

# Metal Complexation in Xylem Fluid<sup>1</sup>

## II. THEORETICAL EQUILIBRIUM MODEL AND COMPUTATIONAL COMPUTER PROGRAM

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### ABSTRACT

Theoretical considerations of metal complex formation in aqueous solutions were used to develop a computer program (CHELATE) to calculate all equilibrium species (free metal ions, metal complexes, etc.) in any user-defined system, such as xylem fluid. Mass-balance equations were established to describe each free metal ion and each free ligand concentration as a function of solution pH, total metal or total ligand, hydrogen-association constants, and the stability constants of known metal complexes. A default data base can be altered by the user to define any desired system covered by the stored equilibrium data. The program can currently handle nine metal ions, 35 ligands, and 500 complex species. The validity of the program was confirmed by using experimental test systems in which free-metal ion activity measurements were made with ion-selective electrodes.

Program CHELATE was used to calculate the distribution of six metals in 0- to 1-hour exudate from soybean (*Glycine max* L. Merr.) and tomato (*Lycopersicon esculentum* Mill.) plants grown in normal and Zn-phytotoxic nutrient solutions. The results indicated that Fe is bound by citric acid, and Cu is bound by several amino acids in the normal-Zn exudate. Most of the Cu in soybean exudate is bound to asparagine and histidine. In tomato, Cu is bound to histidine, glutamine, and asparagine. Zinc, Mn, Ca, and Mg are bound primarily by citric acid and malic acid in both species; the percent bound for these metals is lower than that for Fe and Cu. Zinc phytotoxicity caused equilibrium concentration shifts and resulted in the formation of several additional metal complexes not found in the normal-Zn exudate.

fluid contains many natural complexing compounds and indicates that metal ions should form metal complexes *in vivo* in the xylem sap. There is *in vitro* electrophoretic evidence for stable metal complex formation in xylem fluid (2, 20, 23) but no quantitative estimates of the equilibrium distribution of metals, ligands, metal complexes, and other solutes. In a solution containing several metals and ligands, simultaneous competing equilibria distribute the metals among the ligands, creating numerous complexes. Such a system can be described both qualitatively and quantitatively; however, quantification of all equilibrium species in a multimetal, multiligand system is far more difficult than listing the theoretically permissible (qualitative) species. Although many aqueous species cannot be measured, equilibrium chemistry can be used to estimate metal binding in aqueous solutions.

The degree to which hydrated metal ions associate with various ligands depends primarily on the relative metal and ligand concentrations, solution pH, and the pertinent empirically determined stability constants. These variables have been used to determine equilibrium distributions of metals among ligands in several diverse natural aqueous solutions (6, 9, 21). Most of these systems are too complex to estimate quantitative equilibrium distributions without the aid of computers, and a number of specialized computer programs have been developed (9-11).

The object of building the program reported herein was to generate a model of complexation phenomena in xylem fluid. Only variables considered absolutely necessary to provide a first approximation of metal binding in xylem fluid were chosen, rather than all possible variables. Because a numerical procedure would be used, it was desirable to establish two equations that contained the chosen variables: one for calculating predicted free metal concentrations and one for calculating predicted free ligand concentrations, each in terms of the other. Thus, these equations could be used iteratively to establish free metal and free ligand concentrations that simultaneously satisfied the equilibrium equations for each complex species (*i.e.* metal complexes and protonated ligands) in each defined equilibrium system.

### THEORY

**Derivation of Free Metal and Free Ligand Equations.** Generally, two approaches have been used to solve chemical equilibrium problems. One uses equilibrium constants and mole balance equa-

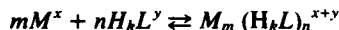
Work reported in a companion paper (24) showed that xylem

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tions (8–10), and the other minimizes the Gibbs free energy equation (15). The equilibrium-constant method was chosen for the present work because equilibrium stability constants have been tabulated for many complexes, and far fewer transformations of raw thermodynamic data are required.

A generalized equilibrium reaction between a metal and a ligand can be described as:



from which the conditional stability constant for the complex is defined as:

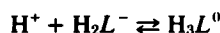
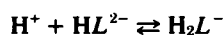
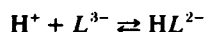
$$\beta_{nm} = \frac{[M_m(H_kL)_n^{x+y}]}{[M^x]^m \cdot [H_kL^y]^n} \quad (1)$$

where  $m$  is the number of metal ions,  $M$  is the metal species,  $x$  is the metal-ion charge,  $n$  is the number of ligands,  $H$  is hydrogen,  $k$  is the number of bound hydrogens,  $L$  is the ligand species,  $y$  is the ligand charge,  $\beta_{nm}$  is the conditional equilibrium stability constant, and  $[ ]$  is the concentration in mol/l. By rearranging equation 1, the inverse relationship between the generalized ligand species ( $[H_kL^y]$ ) and the free metal concentration ( $[M^x]$ ) can be seen:

$$[M^x]^m = \frac{[M_m(H_kL)_n^{x+y}]}{\beta_{nm}[H_kL^y]^n} \quad (2)$$

However, neither  $[H_kL^y]$  nor  $[M_m(H_kL)_n^{x+y}]$  can be measured, so further simplification is necessary.

In an aqueous biological solution, such as xylem sap, a buffer system exists, one in which several acid forms of each ligand are in equilibrium. For example, the following equilibria can be described for a tribasic ligand:



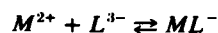
These equilibria can be written to define the hydrogen association/formation constant ( $K$ ), as was done for the conditional stability constant ( $\beta$ ) in equation 1, and then rearranged to show the free ligand concentration (nonprotonated species,  $[L^{3-}]$ ) as a function of the hydrogen ion concentration and its various association constants for the ligand:

$$[L^{3-}] = \frac{[HL^{2-}]}{K_1[H^+]} \quad (3)$$

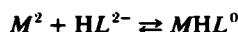
$$[L^{3-}] = \frac{[H_2L^-]}{K_1K_2[H^+]^2} \quad (4)$$

$$[L^{3-}] = \frac{[H_3L^0]}{K_1K_2K_3[H^+]^3} \quad (5)$$

The inverse relationship between the free ligand concentration and the hydrogen ion concentration is shown by equations 3 to 5. Because  $[H^+]$  affects the forms of a ligand present in solution, an equation relating  $[M^x]$  and  $[L^y]$  must include the pH effect. For example, assume that several forms of the above tribasic ligand and a divalent metal ( $[M^{2+}]$ ) have the following reactions:



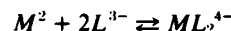
$$\beta_1^a = \frac{[ML^-]}{[M^{2+}] \cdot [L^{3-}]} \quad (6)$$



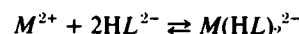
$$\beta_1^b = \frac{[MHL^0]}{[M^{2+}] \cdot [HL^{2-}]} \quad (7)$$



$$\beta_1^c = \frac{[MH_2L^+]}{[M^{2+}] \cdot [H_2L^-]} \quad (8)$$



$$\beta_2^a = \frac{[ML_2^{4-}]}{[M^{2+}] \cdot [L^{3-}]^2} \quad (9)$$



$$\beta_2^b = \frac{[M(HL)_2^{2-}]}{[M^{2+}] \cdot [HL^{2-}]^2} \quad (10)$$

For  $\beta_{nm}^{a,b,c}$ , etc.,  $a$ ,  $b$ , and  $c$  denote the first, second, and third constants for a single metal and a single ligand in a complex. When  $m = 1$ , it is omitted, so increasing  $n$  represents multiple ligands bound to a single metal.

A mass balance equation can be written for the metal, so that the total metal ( $M_T$ ) is equal to the free metal ( $[M^{2+}]$ ) plus all the bound metal:

$$M_T = [M^{2+}] + [ML^-] + [MHL^0] + [MH_2L^+] + [ML_2^{4-}] + [M(HL)_2^{2-}] \quad (11)$$

By rearranging equations 6 to 10 and substituting them into equation 11, the following equation is obtained:

$$M_T = [M^{2+}] + \beta_1^a[M^{2+}][L^{3-}] + \beta_1^b[M^{2+}][HL^{2-}] + \beta_1^c[M^{2+}][H_2L^-] + \beta_2^a[M^{2+}][L^{3-}]^2 + \beta_2^b[M^{2+}][HL^{2-}]^2 \quad (12)$$

And by further rearranging equations 3 to 5 and substituting them into equation 12, one obtains the following equation:

$$M_T = [M^{2+}] + \beta_1^a[M^{2+}][L^{3-}] + \beta_1^bK_1[M^{2+}][H^+][L^{3-}] + \beta_1^cK_1K_2[M^{2+}][H^+]^2[L^{3-}] + \beta_2^a[M^{2+}][L^{3-}]^2 + \beta_2^b(K_1)^2[H^+]^2[M^{2+}][L^{3-}]^2 \quad (13)$$

The final free metal equation is obtained by dividing both sides of equation 13 by  $[M^{2+}]$  and rearranging to get:

$$[M^{2+}] = M_T / (1 + \beta_1^a[L^{3-}] + \beta_1^bK_1[H^+][L^{3-}] + \beta_1^cK_1K_2[H^+]^2[L^{3-}] + \beta_2^a[L^{3-}]^2 + \beta_2^b(K_1)^2[H^+]^2[L^{3-}]^2) \quad (14)$$

Equation 14 illustrates the incorporation of the step-wise equilibrium constants for the addition of both one- and two-ligand molecules (same type) to a single metal ion as well as the pH effect, shows the inverse relationship between free metal ion concentration ( $[M^{2+}]$ ) and free ligand concentration ( $[L^{3-}]$ ), and provides the simplification desired for the generalized free metal equation (equation 2). If several ligands were in the system, then the denominator would be expanded to include all complexes. This would have the effect of further reducing the free metal concentration.

A similar equation can be derived for the free ligand concentration. The total ligand concentration ( $L_T$ ) would be the sum of all the free ligand plus all protonated and metal-bound ligand:

$$L_T = [L^{3-}] + [HL^{2-}] + [H_2L^-] + [H_3L^0] + [ML^-] + [MHL^0] + [MH_2L^+] + 2[ML_2^{4-}] + 2[M(HL)_2^{2-}] \quad (15)$$

Equation 15 can be rewritten in terms of equations 3 to 10 to give:

$$L_T = [L^{3-}] + K_1[H^+][L^{3-}] + K_1K_2[H^+]^2[L^{3-}] + K_1K_2K_3[H^+]^3[L^{3-}] + \beta_1^a[M^{2+}][L^{3-}] + \beta_1^bK_1[H^+][M^{2+}][L^{3-}] + \beta_1^cK_1K_2[H^+]^2[M^{2+}][L^{3-}] + 2\beta_2^a[M^{2+}][L^{3-}]^2 + 2\beta_2^b(K_1)^2[H^+]^2[M^{2+}][L^{3-}]^2 \quad (16)$$

Equation 16 is analogous to equation 13, except the variable  $[L^{3-}]$  occurs as a squared term instead of first order, as  $[M^{2+}]$  does in equation 13. Also, by factoring  $[L^{3-}]$  out of all terms without  $[M^{2+}]$ , a new term is defined:

$$\alpha = 1 + K_1 [H^+] + K_1 K_2 [H^+]^2 + K_1 K_2 K_3 [H^+]^3 \quad (17)$$

This quantity is important because, if the metal were absent, it would define, quantitatively, the amount of free ligand as a function of pH.

Therefore, by using simple mass balance equations for each metal and each ligand in a system (e.g. equations 11 and 15), every free metal species and free ligand species can be described in terms of its total concentration, solution pH, and equilibrium stability and association constants. Once these values have been calculated, they can be used to solve any pertinent equilibrium equations (e.g. equations 6–10). Thus, the entire equilibrium system can be described.

