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Metal Complexation in Xylem Fluid[1]

III. ELECTROPHORETIC EVIDENCE

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ABSTRACT

The capacity of ligands in xylem fluid to form metal complexes was tested with a series of *in vitro* experiments using paper electrophoresis and radiographs. The xylem fluid was collected hourly for 8 hours from soybean (*Glycine max* L. Merr.) and tomato (*Lycopersicon esculentum* Mill.) plants grown in normal and Zn-phytotoxic nutrient solutions. Metal complexation was assayed by anodic or reduced cathodic movement of radionuclides (^{63}Ni, ^{65}Zn, ^{109}Cd, ^{54}Mn) that were presumed to have formed negatively charged complexes.

Electrophoretic migration of Ni, Zn, Cd, and Mn added to xylem exudate and spotted on KCl- or KNO_3-wetted paper showed that stable Ni, Zn, and Cd metal complexes were formed by exudate ligands. No anodic Mn complexes were observed in this test system. Solution pH, plant species, exudate collection time, and Zn phytotoxicity all affected the amount of metal complex formed in exudate. As the pH increased, there was increased anodic metal movement. Soybean exudate generally bound more of each metal than did tomato exudate. Metal binding usually decreased with increasing exudate collection time, and less metal was bound by the high-Zn exudate.

Ni, Zn, Cd, and Mn in exudate added to exudate-wetted paper demonstrated the effect of ligand concentration on stable metal complex formation. Complexes for each metal were demonstratable with this method. Cathodic metal movement increased with time of exudate collection, and it was greater in the high-Zn exudate than in the normal-Zn exudate. A model study illustrated the effect of ligand concentration on metal complex stability in the electrophoretic field. Higher ligand (citric acid) concentrations increased the stability for all metals tested.

Xylem sap contains many potential complexing compounds in the form of organic and amino acids (10). The ability of these ligands to form metal complexes in nonbiological solutions depends on their concentration and on factors such as competing inorganic and organic compounds, metal concentrations, metal hydrolysis, solution ionic strength and pH, and empirically determined stability constants (3, 4). Metal-complex formation in xylem fluid can be estimated by using theoretical equilibrium chemistry and analytically determined solute concentrations (11); however, experimental data are needed to understand fully the nature of metal binding in xylem sap.

There are mainly two types of measurements used to assay metal complexation in xylem fluid. The first is electrophoretic and accounts for almost all available experimental data (2, 6–8). When a metal and a ligand combine, the resulting complex has a net charge that can be negative, positive, or neutral. In addition, formation of a metal complex can change a metal's electrophoretic mobility even if its charge remains the same. This provides a means of detecting metal binding. Tiffin (9) has reviewed the use of this method to test for the existence of metal complexes in xylem sap for many of the transition metals. This work has included *in vitro* metal additions to the sap as well as testing the sap after growing plants in radionuclide-labeled nutrient solutions.

Electrophoresis is well-suited to studying chemical species such as Fe-citrate because they are slowly exchanging (stable) complexes. The major drawback of paper electrophoresis is that the electric field of electrophoresis provides a harsh environment, and weakly bound, rapidly exchanging complexes are separated under its influence. Tiffin (9) discussed the use of exudate-wetted paper to test for rapidly exchanging metal complexes as opposed to using paper wetted with neutral-salt or buffer solutions.

The second type of measurement for metal binding in xylem sap has been used only in the case of Ca binding. Bradfied (1) measured Ca complexation in apple-tree xylem with a Ca-selective electrode. By measuring total Ca by atomic absorption and free Ca activity with the electrode, he determined that "about" 50% of the Ca in the exudate was organically bound and 50% was free. He also measured total citric and malic acids and calculated that most of the Ca was bound to these two acids. This type of measurement has limited use because only a few ion-selective electrodes are currently available for those metals normally found in the exudate.

