth conditions (8), our simulation was carried out over the possible vacuolar pH range of 4 to 7. Data were obtained at 0.25 pH unit intervals.

RESULTS AND DISCUSSION

Role of Cd-Binding Peptides

Sulfhydryl-rich proteins called metallothioneins which bind Cd as well as other heavy metals were observed in animals about 30 years ago. These metal-binding proteins are thought to detoxify toxic metals and serve as a storage form for the micronutrients Zn and Cu. More recently, a family of non-metallothionein, sulfhydryl-rich peptides called Cd-binding peptides, Cd-γ-glutamyl peptides, phytochelatins, etc, was observed in plants. A role for these peptides in the detoxification of Cd has been suggested from recent studies (18). Here, we attempt to assess the importance of these Cd-binding peptides relative to that of organic acids and other ligands in binding Cd in plant vacuoles.

Figure 1 describes Cd species distribution at pH 5, a commonly found vacuolar sap pH and that expected in tobacco cultured cells (9), as a function of Cd-binding peptides from 10^{-7} to $10^{-3.5}$ M. The induction of Cd-binding peptides has been shown to be dependent on the time of exposure and concentration of Cd exposure in several systems. Krotz *et al.* (9) reported that after 4 and 36 h exposure of tobacco cells to 45 μM Cd, about 10 and 30% of Cd present was as Cd-peptide, respectively. Here, we calculated the molar concentration of Cd-peptide using these percentages and the experimentally determined concentration of Cd. This calculation does not assume knowledge of the species distribution of peptides present (average *N* number) or the metal-peptide stoichiometry, both of which are unknown.

Recently, Vögeli-Lange and Wagner (27) showed that all of the Cd-peptide and Cd occurring in leaf protoplasts isolated from tobacco seedlings grown in the presence of 20 μM Cd for 1 week was recovered in isolated vacuoles. Earlier, Krotz *et al.* (9) showed that after 3 to 4 d exposure of Datura cultured cells to 30 to 45 μM Cd most of the metal was recovered in isolated vacuoles. A number of studies using electron microscopy techniques have shown the presence of Cd deposits in vacuoles as well as other sites within tissues of plants exposed to high levels of Cd (5, 19, 25, 26). Thus, available evidence suggests that, at least after exposure to moderate or high levels of Cd for periods of days, both metals and induced Cd-peptides are likely to be found in the vacuole. As shown in Fig. 1, if concentrations of other ligands in the vacuole system are constant, the amount of Cd complexed by peptides will depend on the amount of peptide present. According to the GEOCHEM-PC analysis using data from Table I and including the presence of Cd-peptide to complex 10 and 30% of the total Cd, vacuolar concentration of peptide ligand must reach $10^{-5.5}$ and $10^{-5.1}$ M (Fig. 1) for cultured tobacco cells exposed to 45 μM Cd (contain 0.027 mM Cd after 4 h). This simulated estimate is based on the Cd-peptide formation constant of 10^{19} reported by Reese and Wagner for tobacco cultured cells and leaf peptides (20). It is estimated that it would require concentrations of about $10^{-4.4}$ and $10^{-4.1}$ M of Cd-binding peptide to complex the same percentages of total Cd (0.398 mM) in suspension cultured tobacco cells exposed to 600 μM Cd (simulation not shown).

Table II. *Selected Stability Constants Used in the Simulations with the GEOCHEM-PC Model for Cd and Inorganic Ligands Ascribed to Tobacco Suspension Cell Vacuoles*