**Equilibrium Constant Transformations.** The stability constants are influenced by solution ionic strength because they are calculated as concentration constants. The concentration of any species is actually:

$$C_i = \frac{a_i}{\gamma_i} \quad (18)$$

The activity coefficient ( $\gamma_i$ ) is a function of solution ionic strength ( $\mu$ ). For divalent ions, the effect of  $\mu$  on  $\gamma_i$  is insignificant at  $\mu > 0.1$ ; however, for  $0 < \mu < 0.1$ , there is a strong curvilinear

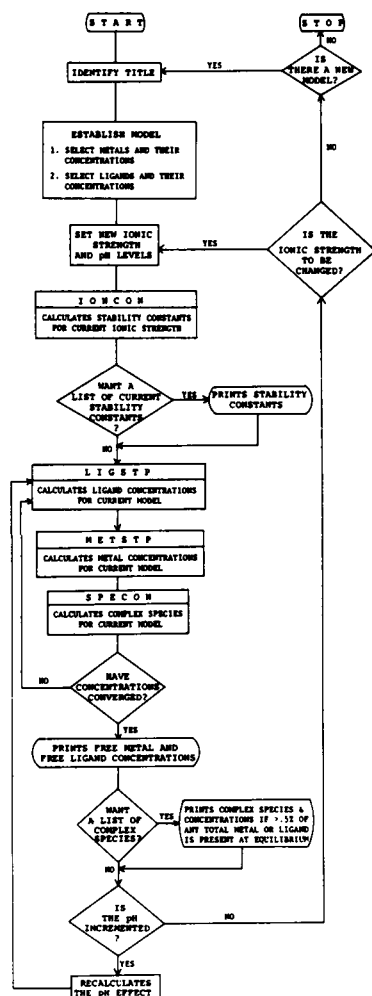


FIG. 1. Program CHELATE flow chart.

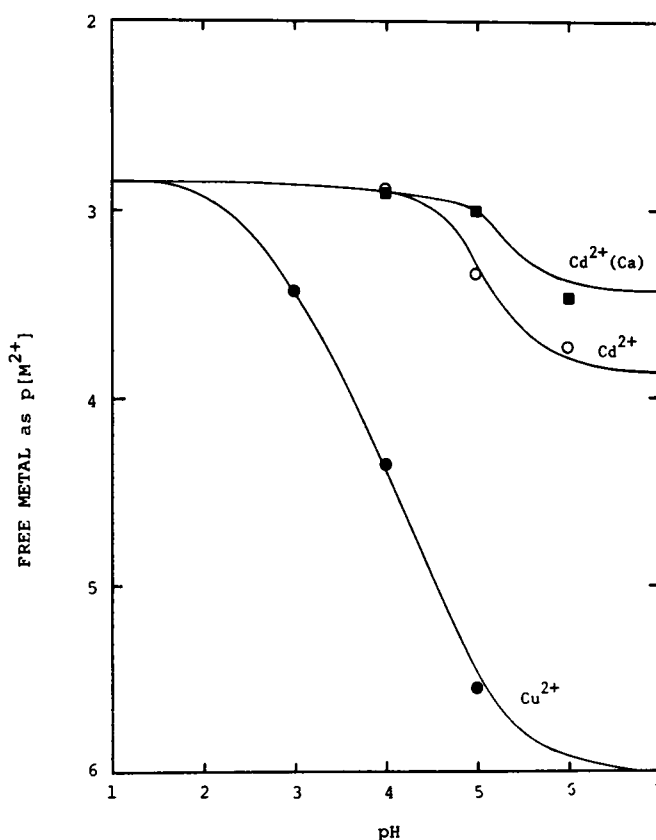


FIG. 2. Effect of pH on free metal concentrations. —, curves generated by the computer model for each of the experimental solutions. Measured values are shown: (●),  $Cu^{2+}$  in a solution of 2.5 mM concentrations of copper, citric acid, and malonic acid; (○),  $Cd^{2+}$  in a solution of 2.5 mM concentrations of cadmium, citric acid, and malonic acid; and (■),  $Cd^{2+}$  in a solution of 2.5 mM concentrations of cadmium, calcium, citric acid, and malonic acid. Each value is the mean of two replications.

relationship and the effect of ionic strength on the value of  $K$  or  $\beta$  can be significant. Because of this relationship, all stability constants used here were transformed to values appropriate to xylem fluid ionic strength.

The following equation was used to transform all concentration equilibrium constants to values corresponding to  $\mu = 0.032$  M:

$$\beta_{nm}^{0.032} = \beta_{nm}^{\mu} \left| \frac{\gamma_{ML}}{\gamma_M^m \cdot \gamma_L^n} \right|_{\mu} \cdot \left| \frac{\gamma_M^m \cdot \gamma_L^n}{\gamma_{ML}} \right|_{0.032} \quad (19)$$

This is the apparent ionic strength of normal-Zn soybean exudate, and the constants were used as part of the default data base for program CHELATE. The apparent ionic strength of xylem fluid was estimated from the following equation:

$$\mu = 0.013 EC_{25} \quad (20)$$

$EC_{25}$  is the electrical conductivity (in mmhos/cm) of the fluid corrected to 25 C. The conversion factor (0.013) was generated from measurements of soil solutions and natural waters (5).

The activity coefficients ( $\gamma_i$ ) were calculated from the Davies equation (a modification of the Debye-Hückel equation) (7):

$$\log \gamma_i = -AZ_i^2 \left| \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} - 0.3 \mu \right| \quad (21)$$

For water at 25 C,  $A = 0.51$ ;  $Z_i$  is the ionic charge. For uncharged compounds (e.g.  $ML^n$ ), a simplifying assumption was made (7):  $\log \gamma_i = b\mu$ , where  $b$  is generally  $0.01 < b < 0.1$ ; so, if  $\mu < 1$ ,  $\gamma_i$  is assumed to be equal to 1.