The work reported herein was conducted to examine the effects of plant species, Zn phytotoxicity, pH, and exudate collection time on *in vitro* metal complexation in xylem exudate. In addition, a

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model study was used to illustrate metal-complex formation as assayed by paper electrophoresis.

MATERIALS AND METHODS

The details of plant culture and exudate collection were reported previously (10). Briefly, soybean (*Glycine max* L. Merr.) and tomato (*Lycopersicon esculentum* Mill.) plants were grown in a growth chamber for approximately 4 weeks in normal (0.5 μM) and Zn-phytotoxic nutrient solutions (20% Johnson's solution). Xylem exudate was collected hourly for 8 h from the topped plants, filtered (0.45 μm), and then frozen until needed.

A horizontal electrophoresis apparatus similar to the one described by Tiffin (5) was used for electrophoretic assay of *in vitro* metal binding. The electric field was provided by a constant current/constant voltage power supply, and the current was low enough not to require cooling of the papers. Whatman No. 3MM paper was used for all experiments. Specific conditions for each electrophoretic experiment varied and are listed with each figure. Metal binding was assayed by the presence of metal species with different mobility than the hydrated ion.

In all experiments, the pH of each sample preparation (0.5 ml) was adjusted with HCl and NaOH to match the pH of the support solution used to wet the paper. In several preliminary experiments, indicator dyes appropriate for each experimental pH were added to each neutral-salt support solution to test for any pH changes during electrophoresis. No pH changes were observed in the areas on the paper where the radionuclides were migrating. Generally, papers were initially wetted with appropriate support electrolytes, and then the moistened papers were equilibrated in the electrophoresis apparatus for 30 min prior to adding the samples. The support solutions were either the neutral salts KCl and KNO_3 or xylem exudate. The electrolyte in the electrode reservoirs was 20 mM Na-acetate buffer, regardless of the solution saturating the paper.

Carrier-free radionuclide stock solutions (^{65}Zn, ^{109}Cd, ^{54}Mn) were diluted with deionized H_2O so that adding 5 μl of the stock to each 0.5-ml sample provided between 2 and 5 μCi activity. Activity-free, stock metal solutions were used to provide metals at 25 μM. These solutions were prepared from either $Cd(NO_3)_2 \cdot 4H_2O$, $Zn(Cl)_2$, or $Mn(Cl)_2 \cdot 4H_2O$. Nuclides with specific activities (all ^{63}Ni and some Zn, Cd, and Mn preparations) were also used; these solutions were diluted so that 5 μl of the stock added to 0.5-ml samples gave 25 μM metal concentrations and variable activities. All samples were prepared, incubated for 24 h at 0 C, and rechecked for pH stability before being added to the paper for electrophoresis. After electrophoresis the papers were dried and radiographed.

RESULTS AND DISCUSSION

Neutral-Salt System. In the electrophoretic testing, complexes with negative charges move toward the positive pole (anode) and those with positive charges move toward the negative pole (cathode). Solution pH strongly affected the metal binding of all four metals. No binding was observed at pH 3 for Ni or Cd (Figs. 1 and 3); Zn showed several cathodically migrating spots in addition to those moving like the control (Zn salt) treatment (Fig. 2). As the pH of the system increased, more of each metal interacted with the exudate ligands.

Nickel was complexed strongly by exudate ligands at pH 5, 7, and 9 (Fig. 1). An anodically migrating spot at 2 cm indicated the formation of a stable, negatively charged complex. This complex has been studied previously (2, 8, 9) and is possibly a mixed-ligand complex. Although the same Ni complex appears to be present in both soybean and tomato, much more Ni was complexed by soybean exudate from the normal-Zn plants. As the sampling time increased, the proportion of anionic Ni in tomato exudate decreased until none was observed in the 6- to 7-h exudate. Soybean exudate retained a fairly high capacity to bind Ni over the entire sampling period.