Ligand	Reaction	log K[a]	Ref.
Chloride	$Cd^{2+} + Cl^- \rightarrow CdCl^+$	1.98 ± 0.03	22
	$Cd^{2+} + 2Cl^- \rightarrow CdCl_2$	2.60 ± 0.10	22
	$Cd^{2+} + 3Cl^- \rightarrow CdCl_3$	2.40 ± 0.10	22
	$Cd^{2+} + 4Cl^- \rightarrow CdCl_4^{2-}$	1.70	22
Sulfide	$Cd^{2+} + S^{2-} \rightarrow CdS(solid)$	27.00 ± 0.10	22
	$Cd^{2+} + HS^- \rightarrow CdHS^+$	7.60 (1.0 M)	22
	$Cd^{2+} + 2HS^- \rightarrow Cd(HS)_2$	14.60 (1.0 M)	22
	$Cd^{2+} + 3HS^- \rightarrow Cd(HS)_3^-$	16.50 (1.0 M)	22
	$Cd^{2+} + 4HS^- \rightarrow Cd(HS)_4^{2-}$	18.90 (1.0 M)	22
Phosphate	$3Cd^{2+} + 2PO_4^{3+} \rightarrow Cd_3(PO_4)_2(solid)$	38.10	11[b]
	$Cd^{2+} + HPO_4^{2-} \rightarrow CdHPO_4$	3.20	11[b]

[a] Values represent the thermodynamic stability constants (no ionic strength indicated) or conditional stability constants (ionic strength indicated in parentheses). Conditional stability constants, to be used as GEOCHEM-PC inputs, were adjusted to thermodynamic constants using the Davies and Helgeson equations (23). [b] Derived from the relevant reactions given by Lindsay (11).

Table III. *Selected Stability Constants Used in the Simulations with the GEOCHEM-PC Model for Cd and Organic Ligands Ascribed to Tobacco Suspension Cell Vacuoles*

Ligand	Reaction	log K[a]	Ref.
Malate	$Cd^{2+} + CH_2COH(COO)_2^{2-} \rightarrow CdCH_2COH(COO)_2$	2.36 (0.1 M)	12
	$Cd^{2+} + CH_2COH(COO)_2H^- \rightarrow CdCH_2COH(COO)_2H^+$	1.34 (0.1 M)	12
Citrate	$Cd^{2+} + (CH_2)_2COH(COO)_3^{3-} \rightarrow Cd(CH_2)COH(COO)_3^-$	5.36	12
	$Cd^{2+} + 2(CH_2)_2COH(COO)_3^{3-} \rightarrow Cd((CH_2)_2COH(COO)_3)_2^{4-}$	4.54 (0.5 M)	12
	$Cd^{2+} + (CH_2)_2COH(COO)_3H^{2-} \rightarrow Cd(CH_2)_2COH(COO)_3H$	2.20 (0.1 M)	12
	$Cd^{2+} + (CH_2)_2COH(COO)_3H_2^- \rightarrow Cd(CH_2)_2COH(COO)_3H_2^+$	0.97 (0.1 M)	12
Oxalate	$Cd^{2+} + (COO)_2^{2-} \rightarrow Cd(COO)_2$(solid)	7.82	3
	$Cd^{2+} + (COO)_2^{2-} \rightarrow Cd(COO)_2$	2.73 ± 0.03 (1.0 M)	13
	$Cd^{2+} + 2(COO)_2^{2-} \rightarrow Cd((COO)_2)_2^{2-}$	4.10 ± 0.10 (1.0 M)	13
	$Cd^{2+} + 3(COO)_2^{2-} \rightarrow Cd((COO)_2)_3^{4-}$	5.10 (1.0 M)	13
	$Ca^{2+} + (COO)_2^{2-} \rightarrow Ca(COO)_2$(solid)	8.59	3

[a] Values represent the thermodynamic stability constants (no ionic strength indicated) or conditional stability constants (ionic strength indicated in parentheses). Conditional stability constants, to be used as GEOCHEM-PC inputs, were adjusted to thermodynamic constants using the Davies and Helgeson equations (23).