Table 1. Mean Analytical Concentrations<sup>†</sup> of the 0–1 h Fraction of Soybean and Tomato Xylem Exudate Taken from Plants Grown in Normal<sup>‡</sup> and High-Zn Nutrient Solutions

Element or Compound	Normal Zn Solution		High Zn Solution	
	Soybean	Tomato	Soybean	Tomato
	-----μM-----		-----μM-----	
Ca	2,605	2,644	2,789	1,527
Cu	5.0	5.1	5.8	2.3
Fe	5.7	6.8	3.9	4.5
Mg	1,132	936	1,276	578
Mn	8.1	6.1	8.3	5.0
NH <sub>4</sub> <sup>+</sup>	706	750	335	794
PO <sub>4</sub> <sup>3-</sup>	575	160	471	191
Zn	5.9	6.5	28	77
Citric	1,706	301	1,104	328
Fumaric	66	-	58	76
Malic	890	628	502	817
Malonic	264	-	126	-
Succinic	42	-	34	-
Tartaric	40	-	24	-
Alanine	49	22	72	24
β-Alanine	-	-	18	-
γ-Aminobutyric	-	-	4	2
Arginine	30	10	22	4
Asparagine	2,583	242	917	114
Aspartic	159	76	154	75
Cysteine	2	-	8	-
Ethanolamine	-	28	34	12
Glutamic	18	51	6	38
Glutamine	132	522	46	353
Glycine	4	4	15	10
Histidine	62	18	42	8
Isoleucine	24	24	22	20
Leucine	28	24	34	22
Lysine	86	62	59	16
Methionine	12	-	8	-
Phenylalanine	10	14	20	12
Proline	20	8	40	2
Serine	29	23	49	30
Threonine	42	34	28	25
Tyrosine	7	2	8	6
Valine	78	45	57	37

<sup>†</sup> Inorganic species = three replications; organic species = two replications.

<sup>‡</sup> The normal nutrient Zn level for both species was 0.5 μM.

The high Zn levels were: for soybean, 3 μM for the first 12 days and 8 μM until harvest (12 days later); for tomato, 30 μM for the first 12 days and 80 μM until harvest (20 days later).

Table II. Predicted Equilibrium Distribution of Species Formed at Three pH Levels from Initial Concentrations of 2.500  $\mu\text{M}$  of Cu, Citric Acid, and Malonic Acid

Species	pH 3		pH 4		pH 5	
	Equilibrium	Fraction of	Equilibrium	Fraction of	Equilibrium	Fraction of
	concentration	total metal	concentration	total metal	concentration	total metal
	$\mu\text{M}$	%	$\mu\text{M}$	%	$\mu\text{M}$	%
$\text{Cu}^{2+}$	668†	26.7†	72	2.9	5	.2
$\text{Citrate}^{3-}$	<1	—	4	—	105	—
$\text{H}\cdot\text{Citrate}^{2-}$	35	—	363	—	849	—
$\text{H}_2\cdot\text{Citrate}^-$	1110	—	1150	—	269	—
$\text{H}_3\cdot\text{Citrate}^0$	977	—	101	—	2	—
$\text{Cu}\cdot\text{Citrate}^-$	65	2.6	725	29.0	1250	50.0
$\text{Cu}\cdot\text{H}\cdot\text{Citrate}^0$	123	4.9	137	5.5	24	.9
$\text{Cu}\cdot\text{H}_2\cdot\text{Citrate}^+$	191	7.6	21	.8	<1	<.1
$\text{Malonate}^{2-}$	2	—	29	—	278	—
$\text{H}\cdot\text{Malonate}^-$	676	—	854	—	822	—
$\text{H}_2\cdot\text{Malonate}^0$	367	—	46	—	4	—
$\text{Cu}\cdot\text{Malonate}^0$	1084	43.4	1470	58.8	1042	41.7
$\text{Cu}\cdot\text{H}\cdot\text{Malonate}^+$	367	14.7	50	2.0	4	.1
$\text{Cu}\cdot(\text{Malonate})_2^{2-}$	2	.1	26	1.0	175	7.0

† All concentrations have been rounded to the nearest  $\mu\text{M}$ .

‡ Values within a column may not sum to 100% because of rounding.

## MATERIALS AND METHODS

**Program CHELATE.** The computer program CHELATE calculates equilibrium concentrations of free metals, free ligands, and all hydrogen and metal-ligand complexes of user-defined systems. It is an interactive FORTRAN program designed to provide time-sharing terminal access, as well as manipulation of system variables such as pH and ionic strength. It interacts with a default data base that can be altered by the user for any particular user-defined equilibrium system covered by the data base. The data base currently contains information on approximately 400 equilibrium equations,<sup>3</sup> including stability constants. The equations are based on eight metals and 30 ligands.

Program CHELATE can currently handle nine metals, 35 ligands, and 500 complex species. It can easily be expanded to handle greater numbers. The current default concentrations (data base) of metals and ligands are based on analytical values from 0- to 1-h soybean-stem exudate. The interactive feature allows these concentrations to be changed for any particular run. The stability constants in the data base have all been standardized to an ionic strength of 0.032 M, the ionic strength of the 0- to 1-h soybean exudate. Any ionic strength can be chosen for a given system, and the stability constants are transformed accordingly. If the option is not used, then the default ionic strength is 0.032 M.

A general flow chart for program CHELATE is given in Figure 1. The program is made up of several subroutines and functions that interact with each other and the main program. Complex

species are either metal complexes or nonmetal, protonated ligands.

IONCON utilizes either the default ionic strength or the new value entered for the current model to calculate the stability constants for the current model.

LIGSTP calculates the free ligand concentrations from an initial estimate based on either the pH effect or the amount of total metals. If the number of ligands bound to a metal is two or less, then either a linear or a quadratic solution is used; if the number is greater than two, then Newton-Raphson's (3) solution is used. In finding the ligand concentrations, LIGSTP uses a function (DPOLY) and two subroutines (PINPT and DSDIV). DPOLY calculates, in conjunction with DSDIV, the derivatives of the current polynomial. These then are used to find the positive root of the equation; this is used as the current estimate of the free ligand concentration. PINPT identifies which metal and which ligand are in any given complex species.

METSTP calculates the free metal concentrations based on the input from LIGSTP and PINPT. METSTP and LIGSTP interact iteratively under the commands of the general program (CHELATE), eventually stabilizing at free metal ion and ligand concentrations that satisfy all equilibria in the current model and change less than 12 decimal places for subsequent iterations.

SPECON calculates the concentrations of all complex species in the model, based on values calculated in LIGSTP and METSTP. SPECON also uses PINPT to establish which metals and which ligands are in all complex species.

**Ligand-Metal Test Solutions.** Several test solutions were used to examine the validity of the computer model by measuring free metal activities. These test solutions were chosen because of the

<sup>3</sup> Copies of the equilibrium equations and stability constants, as well as the FORTRAN program, are available from M. C. W.

