Low Molecular Weight Complexes of Zinc and Other Trace Metals in Lettuce Leaf

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A low molecular weight Zn-containing fraction has been partly purified from lettuce (*Lactuca sativa* L.) leaf by ultrafiltration, gel permeation chromatography, and ion-exchange chromatography. Comparative observations were made on the distribution of Zn and other trace elements among low molecular weight fractions. Other metals (Cu, Fe, Mn, Co, Ni, Ca, Mg) were associated with the Zn-binding fractions under study, and certain metals also occurred in other low molecular weight fractions (Fe, Mn, Ca, Mg). The major Zn-binding fraction had a molecular weight of about 1250; it contained S, reducing sugars, ninhydrin-positive components, and a trace of P. It was labile to mild acids and dissociated when chromatographed on an amino acid analyzer or a polar HPLC media. The isolated low molecular weight fractions were 73% of the total soluble Zn, indicating that they may have nutritional importance. The significance of such abundant low molecular weight trace-metal complexes in plant tissues is discussed.

Functional metalloproteins are a small proportion of the total trace-metal content in living organisms (Tinker, 1981). Typically, the majority of a trace metal is distributed among low molecular weight complexes, storage metalloproteins, and insoluble forms associated with cell wall and other cell surfaces and structures. Of these, low molecular weight complexes are frequently in greatest abundance and of considerable importance both in the plant's metabolism and to animal and human nutrition.

In higher plants, considerable interest centers on uptake and translocation processes for trace elements (Price et al., 1972; Tiffin, 1977). Electrophoretic and chromatographic experiments have demonstrated the presence of anionic trace-metal complexes of low molecular weight in many plant species. Tiffin (1970) described a complex with the properties of Fe(III) citrate in xylem exudates of soybean. Research with soybean and tomato phloem exudates also indicated this association and presented further evidence suggesting the association of Zn and Cu with organic acids other than citrate (White et al., 1981). Anionic complexes, possibly with amino acids, have been reported for Cu and Zn in ryegrass extracts (Bremner and Knight, 1970), with Ni in peanut extracts (Thompson and Tiffin, 1974), and with Ni, Co, and Zn in phloem exudates of *Ricinus sp.* (Van Goor and Wiesma, 1976; Wiesma and Van Goor, 1979).

A heterocyclic compound—nicotianamine—was found to be the absent factor in a mutant tomato (cv. Chloronerva) having a defective Fe translocation mechanism in its leaves (Budesinsky et al., 1980). Nicotianamine has been widely found in higher plants (Noma and Naguchi, 1976; Ripperger and Schreiber, 1982). Similar compounds (e.g., mugineic acid, 2-deoxymugeineic acid, and avenic acid) have been found in root washings of Fe-deficient cereals (Sugiura et al., 1981; Fushiya et al., 1980; Ripperger and Schreiber, 1982). These compounds form stable anionic complexes with several trace metals, although published evidence on their physiological role is limited to studies on Fe (Sugiura and Nomoto, 1984).

Recently, Grill et al. (1985) identified a new class of relatively low molecular weight heavy-metal-binding peptides in a wide range of higher plants. They named these compounds "phytochelatins" and suggest that these compounds are the principal heavy-metal-complexing peptides in plants. The structure of these peptides was determined to be (γ-glutamylcysteine)-*n*-glycine (n = 3–7). They reported that the synthesis of these peptides is induced in plant cells in response to excessive exposure to heavy metals (i.e., Cd, Cu, Hg, Pb, and Zn). These peptides are linear polymers of the γ-Glu-Cys portion of glutathione and may be formed from glutathione in vivo. These authors suggested that phytochelatins play a role in heavy-metal homeostasis and detoxification through metal thiolate formation.