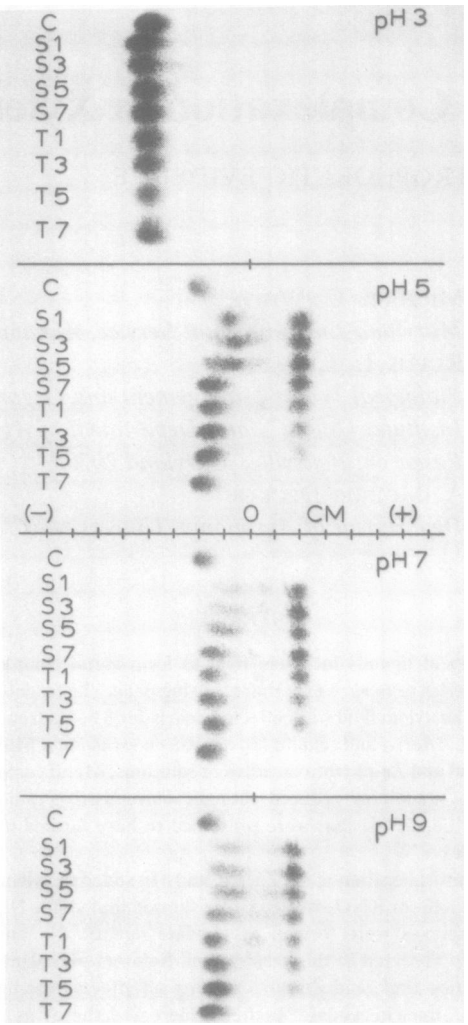

FIG. 1. Radiograph of electrophoretically distributed (neutral salt electrolyte) ^{63}Ni in inorganic controls (C), and soybean (S) and tomato (T) stem exudates collected at 0 to 1, 2 to 3, 4 to 5, and 6 to 7 h after severing the plant stems. Conditions: 10 μl of each pH-adjusted sample [containing 0.09 μCi ^{63}Ni and 14.7 ng Ni/10 μl (25 μM)] added to Whatman No. 3MM paper; papers previously wetted with pH-adjusted (3, 5, 7, or 9) 20 mM KCl; 20 mamp (320–360 v); 2 h; room temperature. X-ray film was exposed for 48 h.

Zinc also formed stable complexes that moved anodically (Fig. 2). However, unlike Ni the anodic Zn spots were found only at pH 7 and 9, and primarily in soybean exudate. The cathodic streaking by Zn illustrates metal complex separation in the electric field. Tiffin's (6) report that he found no electrophoretic [Na-acetate buffer (pH 5.4)] evidence of Zn binding in tomato exudate is confirmed by our work. As suggested in Figure 2, Zn complexes in exudate are not stable at lower pH values in this test system but are quite stable at pH 9.

Like Ni and Zn, Cd formed anodically migrating complexes at the higher pH levels (Fig. 3). The spots on the right-hand side of Figure 3 were exposed longer than the rest of the film during printing to highlight the spots. The anodic Cd complexes moved much further (8.5 cm) than did the anodic Zn (3 cm) and Ni (2 cm) complexes. The anodic Cd spots were much greater at pH 7 and 9 than at pH 5 and, at pH 7, more bound Cd was found in the 0- to 1-h samples of both soybean and tomato (Fig. 3) than in