Table III. Predicted Equilibrium Distribution of Species Formed at Three pH Levels from Initial Concentrations of 2,500  $\mu\text{M}$  of Cd, Citric Acid, and Malonic Acid

Species	pH 4		pH 5		pH 6	
	Equilibrium	Fraction of	Equilibrium	Fraction of	Equilibrium	Fraction of
	concentration	total metal	concentration	total metal	concentration	total metal
	$\mu\text{M}$	%	$\mu\text{M}$	%	$\mu\text{M}$	%
$\text{Cd}^{2+}$	1873†	74.9†	796	31.9	316	12.6
Citrate <sup>3-</sup>	6	-	95	-	360	-
H·Citrate <sup>2-</sup>	454	-	763	-	290	-
H <sub>2</sub> ·Citrate <sup>-</sup>	1440	-	242	-	9	-
H <sub>3</sub> ·Citrate <sup>0</sup>	127	-	2	-	<1	-
Cd·Citrate <sup>-</sup>	168	6.7	1202	48.1	1812	72.5
Cd·H·Citrate <sup>0</sup>	270	10.8	193	7.7	29	1.2
Cd·H <sub>2</sub> ·Citrate <sup>+</sup>	36	1.4	2	.1	<1	<.1
Malonate <sup>2-</sup>	73	-	522	-	1664	-
H·Malonate <sup>-</sup>	2160	-	1630	-	492	-
H <sub>2</sub> ·Malonate <sup>0</sup>	117	-	9	-	<1	-
Cd·Malonate <sup>0</sup>	89	3.5	285	11.4	341	13.6
Cd·H·Malonate <sup>+</sup>	64	2.6	21	.8	2	.1

† All concentrations have been rounded to the nearest  $\mu\text{M}$ .

‡ Values within a column may not sum to 100% because of rounding.

relative nonspecificity (among metals) of oxygen for donating electrons. The stability constants of citric and malonic acids for most divalent metals have been determined accurately, and direct measurements of  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  activities are possible with the electrodes.

The first test solution consisted of 2.5 mM Cd as  $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  plus 2.5 mM citric acid plus 2.5 mM malonic acid; the pH was adjusted to 4.0, 5.0, and 6.0 in separate 1-liter solutions, and the ionic strength was adjusted to 0.032 M with 6 N  $\text{NaNO}_3$ . The appropriate  $\text{NaNO}_3$  additions were determined from the difference between 0.032 M and the contribution to the ionic strength by all calculated equilibrium species at each pH (where  $\mu = \sum c_i z_i^2$ ).

The second test solution consisted of 2.5 mM Cu as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  plus 2.5 mM citric acid plus 2.5 mM malonic acid; the pH levels were adjusted to 3.0, 4.0, and 5.0 in separate 1-liter solutions. The ionic strength was adjusted to 0.032 M with 6 N  $\text{NaNO}_3$  by the method listed for Cd.

The third test solution consisted of 2.5 mM Cd as  $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  plus 2.5 mM Ca as  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  plus 2.5 mM citric acid plus 2.5 mM malonic acid. The pH levels were adjusted to 4.0, 5.0, and 6.0 in separate 1-liter solutions, and the ionic strength was adjusted to 0.032 M with 6 N  $\text{NaNO}_3$ .

Standard solutions were prepared from the same metal salts and had their pH and ionic strengths adjusted to match the test solutions. The range of standard curve concentrations was four 10-fold increments between  $10^{-5}$  and  $10^{-2}$  M for Ca and Cd and five 10-fold increments between  $10^{-6}$  and  $10^{-2}$  M for Cu. Semi-log plots of mv versus standard concentrations showed highly linear responses with slopes matching the manufacturer's recommended values. All ion measurements were made with an Orion model 901 microprocessor ionalyzer and Orion Cd and Cu ion-selective

electrodes. All experiments were repeated twice.

Program CHELATE was used to estimate the equilibrium distribution of six metal species in the 0- to 1-h tomato and soybean exudates collected from plants grown in normal and high-Zn nutrient solutions (24). All metals (Ca, Cu, Fe, Mg, Mn, and Zn), and inorganic and organic solutes were analytically determined as reported elsewhere (24) and are listed in Table I.

## RESULTS AND DISCUSSION

**Calculated Test Systems.** For the first solution, the calculated distributions of equilibrium species at several pH levels are given in Table II. At pH 3, Cu exists primarily as Cu-malonate species (58%) and free Cu (27%). Hydrogen competes effectively with Cu at this pH for association with citric acid. As the pH increases, citric acid becomes more effective at complexing Cu until, at pH 5, the competitive advantage of malonic acid is lost, and both acids bind approximately equal amounts of Cu. This system clearly demonstrates the strong competitive ability of both  $\text{H}^+$  and individual ligands.

A similar system (Table III) was used to illustrate the equilibrium distribution of Cd, a weaker-binding metal than Cu. At pH 4, citric acid binds Cd more effectively than malonic acid, and a much lower percentage of Cd than Cu is complexed at pH 4. More Cd is bound as the pH increases, until only 13% of the total Cd remains free at pH 6.

In a system containing both Cd and Cu at pH 4 and 5, Cu partially displaces Cd from both citric and malonic acid complexes, resulting in free Cd increases from 75 to 84% at pH 4 and from 13 to 53% at pH 5 (data not shown). The large increase at pH 5 illustrates the strong competition that can occur between metals when  $\text{H}^+$  is not important competing cation ( $\text{OH}^-$  would

Table IV. Predicted Equilibrium Distribution of Species Formed at Three pH Levels from Initial Concentrations of 2,500  $\mu\text{M}$  Each of Cd, Ca, Citric Acid, and Malonic Acid