Jackson et al. (1985) have also isolated two metal-binding complexes from a Cd resistant strain of *Datura innoxia*. The smaller of the two complexes is very similar to the phytochelatins reported by Grill et al. (1985). It contained seven amino acids—one glycine, three cysteine, and three glutamate residues. This compound bound with Cd to form a dimer and tetramer having a molecular weight of either 1800 or 4000, respectively. Most of the Cd in the plant cells was bound to the smaller of the two complexes that were isolated (i.e., 81% of the cellular Cd was in this form). The amount of these complexes in the plant cells was positively correlated to the amount of Cd supplied. They suggested that these complexes are involved in Cd tolerance in plants.

Another group of compounds, α-, β-, and γ-thujaplicins, are complexers of Fe found in extracts of wood from several tree species (Akers et al., 1980). A similar compound, ellagic acid, was isolated as a Zn-binding ligand from leaves of *Vaccinium myrtillus* L. (Gomah and Davies, 1974).

Very little information is available on the competitive associations of trace elements of related chemical properties among such low molecular weight complexes. We have observed comparable chromatographic profiles of several trace metals in extracts of soybean seed, using ICP (inductively coupled argon plasma emission spectrometer) for rapid multielement analysis (Walker and Welch, 1981). ICP can provide insight into trace-element interactions, even when little is known of their metabolism, e.g. Ni (Welch, 1981) and B (Price et al., 1972).

In this paper we demonstrate that low molecular weight trace-metal coomplexes are in abundance in lettuce leaf extracts. We describe chromatographic fractionation of these extracts, focusing on the low molecular weight species binding Zn and Cu, as well as chromatographic properties of other nutritionally important elements.

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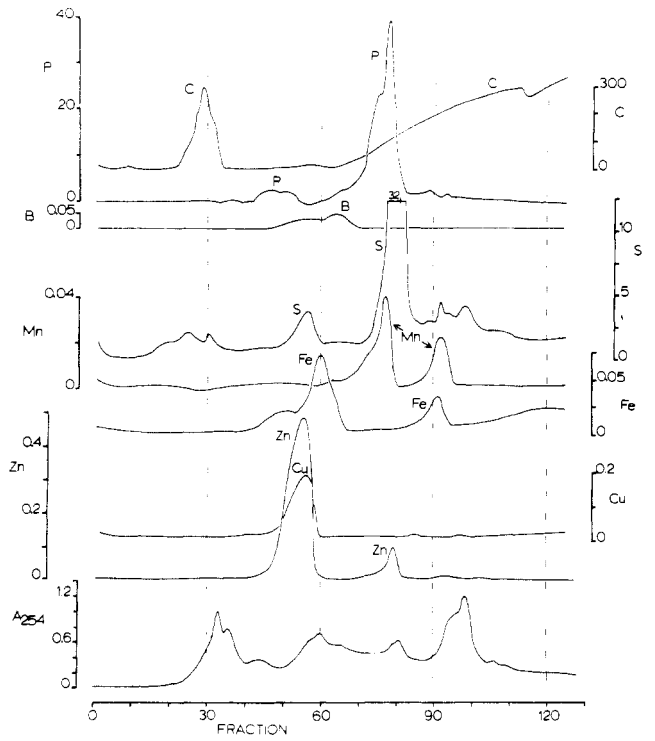

Figure 1. Chromatogram of 1000–10 000 Da ultrafiltration fraction of lettuce leaf extract on DEAE-cellulose (Whatman DE-52) with a gradient of ammonium bicarbonate (0–0.3 M). Concentrations of all elements are given in micrograms/milliliter, except for C which is in millimoles/liter. A_{254} is spectrophotometer absorbance at 254 nm. Element symbols adjacent to peaks are to eliminate ambiguities of scale to curve assignments. Overscale S peak reached a maximum of 32 μg mL^{-1}.