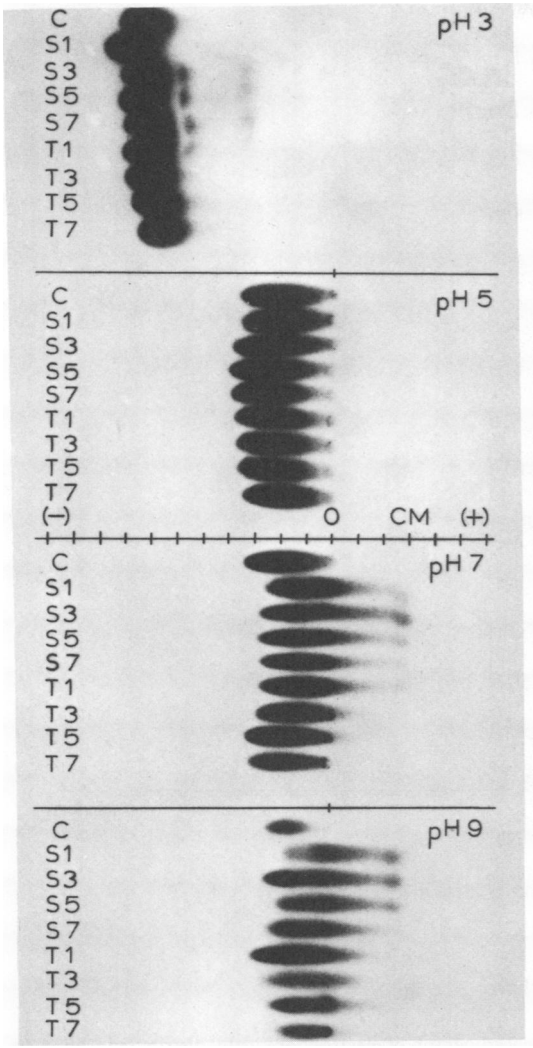

FIG. 2. Radiograph of electrophoretically distributed (neutral salt electrolyte) ^{65}Zn in inorganic controls (C), and soybean (S) and tomato (T) stem exudates collected at 0 to 1, 2 to 3, 4 to 5, and 6 to 7 h after severing the plant stems. Conditions: 10 μl of each pH adjusted sample [containing 0.09 μCi ^{65}Zn and 16.4 ng Zn/10 μl (25 μM)] added to Whatman No. 3MM paper; papers previously wetted with pH-adjusted (3, 5, 7, or 9) 20 mM KCl; 20 mamp (360–400 v); 2 h; room temperature. X-ray film was exposed for 24 h.