Species	pH 4		pH 5		pH 6	
	Equilibrium	Fraction of	Equilibrium	Fraction of	Equilibrium	Fraction of
	concentration	total metal	concentration	total metal	concentration	total metal
	$\mu\text{M}$	%	$\mu\text{M}$	%	$\mu\text{M}$	%
$\text{Cd}^{2+}$	1929†	77.2‡	1098	43.9	724	29.0
$\text{Ca}^{2+}$	2092	83.7	1478	59.1	1134	45.4
$\text{Citrate}^{3-}$	5	—	50	—	99	—
$\text{H}\cdot\text{Citrate}^{2-}$	389	—	404	—	80	—
$\text{H}_2\cdot\text{Citrate}^{-}$	1230	—	128	—	2	—
$\text{H}_3\cdot\text{Citrate}^0$	109	—	1	—	<1	—
$\text{Cd}\cdot\text{Citrate}^{-}$	148	5.9	877	35.1	1146	45.8
$\text{Cd}\cdot\text{H}\cdot\text{Citrate}^0$	238	9.5	141	5.6	18	.7
$\text{Cd}\cdot\text{H}_2\cdot\text{Citrate}^{+}$	31	1.3	2	.1	<1	<.1
$\text{Ca}\cdot\text{Citrate}^{-}$	102	4.1	744	29.8	1131	45.3
$\text{Ca}\cdot\text{H}\cdot\text{Citrate}^0$	205	8.2	150	6.0	23	.9
$\text{Ca}\cdot\text{H}_2\cdot\text{Citrate}^{+}$	41	1.6	3	.1	<1	<.1
$\text{Malonate}^{2-}$	71	—	502	—	1294	—
$\text{H}\cdot\text{Malonate}^{-}$	2100	—	1480	—	382	—
$\text{H}_2\cdot\text{Malonate}^0$	114	—	8	—	<1	—
$\text{Cd}\cdot\text{Malonate}^0$	89	3.6	357	14.3	607	24.3
$\text{Cd}\cdot\text{H}\cdot\text{Malonate}^{+}$	64	2.6	26	1.0	4	.2
$\text{Ca}\cdot\text{Malonate}^0$	21	.8	105	4.2	208	8.3
$\text{Ca}\cdot\text{H}\cdot\text{Malonate}^{+}$	39	1.6	20	.8	4	.2

† All concentrations have been rounded to the nearest  $\mu\text{M}$ .

‡ Values within a column may not sum to 100% because of rounding.

provide similar competition at higher pH levels).

The equilibrium distribution of a system similar to that shown in Table III, plus Ca (2.5 mM), was used at several pH levels to examine the competitive effects of a weakly binding metal (Ca) (Table IV). By comparing Tables III and IV it can be seen that, at each pH level: more Cd remains unbound in the presence of Ca; Ca competes with Cd for citric acid, particularly at the higher pH levels; and Ca alters the Cd-malonic acid equilibrium.

The calculated distributions shown in Tables II to IV illustrate interactions that can occur in solutions such as xylem fluid. Strongly competing cations (Cu, and  $\text{H}^+$  at low pH) can dominate the distribution of weaker-binding metals such as Cd and Ca. Even among the weaker-binding metals a hierarchy exists, and metals such as Cd and Ca also interact to establish unique distributions that depend on factors such as types of ligands present and solution pH. Changes in pH, metals, and ligands result in the establishment of successively unique equilibrium systems.

The calculated values for these simple solutions are based on a theoretical development of system equations suitable for iterative solutions. The fundamental assumptions underlying these equations are that equilibrium is reached quickly, without important kinetic hindrance, and that the stability constants accurately summarize the potential of a given complex being formed. It was also

assumed that precipitation does not occur. Although no automatic checks for solubility product violations are built into the computer model at this time, numerous hand calculations (using equilibrium concentrations) have shown no violations at the chosen pH levels for a number of metals.

An obvious limitation to the model is a lack of information about all possible complexes that might be formed. Because key species might not be represented, the distribution of metals could be erroneously assigned to less important species. Sarkar (14) recently described a means of obtaining conditional, mixed-complex equilibrium constants and overcoming this problem.

**Measured Test Systems.** The relationship between measured and calculated metal ion activity is shown in Figure 2. The curves show free metal concentration as a function of solution pH. The computer model closely predicted the free metal concentrations for these systems. Measured Cu values fit the curve especially well. Free Cd concentrations for both Cd solutions lie very close to the theoretical curves. Small deviations such as that observed for free Cd in the Cd + Ca solution at pH 6 might be the result of not including possible mixed-ligand species in the model or the result of Cd-chloride complex formation from Cl added during pH adjustment.

**Calculated Metal Distributions in 0- to 1-h Stem Exudate.** For soybean at the normal-Zn treatment, six metals and 26 ligands

Table V. Calculated† Equilibrium Distribution of Metal Species in Soybean‡ and Tomato‡ Stem Exudate from Plants Grown in Normal Zn (0.5  $\mu$ M) Nutrient Solutions

Species§	Soybean		Tomato	
	Equilibrium	Fraction of	Equilibrium	Fraction of
	concentration	total metal	concentration	total metal
	$\mu$ M	%	$\mu$ M	%
Ca <sup>2+</sup>	1,266	48.6	2,217	83.8
Cu <sup>2+</sup>	$3.3 \times 10^{-4}$	<.01	$1.5 \times 10^{-3}$	.03
Fe <sup>3+</sup>	$5.4 \times 10^{-8}$	<.01	$4.9 \times 10^{-7}$	<.01
Mg <sup>2+</sup>	648	57.2	831	88.7
Mn <sup>2+</sup>	2.9	36.6	4.4	72.2
Zn <sup>2+</sup>	0.2	3.8	1.4	20.8
Ca·Citrate <sup>-</sup>	1,118	42.9	220	8.3
Ca·Malate <sup>0</sup>	152	5.8	185	7.0
Ca·Malonate <sup>0</sup>	31	1.2	-	-
Cu·Asn <sup>+</sup>	.18	3.5	.19	3.7
Cu·(Asn) <sub>2</sub> <sup>0</sup>	3.9	78.5	.99	19.4
Cu·Citrate <sup>-</sup>	.06	1.3	-	-
Cu·Gln <sup>+</sup>	-	-	.16	3.1
Cu·(Gln) <sub>2</sub> <sup>0</sup>	-	-	1.1	21.9
Cu·His <sup>+</sup>	.14	2.8	.52	10.2
Cu·(His) <sub>2</sub> <sup>0</sup>	.63	12.6	1.9	37.5
Fe·Citrate <sup>0</sup>	5.7	99.5	6.5	96.3
Fe·Glu <sup>+</sup>	-	-	.2	2.4
Mg·Citrate <sup>-</sup>	405	35.8	58	6.2
Mg·Malate <sup>0</sup>	43	3.8	31	4.1
Mg·Malonate <sup>0</sup>	20	1.8	-	-
Mn·Citrate <sup>-</sup>	3.9	47.8	.6	10.6
Mn·Malate <sup>0</sup>	.9	11.5	1.0	15.7
Mn·Malonate <sup>0</sup>	.3	3.4	.08	1.3
Zn·Citrate <sup>-</sup>	5.3	89.1	3.6	55.6
Zn·Malate <sup>0</sup>	.2	4.2	1.06	16.3
Zn·Malonate <sup>0</sup>	.7	1.2	.08	1.3