Role of Organic Acids

Mathys (14) proposed a model to explain tolerance of certain plant clones to growth in the presence of potentially phytotoxic concentrations of Zn. This model suggests that malate-Zn complexes formed in the cytosol facilitate transfer of Zn to the vacuole where the metal is exchanged with oxalate to finally sequester the metal in the vacuole as stable Zn-oxalate (14). Although intriguing, this model has been questioned because of a lack of direct evidence concerning compartmentation and the nature of ion species in plant vacuoles (30). The Mathys model has apparently not been tested for Cd accumulation. We argued that there is sufficient organic acid in tobacco suspension cells grown in growth-inhibiting and noninhibiting levels of Cd to sequester this metal (9). We also speculated that accommodation of such cells to high Cd exposure in the absence of Cd-binding peptide may be via vacuole complexation with organic acids (9).

Figure 2 shows a computer-simulated speciation of Cd as a function of vacuolar pH based on the concentrations of constituents ascribed to vacuoles (Table I) and to a level of Cd-induced peptide that corresponds to 10% complexation of the total vacuolar Cd after 4 h exposure to 45 μM Cd as described in ref. 9. As shown in Figure 2, malate and oxalate complex about 20 and 7% of total Cd at pH 4, respectively. On the other hand, citrate sequesters 17% of total Cd. Moreover, citrate becomes predominant in complexing vacuolar Cd above pH 4.3. This result strongly suggests the potential of citrate for sequestering Cd in the absence of high levels of Cd-binding peptide. Exposure of tobacco suspension cells to higher levels of Cd (90–600 μM) caused growth inhibition but continued, more rapid accumulation of Cd-peptide after 4 h (data not shown) and presumably higher levels of Cd-peptide in the vacuole. We have observed after gel filtration that extracts of cells exposed to such levels of metal for several days contained only peptide-bound Cd and no free metal (G.J. Wagner, unpublished data). Results shown in Figures 1 and 2, our unpublished observations, and the lack of evidence for such substantial Cd-peptide in extracts of plants grown in a natural environment or under conditions of low Cd exposure (0.1 μM Cd) suggest that under these conditions vacuolar sequestration of Cd may be principally as Cd-citrate. We note that concentrations of Cd-peptides (measured as thiol content) in maize roots exposed to 1 μM Cd were $<5 \times 10^{-7}$ M (24). At such low concentration, little metal would be bound to pep-

Table IV. *Selected Thermodynamic Protonation Constants Used in the Simulations With the GEOCHEM-PC Model*

Ligand	Reaction	log K	Ref.
Malate	$H^+ + CH_2COH(COO)_2^{2-} \rightarrow CH_2COH(COO)_2H^-$	5.10	12
	$H^+ + CH_2COH(COO)_2H^- \rightarrow CH_2COH(COOH)_2$	3.46	12
Citrate	$H^+ + (CH_2)_2COH(COO)_3^{3-} \rightarrow (CH_2)COH(COO)_3H^{2-}$	6.40 ± 0.00[a]	12
	$H^+ + (CH_2)_2COH(COO)_3H^{2-} \rightarrow (CH_2)_2COH(COO)_3H_2^-$	4.76 ± 0.00[a]	12
	$H^+ + (CH_2)_2COH(COO)_3H_2^- \rightarrow (CH_2)_2COH(COOH)_3$	3.13 ± 0.07	12
Oxalate	$H^+ + (COO)_2^{2-} \rightarrow (COO)_2H^-$	4.27 ± 0.00[a]	12
	$H^+ + (COO)_2H^- \rightarrow (COOH)_2$	1.25	12
Sulfide	$H^+ + S^{2-} \rightarrow HS^-$	13.90 ± 0.10	22
	$H^+ + HS^- \rightarrow H_2S$	7.02 ± 0.04	22
Phosphate	$H^+ + PO_4^{3-} \rightarrow HPO_4^{2-}$	12.35 ± 0.02	22
	$H^+ + HPO_4^{2-} \rightarrow H_2PO_4^-$	7.20	22
	$H^+ + H_2PO_4^- \rightarrow H_3PO_4$	2.15	22

[a] ±0.00 means that the constants from independent original publications are the same (12, 22).