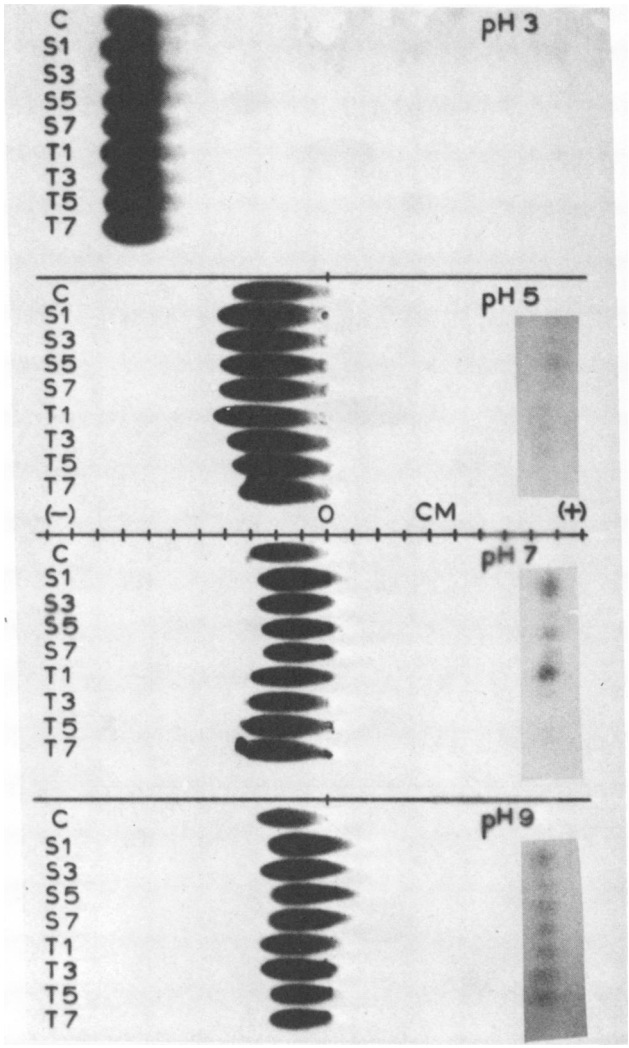

FIG. 3. Radiograph of electrophoretically distributed (neutral-salt electrolyte) ^{109}Cd in inorganic controls (C), and soybean (S) and tomato (T) stem exudates collected at 0 to 1, 2 to 3, 4 to 5, and 6 to 7 h after severing the plant stems. Conditions: 10 μl of each pH-adjusted sample [containing 0.1 μCi ^{109}Cd and 28 ng Cd/10 μl (25 μM)] added to Whatman No. 3MM paper; papers previously wetted with pH-adjusted (3, 5, 7, or 9) 20 mM KNO_3; 20 mamp (360–400 v); 2 h; room temperature. X-ray film was exposed for 7 h. The sections on the right-hand side of the pH 5, 7, and 9 strips were isolated and overexposed during printing to highlight the spots.

later samples. At pH 9, tomato exudate appeared to complex slightly more Cd than soybean exudate. No Mn binding was observed for either soybean or tomato exudate at any of the pH values tested (results not shown). Similar experiments were used to examine the binding of the same four metals by exudate from plants grown in high-Zn nutrient solutions. Generally, much less metal binding (*i.e.* anodic movement) was found in exudate from plants grown in normal-Zn nutrient solutions (results not shown).

These neutral-salt *in vitro* studies have demonstrated that fairly stable metal complexes are formed in xylem sap by natural ligands. However, a number of variables can alter the degree of binding. The individual metal, plant species, system pH, Zn phytotoxicity, and time after cutting the stem all influence metal-complex formation in exudate.

Exudate System. Radionuclide-labeled Ni, Zn, Cd, and Mn in exudate were added to exudate-wetted paper to test for evidence of metal-complex formation in a system that assures a continuous supply of binding ligands.

The effects of time of exudate collection and Zn treatment on the movement of the four metals in soybean exudate are shown in Figure 4. In the 0- to 1-h exudate sample (normal Zn), the anodic Ni complex appears to saturate so that the excess Ni runs cathodically, Zn migrated anodically as a compact negative complex, Cd streaked cathodically from the origin, and Mn moved cathodically in a compact spot. These metals showed similar movement in tomato exudate from the same sampling time and Zn treatment (Fig. 5A, 0–1 h).

At 6 to 7 h, only a small amount of Ni moved anodically in soybean (Fig. 4A), and none moved anodically in tomato (Fig. 5A). The cathodic movement of Cd and Mn was greater at the later sampling time for both soybean and tomato (Figs. 4A and 5A), and Zn changed from an anodic complex to a cathodic complex in soybean and tomato. The excess Ni that ran cathodically in the 0- to 1-h exudate (Figs. 4A and 5A) moved even further toward the negative pole in the later exudate (Figs. 4A and 5A, 6–7 h).