+ Calculations were based on analytical concentrations of 0-1 hr stem exudate samples.

‡ For soybean, the ionic strength was 0.032 M and the pH was 6.1; for tomato, these values were 0.024 M and 6.5, respectively.

§ Only the free metal species and metal-ligand complexes having >1% of the total metal are listed. See Table 1 for the 0-1 hr analytical concentrations of each metal.

were included in the calculations; this resulted in 237 equilibrium species. For tomato at the same Zn concentration, there were six metals and 23 ligands, resulting in 208 equilibrium species. Only those metal complexes having >1% of the total metal are shown (Table V); all free metal concentrations are listed.

According to the model, Cu and Fe are nearly 100% bound in both soybean and tomato exudate (Table V). Essentially, none of either metal exists as an unbound hydrated ion. Most of the Cu in soybean exudate is bound to asparagine (82%) and histidine (15%). In tomato exudate, Cu should be bound primarily to histidine (48%), glutamine (25%), and asparagine (23%). Fe should be bound to citric acid in both soybean (99.5%) and tomato (96.3%). Thus, Fe and Cu apparently have quite different equilibrium

distributions in xylem exudate.

Tiffin (20) reported that Cu in soybean, tomato, cucumber, and peanut xylem sap was negatively charged when tested electrophoretically. However, the major Cu-amino acid complexes identified by CHELATE are either neutral or positively charged. This discrepancy might be an example of the model's inability to account for mixed-ligand complexes. Copper has a higher affinity for amino acids than other metals. Nickel binding by histidine is similar to that of Cu (preference for amino acids), and Tiffin has electrophoretically tested Ni binding by histidine at pH 5.4 in Na-acetate (unpublished data). Histidine bound Ni in a stable, positively charged complex that moved cathodically. Copper would be expected to react similarly. Thus, the electrophoretically ob-



Table VI. Calculated† Equilibrium Distribution of Metal Species in Soybean‡ and Tomato‡ Stem Exudate from Plants Grown in High Zn§ Nutrient Solutions

Species¶	Soybean		Tomato	
	Equilibrium concentration	Fraction of total metal	Equilibrium concentration	Fraction of total metal
	$\mu\text{M}$	%	$\mu\text{M}$	%
$\text{Ca}^{2+}$	1,882	67.5	1,174	76.9
$\text{Cu}^{2+}$	$7.8 \times 10^{-4}$	.01	$1.9 \times 10^{-3}$	.08
$\text{Fe}^{3+}$	$8.4 \times 10^{-8}$	<.01	$2.0 \times 10^{-7}$	<.01
$\text{Mg}^{2+}$	951	74.5	484	83.7
$\text{Mn}^{2+}$	4.6	55.8	3.2	63.3
$\text{Zn}^{2+}$	2.2	7.9	11.2	14.4
$\text{Ca}\cdot\text{Citrate}^-$	741	26.6	196	12.8
$\text{Ca}\cdot\text{Malate}^0$	114	4.1	149	9.7
$\text{Cu}\cdot\text{Asn}^+$	.23	4.0	.12	5.1
$\text{Cu}\cdot(\text{Asn})_2^0$	2.9	49.7	.29	12.5
$\text{Cu}\cdot\text{Asp}^0$	-	-	.06	2.6
$\text{Cu}\cdot\text{Citrate}^-$	.07	1.2	.07	3.1
$\text{Cu}\cdot\text{Gln}^+$	-	-	.14	6.1
$\text{Cu}\cdot(\text{Gln})_2^0$	-	-	.66	28.9
$\text{Cu}\cdot\text{His}^+$	.39	6.7	.31	13.6
$\text{Cu}\cdot(\text{His})_2^0$	2.1	36.3	.53	23.0
$\text{Cu}\cdot\text{Thr}^+$	-	-	.02	1.0
$\text{Fe}\cdot\text{Citrate}^0$	3.9	99.3	4.4	98.1
$\text{Fe}\cdot\text{Glu}^+$	-	-	.05	1.1
$\text{Mg}\cdot\text{Citrate}^-$	265	20.8	57	9.9
$\text{Mg}\cdot\text{Malate}^0$	32	2.5	34	5.8
$\text{Mg}\cdot\text{Malonate}^0$	13	1.0	-	-
$\text{Mn}\cdot\text{Citrate}^-$	27	32.5	.78	15.6
$\text{Mn}\cdot\text{Malate}^0$	.73	8.8	1.0	20.9
$\text{Mn}\cdot\text{Malonate}^0$	.20	2.4	-	-
$\text{Zn}\cdot\text{Citrate}^-$	24	83.3	50	65.0
$\text{Zn}\cdot\text{Malate}^0$	1.3	4.4	13	17.1
$\text{Zn}\cdot\text{Malonate}^0$	.32	1.1	-	-
$\text{Zn}\cdot\text{H}\cdot\text{Phosphate}^0$	-	-	.74	1.0

† Calculations were based on analytical concentrations of 0-1 hr stem exudate samples.

‡ For soybean, the ionic strength was 0.034 M and the pH was 6.3; for tomato, these values were 0.019 M and 6.5, respectively.

§ At harvest, Zn levels in the nutrient solutions were 8  $\mu\text{M}$  (soybean) and 80  $\mu\text{M}$  (tomato).