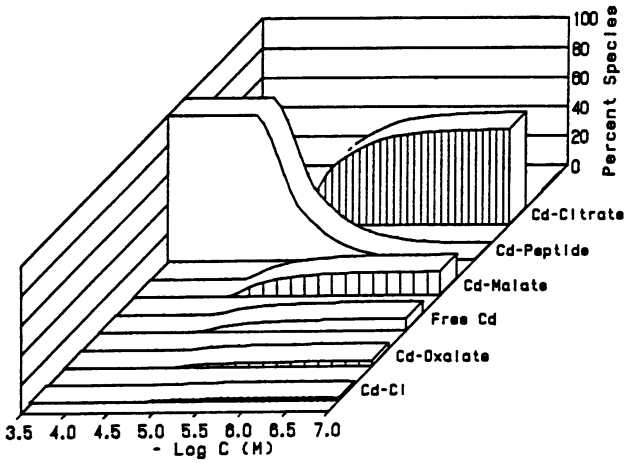

Figure 1. Simulated vacuolar distribution of Cd species with respect to concentration of Cd-binding peptide in the vacuole at pH 5 for cultured tobacco cells exposed to 45 μM Cd for 4 h. All other ligands are assumed to occur at concentrations given in Table I. C (M) is the molar concentration of Cd occurring as peptide-bound Cd as determined by Krotz *et al.* (9) (see "Materials and Methods").

tide in the presence of abundant citrate (Fig. 1). Plants exposed to high levels of the metal in high-exposure experimental and high-pollution conditions may contain substantial Cd-peptide. We note that because protonation constants for peptide were not available, the model assumed 100% unprotonated peptide, and therefore, simulation of Cd-peptide is constant, even at low pH. Based on previous studies of the relationship between pH and binding of Cd to peptide (20), we anticipate that, if protonation constants were considered, the percentage of Cd bound to peptide would decrease below pH 5 and additional metal would occur as free Cd. This would slightly modify Cd-peptide data of Figures 2 and 4 below about pH 4.5.

Figure 2 shows a dominance of citrate in the complexation of Cd, but it does not give the stoichiometry of the complexes. To evaluate the stoichiometry of Cd-citrate complexation, we further simulated the species distribution of Cd-citrate complexes as a function of vacuolar pH and the results are presented in Figure 3. The citrate-coordinated Cd complexes are expressed by three digit numbers on the *z* axis. The first, second, and third digits assigned to coordination complexes represent the number of metal ions, number of ligands, and the number of protons in each complex, respectively. Figure 3 shows that Cd-citrate could be in three of the four coordination modes in the vacuole between pH 4 and 7. This is especially true at pH 4 (60.6, 32.3, and 7.0% of the total Cd for complex species 110, 111, and 112, respectively). However, species 110 is predominant over the entire range of possible vacuolar pH.

Godbold *et al.* (4) reported that the total Zn to citrate ratio in roots of nontolerant *Deschampsia caespitosa* was 1 and that in Zn-tolerant clones the ratio was 1.5 to 2. However, they found, in their attempt to determine the chemical form of Zn, that root saps of both ecotypes showed a similar 1:1 molar ratio of Zn to citrate after gel filtration chromatography. Studies similar to those of Godbold *et al.* (4) have not been reported for Cd tolerant *versus* nontolerant plants. The simulated result shown in Figure 3 suggests that Cd may have a similar complexation stoichiometry with citrate to Zn in cultured tobacco cells exposed to 45 μM for 4 h.