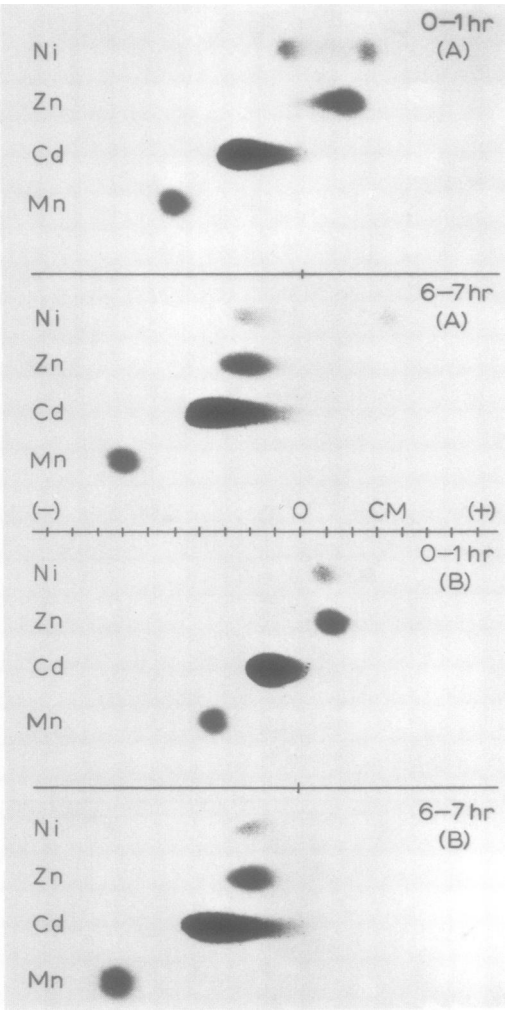

FIG. 4. Radiograph of electrophoretically distributed ^{63}Ni, ^{65}Zn, ^{109}Cd, and ^{54}Mn in soybean exudate added to exudate-wetted paper. The exudate was collected (0–1 and 6–7 h after severing the stems) from plants grown in normal (A)- and high (B)-Zn nutrient solutions. Each metal was added at 25 μM to the original sample so that the following were actually added in the 10-μl additions to the paper: 0.09 μCi ^{63}Ni and 14.7 ng Ni; 0.03 μCi ^{65}Zn and 16.4 ng Zn; 0.05 μCi ^{109}Cd and 28 ng Cd; 0.05 μCi ^{54}Mn and 13.7 ng Mn. Conditions: Whatman No. 3MM paper, pre-wetted with the appropriate exudate [0–1 h = pH 6.1 (A) and 6.3 (B); 6–7 h = pH 5.5 (A) and 5.6 (B)]; 20 mamp (560–680 v); 1 h; room temperature. X-ray film was exposed for 24 h.