¶ Only the free metal species and metal-ligand complexes having &gt;1% of the total metal are listed. See Table 1 for the 0-1 hr analytical concentrations of each metal.

served Cu-containing, negatively charged complex in exudate (20) may include histidine (which has a high stability constant with Cu); however, other ligands are probably coordinated simultaneously in the complex, giving the complex a net negative charge. There is some evidence for this type of multiligand coordination with Ni in xylem exudate. Four separate amino acids were found associated with a negatively charged, stable Ni complex in exudate (L. O. Tiffin, personal communication).

Copper has been shown to form stable mixed-ligand complexes with amino acids readily in simple aqueous solutions (4). Hallman *et al.* (6) computed Cu and Zn distributions among amino acids in simulated blood plasma (pH 7.4) and found that mixed ligand

Cu complexes were the most important equilibrium Cu species. In exudate, mixed-ligand complexes of Cu and amino acids, or even amino acids plus carboxylic acids, could be an important aspect of metal equilibria in the sap.

Tiffin (18, 19) has demonstrated a strong correlation between Fe and citric acid in exudate from numerous plants. The computer model (CHELATE) confirms that Fe-citrate is the chemical form of Fe in normal xylem sap. However, there is no conclusive information about the molecular structure and ionic charge. Tiffin found that the Fe-citrate complex moved anodically 14 to 17 cm as a negatively charged complex when tested electrophoretically. The Fe-citrate complexes included in program CHELATE are

either positive ( $\text{FeHL}^+$ ) or neutral ( $\text{FeL}^0$ ). Uncharged molecules can move in an electric field by electroosmosis (7), but nowhere near the distances/rates noted by Tiffin. Apparently the charged complex observed by Tiffin either contains several molecules of citric acid, or the hydroxyl group dissociates to give the complex a net negative charge. In nonbiological solutions, there is evidence for this type of hydrogen dissociation for Fe-citrate (16, 17, 22). Citrate has also been shown to bind Fe in biological fluids other than exudate (13).

Therefore, even though the multiple Fe/citrate complexes were not included in the model, the high Fe-citrate stability constants and the high citrate to Fe ratios found in exudate both assure preferential Fe binding to citric acid. In the exudate, the actual complexes may not be  $\text{FeHL}^+$  or  $\text{FeL}^0$ , but rather complexes like  $\text{Fe}(\text{cit.})^-$ ,  $[\text{Fe}(\text{cit.})_2]^{5-}$ ,  $[\text{Fe}_2(\text{cit.})_2]^{2-}$ .

According to the model, Zn is almost completely complexed in soybean and 79% complexed in tomato (Table V). The major Zn complexes in soybean and tomato are Zn-citrate and Zn-malate. In soybean, Zn-citrate (89%) is greater and Zn-malate (4%) less than that found in tomato; total citrate is approximately 7-fold higher in soybean than in tomato at this Zn treatment.

Amino acids do not bind Zn very effectively at the acidic pH values of xylem fluid. However, test calculations with CHELATE show that amino acids would compete increasingly with the organic acids for coordination with Zn as exudate pH increases. Amino acid-bound Zn is important in some higher pH biological solutions. For example, Zn coordination with amino acids has been demonstrated in human blood serum (pH 7.4), where Zn-amino acid complexes are part of a "labile pool" of Zn (12).

Calcium, Mg, and Mn are not as extensively complexed as are Cu, Fe, and Zn at this Zn treatment (Table V). It is difficult to measure the degrees of metal complex formation of these weakly binding metals. Bradfield (1) correlated the fluctuations of Ca activity (electrode measurements) with citric and malic acids in apple tree xylem sap and calculated that 58% of the Ca was bound to citric and malic acids. This value is similar to that calculated by CHELATE for soybean (49%) but not for tomato (15%) (Table V). Bradfield also calculated that 30% of the total Mg was bound to citric and malic acids.

The calculated equilibrium distributions of these same six metals in exudate from plants grown in high-Zn nutrient solutions are given in Table VI. For soybean, six metals and 29 ligands were included in the calculations, resulting in 259 equilibrium species. For tomato, six metals and 23 ligands resulted in 201 equilibrium species.

In the high-Zn exudate, Cu and Fe are largely bound to the same classes of ligands as those found for the normal-Zn treatment. In soybean, however, Cu-asparagine complexes decreased from 82 to 54%, and Cu-histidine complexes increased from 15 to 43% (Tables V and VI). In tomato, Cu-glutamine complexes increased and Cu-histidine complexes decreased. Copper-aspartate and Cu-threonine complexes are also present in tomato exudate at the high-Zn treatment. Iron citrate remains the dominant complex in both species at this Zn treatment (Table VI).

Zn is also predominately bound to citric acid under Zn-toxic conditions in both soybean (83%) and tomato (65%) (Table VI). A small amount of soluble Zn-phosphate is present in tomato sap. In addition, free-Zn concentrations are higher in soybean exudate from the high-Zn treatment than the normal-Zn treatment. Free-Zn concentrations were lower in tomato exudate at the high Zn treatment than the normal-Zn treatment (Tables V and VI). Although the concentrations are different, the metal complexes formed by the weaker-binding metals (Ca, Mg, and Mn) are similar for both Zn treatments (Tables V and VI).

Program CHELATE provides an efficient and valuable method

for calculating the equilibrium distributions of metals among ligands in a system like xylem sap. The program's ability to predict accurately the major metal complexes in any system depends on the completeness of its data base. For example, mixed-ligand complexes will probably have to be included to account for the discrepancy between electrophoretic migration of Cu and Ni complexes and the descriptive complexes now in the model. Nevertheless, information such as that shown in Tables V and VI does provide an initial estimate of possible metal binding in the exudate and shows how the system might change if concentrations of metals or ligands are altered or if new metals or ligands are introduced. An example of this is provided by the changes found for several of the Cu complexes in soybean exudate as a result of Zn phytotoxicity. Even though histidine has greater stability constants with Cu than asparagine, the much higher concentration of asparagine in the normal-Zn exudate results in more Cu-asparagine than Cu-histidine complexes. However, when the asparagine to Cu ratio changed from 517 to 158 as a result of Zn phytotoxicity, histidine held a much higher portion of the Cu as a result of stronger competition.

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