We emphasize that results reported here represent ion types and concentrations found in cultured tobacco (cv Wisconsin 38) cells exposed to Cd as described in ref. 9. Inorganic and organic ion contents are known to vary substantially from plant species to species and according to the type of mineral (particularly N) nutrition conditions (6). However, high or-

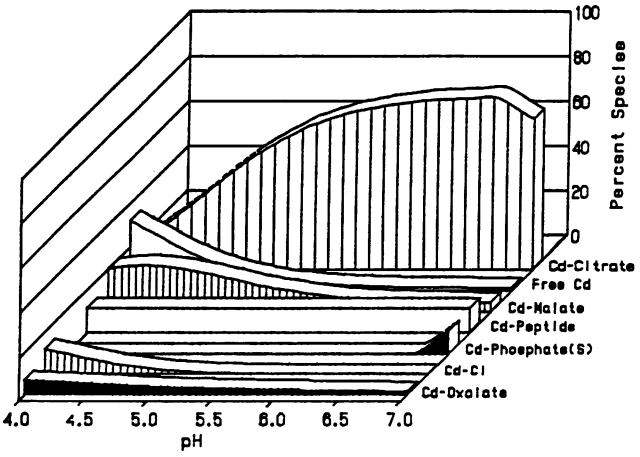

Figure 2. Simulated vacuolar species distribution of Cd complexes as a function of pH in the presence of Cd-binding peptide for cultured tobacco cells exposed to 45 μM Cd for 4 h (vacuole composition described in Table I). Under these conditions, Cd-peptide-bound Cd ($10^{-5.5}$ M) accounts for 10% of the total Cd (see ref. 9 and "Materials and Methods"). S, Insoluble complexes.

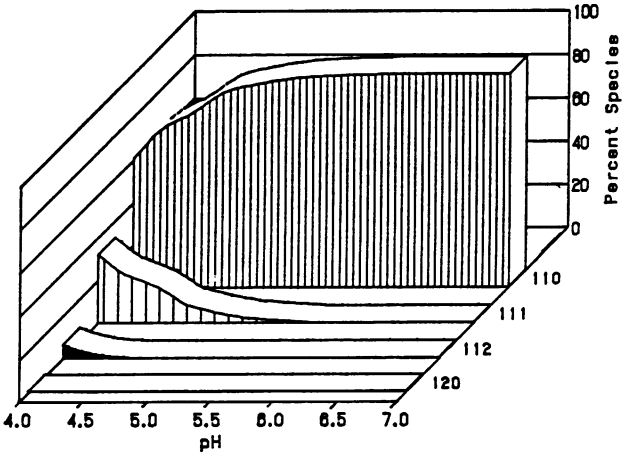

Figure 3. Simulated vacuolar distribution of Cd-citrate species as a function of pH for cultured tobacco cells exposed to 45 μM Cd for 4 h (vacuole composition described in Table I). *z* axis, coordination numbers of the complex. The first digit, from left to right, denotes the number of metal ions in the complex, the second the number of ligands, and the third the number of protons on the ligands.

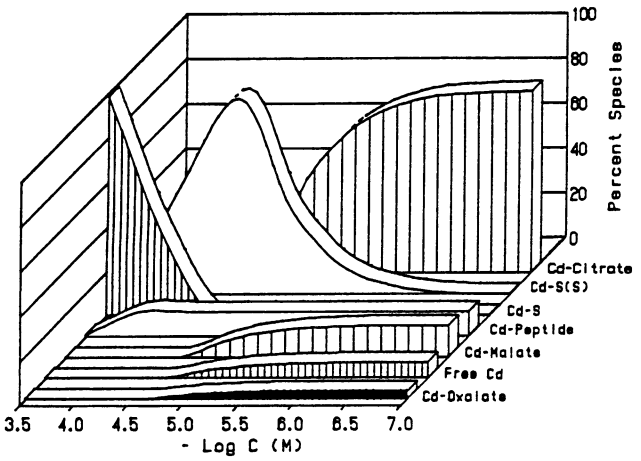

Figure 4. Predicted vacuolar distribution of Cd species with respect to sulfide concentration (if present) in the presence of Cd-binding peptide ($10^{-5.5}$ M) at pH 5, with all other ligands constant at concentrations given in Table I. S, Insoluble complexes.