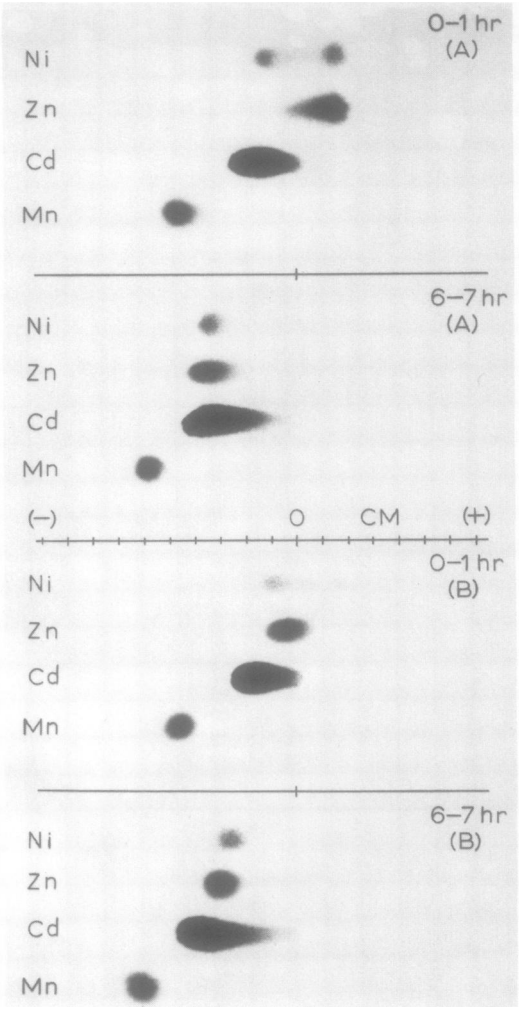

FIG. 5. Radiograph of electrophoretically distributed ^{63}Ni, ^{65}Zn, ^{109}Cd, and ^{54}Mn in soybean exudate added to exudate-wetted paper. The exudate was collected (0–1 and 6–7 h after severing the stems) from plants grown in normal (A)- and high (B)-Zn-nutrient solutions. Each metal was added at 25 μM to the original sample so that the following were actually added in the 10-μl additions to the paper: 0.09 μCi ^{63}Ni and 14.7 ng Ni; 0.03 μCi ^{65}Zn and 16.4 ng Zn; 0.05 μCi ^{109}Cd and 28 ng Cd; 0.05 μCi ^{54}Mn and 13.7 ng Mn. Conditions: Whatman No. 3MM paper, pre-wetted with the appropriate exudate [0–1 h = pH 6.1 (A) and 6.3 (B); 6–7 h = pH 5.5 (A) and 5.6 (B)]; 20 mamp (560–680 v); 1 h; room temperature. X-ray film was exposed for 24 h.

Thus, in the normal-Zn exudate, Ni and Zn clearly showed evidence of the formation of stable, negatively charged metal complexes. The metals ran cathodically in the later samples, which suggests that the concentrations of the binding ligands were too low. The fact that Cd and Mn moved farther cathodically in the later exudate sample may mean that they too were interacting with the exudate ligands in the 0- to 1-h sample, but either their complexes did not have negative charges or they were not bound a high enough percentage of time to have a net negative charge at exudate pH.

In the high-Zn exudate at 0 to 1 h, only one anodic Ni spot was observed in soybean (Fig. 4B), and one cathodic spot in tomato (Fig. 5B). This suggests that Zn phytotoxicity reduced the Ni-binding ligand(s) in the tomato exudate so that insufficient amounts were available to form the stable anodic complex. The single anodic Ni spot in soybean exudate suggests that, in this species, Zn phytotoxicity increased the Ni-binding ligand(s) in exudate, resulting in complete Ni complexation. In addition, Cd and Mn cathodic migration was slightly less than that found at the normal Zn, 0- to 1-h treatment for soybean. In tomato, Cd and Mn movement was similar for both Zn treatments. Zinc movement changed from anodic at 0 to 1 h to cathodic in tomato (Fig. 5) and was unchanged in soybean exudate (Fig. 4).

The changes in electrophoretic metal movement shown in Figures 4 and 5 demonstrate that all four metals interact, to varying degrees, with the exudate ligands. Changes in ligand concentrations due to Zn phytotoxicity or exudate collection time alter the exudate's capacity to form metal complexes. The plant species differ in the ability of their exudate to bind the metals under conditions of Zn phytotoxicity.

Metal-Ligand Interaction Model. Citric acid and divalent metals form a complex having a net negative charge. This acid was selected as a model to provide interpretive information about the apparent dependence of a metal's electrophoretic mobility on exudate ligand concentrations.