MATERIALS AND METHODS

Plant Culture and Harvesting. Lettuce plants (*Lactuca sativa* L. var Black-seeded Simpson) were grown in a growth chamber under a 12-h day–night cycle provided with 250 einstein m^{-2} s^{-1} of photosynthetically active radiation and a day–night temperature cycle of 24/18 °C. Nutrients were supplied as a modified Hoagland's nutrient solution (Johnson et al., 1957) in the following elemental concentrations: 16 mM N, 6 mM K, 4 mM Ca, 2 mM P, 1 mM Mg, 1 mM S, 50 μM Fe, 12.5 μM Cl, 12.5 μM B, 1 μM Mn, 0.5 μM Zn, 0.5 μM Cu, 0.5 μM Mo. The nutrient solutions were changed weekly after the initial 3 weeks of growth. Zinc was supplied as radiolabeled ^{65}Zn in 0.5 μM $ZnSO_4$ and Fe as NH_4–Fe^{3+}–diethylenetriaminepentaacetic acid. Plants were harvested after 71 days of growth, frozen in liquid N_2, freeze-dried, and stored in a freezer at −15 °C.

Isolation of Low Molecular Weight Zn Fractions. The tissue was blended with 20 volumes of N_2-purged, 30 mM ammonium bicarbonate (NH_4HCO_3), squeezed through Miracloth (Calbiochem), transferred to centrifuge tubes, and centrifuged at 21000*g* for 1 h; with all operations performed under N_2 and at 4 °C. The supernatant was filtered (under N_2 pressure) through an ultrafiltration membrane (Amicon Diaflo PM10) with 10 000-Da nominal cutoff; the filtrate was recovered and then filtered through an ultrafiltration membrane (Amicon Diaflo UM2) with a 1000-Da cutoff; the retentate was recovered and freeze-dried. The sample, redissolved in deionized water, was chromatographed on DEAE-cellulose (Whatman DE-52 packed in a 2.5 × 90 cm column, flow rate 30 mL h^{-1}, 5-mL fractions collected) with an NH_4HCO_3 gradient (0–0.3 M, pH 8.5; Figure 1). The fractions containing the majority of the ^{65}Zn radioactivity were collected, freeze-dried, and

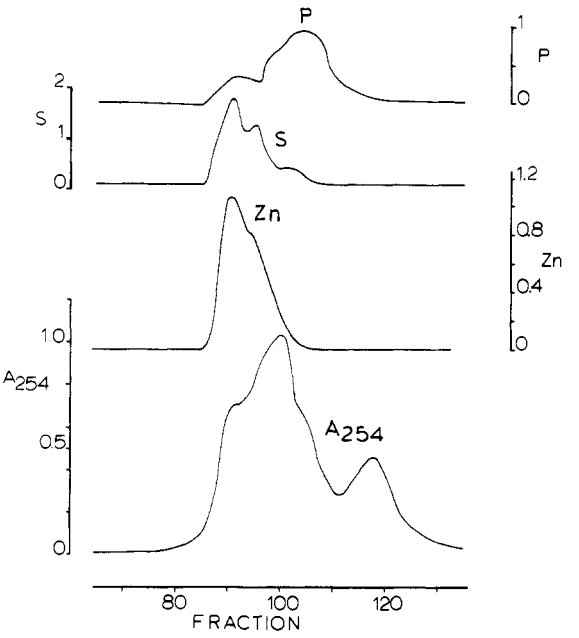

Figure 2. Chromatogram of Zn-binding fraction on Sephadex G-25. See Figure 1 for details of presentation. Samples were eluted with 0.1 M ammonium formate (see text for other chromatographic conditions).