ganic acid content is common in plants (9) and often substantial citric as well as malic acid occurs. Therefore, results reported here regarding the potential of vacuolar citric acid and Cd-binding peptide for complexing Cd may be relevant to most plant cells. Both direct (vacuolar-extravacuolar distribution; 9, 10, 27) and indirect (efflux and pulse-chase; 15) analyses indicate that a large proportion of major inorganic and organic ion species are contained in the vacuole of plant cells.

Role of Inorganic Ligands

Although organic acids appear to play a significant part in sequestering vacuolar Cd, it is interesting to know whether inorganic anions in the system have a role. As shown in Figure 2, some Cd is complexed as Cd-Cl (12.4% at pH 4). It is known that only 0.5% of Cl^- participates in Cd complexation. Apparently, this result is due to the relatively high concentration of Cl^- ($\simeq$0.01 M) encountered in tobacco cell vacuoles (Table I; see ref. 9). Phosphate, on the other hand, precipitates some of the Cd (10%) at vacuolar pH 7. Because the simulation shown in Figure 2 was made in the presence of Cd-binding peptide at a level that complexes 10% cellular Cd, the results may suggest, depending on the vacuolar pH, a possible role for Cl^- and phosphate in quenching Cd ion activity in a plant cell having low vacuolar Cd-binding peptide content.

Figure 4 shows simulated vacuolar Cd speciation as a function of possible vacuolar sulfide. Heuillet *et al.* (5), using x-ray microanalysis, found that the Cd/S ratio in vacuoles of the marine alga *Dunaliella bioculata* after exposure to very high levels of Cd (890 μM) for 20 d was closer to that of mammalian metallothionein than to inorganic CdS. They concluded that complexes of Cd-binding proteins and sulfide were formed. These deposits were not observed after exposure of cells to 8.9 μM Cd for 20 d. Cd-peptide-sulfide complexes have been recently identified and characterized in yeasts (*Candida glabrata* and *Schizosaccharomyces pombe*) exposed to very high levels (1 mM) of Cd (2, 21). The presence of sulfide stabilizes Cd binding to peptides and occurs in crystallites of CdS with Cd-peptides as surface ligands. It is not known whether low-level Cd exposure can result in significant sulfide accumulation in higher plants. A recent electron probe x-ray analysis of *Lemna minor* L. exposed to 30 μM Cd for 8 d suggested the occurrence of deposits containing Cd and S (25), but the chemical composition of these deposits was not determined. As shown in Figure 4, sulfide, if present, could have a dramatic impact on Cd sequestration in the tobacco cultured cell vacuoles. Sulfide was not quantitatively monitored in tobacco cells studied here. However, acidification of extracts of cells exposed to 45 μM Cd for 4 h did not appear to cause evolution of substantial H_2S. Similar cells exposed to 600 μM Cd or 45 μM Cd for long periods produced significant H_2S during acidification of extracts (G.J. Wagner, unpublished data). At pH 5 about $10^{-5.5}$ M sulfide could precipitate 10% of vacuolar Cd in cells exposed to 45 μM Cd for 4 d. This simulation indicates that sulfide can sequester vacuolar Cd as efficiently as Cd-binding peptide (compare Figs. 1 and 4). Figure 4 also suggests that sulfide can form soluble Cd-S complexes if vacuolar sulfide is greater than $10^{-4.3}$ M. Sulfate and nitrate were found to have no significant complexation potential (data not shown).

Cultured tobacco cells exposed to 45 μM Cd for 4 h also contain Zn at 0.05 μM (Table I; see ref. 9). Figure 5 shows the possible effect of sulfide on Zn speciation. Sulfide could precipitate Zn if its concentration reaches $10^{-4.7}$ M or greater. Again, one can only consider a role of sulfide under low-level Cd exposure conditions if its presence can be demonstrated.