Figure 6 shows the effects of increasing citric acid concentrations

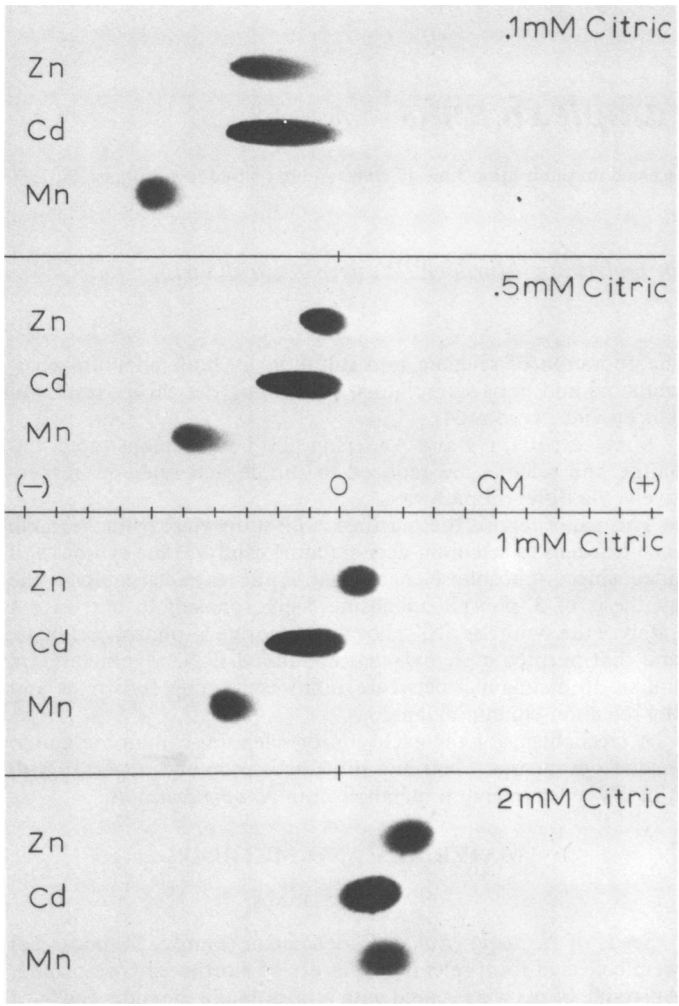

FIG. 6. Radiograph showing the effect of increasing citric acid concentrations on the electrophoretic mobility of 100 μM Zn, Cd, and Mn at pH 6. The support electrolyte was 20 mM KNO_3 (plus citric acid) and each metal solution was prepared so that it matched the pH and concentration of each wetted paper. A 10-μl addition of a metal solution contained one of the following: 0.1 μCi ^{65}Zn and 65.4 ng Zn; 0.05 μCi ^{109}Cd and 112 ng Cd; 0.1 μCi ^{54}Mn and 54.7 ng Mn. Conditions: Whatman No. 3MM paper; 20 mamp (440–460 v); 2 h; room temperature. X-ray film was exposed for 42 h.

(at pH 6) on the electrophoretic mobility of 100 μM concentrations of Zn, Cd, and Mn. The cathodic migration of each metal was completely reversed by the increasing citric acid concentrations. Thus, for each metal there are critical concentrations of a given ligand that will cause a rapidly exchanging metal complex to retain a negative charge. For example, at this pH, Zn required a continuous supply of approximately 1 mM citric acid to stabilize a complex that retained a net negative charge. Cd and Mn required twice this concentration of citric acid (Fig. 6).

Thus, in the exudate-wetted paper experiments (Figs. 4 and 5), the metals moved farther cathodically because the binding ligands in the later exudates and in the high-Zn exudates have decreased below critical concentrations for each metal. The *in vivo* xylem sap ligand concentrations in the high-Zn plants may be lower than in the normal-Zn plants, but the exudate ligands might be high enough to bind considerable amounts of the metals. Test calculations with a computer program showed that much lower concentrations of citric acid than 1 mM are adequate to bind most of the Zn in the presence of Ca and the other competing cations. Therefore, the electrophoretic results do not show a 1:1 correspondence to calculated equilibrium distributions of these metal complexes, and excess ligand is needed to see anionic species.

The work presented here has demonstrated the formation in xylem exudate of slowly exchanging complexes such as the anodic Ni, Zn, and Cd complexes assayed by the neutral-salt test system. Less stable, rapidly exchanging complexes (Mn) were demonstrable only by the more physiologically representative exudate-wetted paper system. The uniqueness of some complexes was also illustrated by this system. The ligands that formed the Ni complex in the 0- to 1-h normal-Zn exudate were saturated and the excess Ni ran cathodically. In contrast, an equal concentration of Zn was fully complexed.

The results of these and related experiments (10, 11), and the work of Tiffin (9) and Cataldo *et al.* (2) provide convincing evidence that unique, labile metal-complex systems exist in xylem fluid for most cations. The micronutrient cations are probably translocated in the xylem sap as metal complexes; the per cent complexed at any instant depends on whether a given metal complex is slowly exchanging or rapidly exchanging under normal *in vivo* conditions. Further, the *in vivo* equilibrium system can be altered by a condition such as Zn phytotoxicity that changes the effective concentrations of potential complexing compounds.

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