CONCLUSION

In summary, computer simulations using the concentrations of ions and organic acids determined for tobacco suspension cells exposed to 45 μM Cd for 4 h and literature values for complex stability constants suggest the following:

1. Cd-binding peptides have high potential for forming

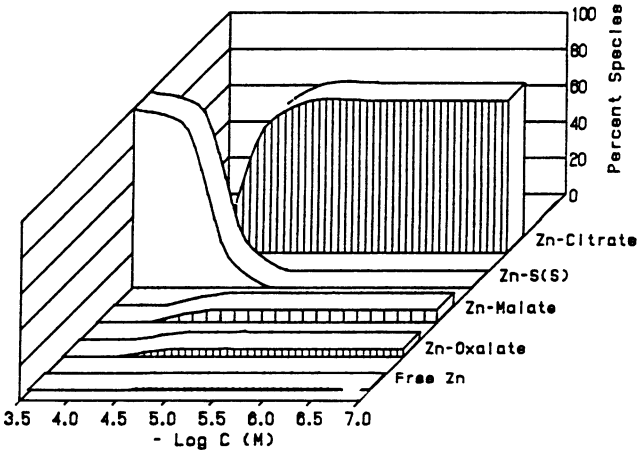

Figure 5. Predicted vacuolar distribution of Zn species with respect to sulfide concentration (if present) in the presence of Cd-binding peptide ($10^{-5.5}$ M) at pH 5, with all other ligands constant at concentrations given in Table I. S, Insoluble complexes.

soluble complexes with Cd under vacuole solute conditions modeled in this study. At high concentrations of peptide ligand, saturation of this ligand is predicted. At low ligand concentrations abundant citrate effectively competes for Cd.

2. Citrate has high potential for complexing Cd between vacuolar pH 4.3 and 7.0 (60% of total Cd complexed by citrate at pH 5). At pH 5, the predicted predominant Cd-citrate species is 1 mol of Cd/1 mol of citrate.

3. Although malate is the most abundant vacuolar organic acid present in the tobacco cultured cells studied, it becomes significant in sequestering Cd only at vacuolar pH <4.5. Oxalate demonstrates little complexation potential with Cd in the vacuole.

4. Inorganic sulfide (if present), phosphate, and chloride also have potential for forming complexes with Cd. Although formation of Cd-Cl is caused by a relatively high concentration of Cl^- ($\simeq$0.01 M encountered in the vacuole), precipitation of CdS and Cd-phosphate is due to high formation constants of the ligands and Cd (Table II).

5. Simulation indicates that Zn present in Cd-treated tobacco cells can also form precipitates with sulfide if vacuolar sulfide is $>10^{-4.7}$ M.

Based on current information in the literature and observations presented here, we speculate that tobacco cells exposed to low levels of Cd as generally occurs in agricultural production would contain little or no sulfide-bound Cd and little or no peptide-bound Cd. Under these conditions, vacuolar citrate would be the predominant form of accumulated Cd. Exposure to high levels of metal as in a high Cd-polluted or high-Cd experimental environment would probably result in synthesis of Cd-binding peptide and significant occurrence of vacuolar Cd-peptide as the major Cd accumulation form. Very high-level Cd exposure (probably growth inhibitory) may result in occurrence of sulfide and the precipitation of CdS in vacuoles and perhaps in other compartments.

Malic acid is often the most abundant organic acid in plants, and citric acid is usually also present in substantial amounts (1). In certain plants, isocitric, aconitic, succinic, oxalic, malonic, or other organic acids may be abundant (ref. 1 and references therein). The metal complexation potential of predominant vacuolar acids may profoundly affect the capacity of a plant species to accommodate/sequester heavy metals. Further study is needed to simulate vacuolar speciation in other plant systems and under various growth conditions.

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