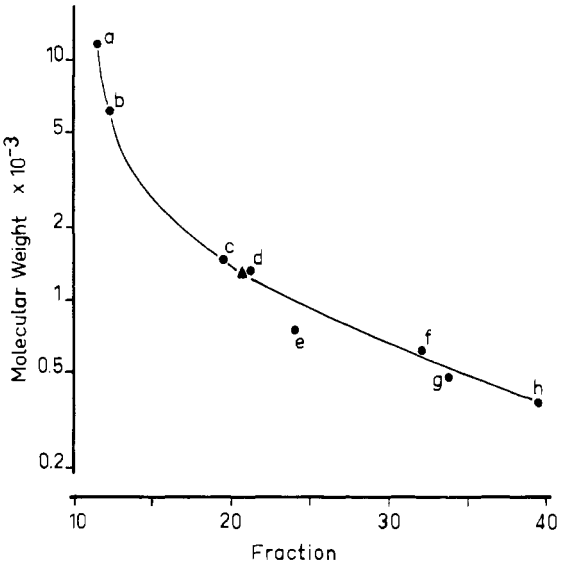

Figure 3. Peak elution fractions of molecular weight standards and Zn-binding fraction (filled triangle) on a 1.6 × 90 cm column bed of Sephadex G-25. Fraction volumes were 6 mL. Standards: (a) cytochrome *c*; (b) insulin; (c) bacitracin; (d) actinomycin D; (e) erythromycin; (f) neomycin; (g) oxytetracycline; (h) N^{α}-benzoyllysine-*p*-nitroanilide hydrochloride.

chromatographed on gel permeation dextran media (Sephadex G-25 packed in a 5.0 × 90 cm column, flow rate 53 mL h^{-1}, 16-mL fractions collected) in 0.1 M ammonium formate (NH_4COOH), pH 6.5 (Figure 2). Before use, the Sephadex G-25 media was treated with 0.1 g of solid sodium borohydride ($NaBH_4$)/100 mL of swollen media, while refluxing in water overnight to chemically reduce any residual metal-binding carboxylate groups that might be present. The molecular weight of the Zn ligand was determined from its elution position (on a 1.6 × 90 cm column of Sephadex G-25) compared to those of standards of known molecular weight (Figure 3). The chromatography fractions containing Zn were again recovered and freeze-dried. This isolate was chromatographed on

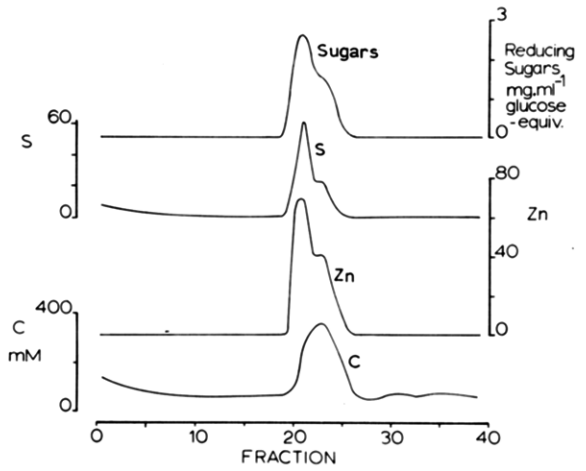

Figure 4. Chromatogram of Zn-binding fraction on DEAE-cellulose (Whatman DE-51) in 0.1 M ammonium formate. See Figure 1 for details.

DEAE-cellulose (Whatman DE-51 packed in a 1.5 × 80 cm column, flow rate 24 mL h^{-1}, 6-mL fractions collected) in 0.1 M NH_4COOH (Figure 4), and the Zn fractions were recovered, freeze-dried, and dissolved in a minimum amount of water.

Other Methods. Free sulfhydryls were estimated by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman, 1959). An aliquot of 0.1 mL of 120 mM DTNB in methanol was added to 0.5 mL of a chromatogram fraction, the volume was made to 3 mL with 0.2 M Tris, pH 8.2, and the absorbance at 412 nm was compared to a curve for appropriately prepared cysteine standards. Reducing sugars were estimated by their furfural reaction with α-napthol in concentrated sulfuric acid. Aliquots of 0.1–0.01 mL of chromatogram fractions were made to 1 mL with water, mixed with 4 mL of 96% sulfuric acid (H_2SO_4), and placed in a 40 °C water bath. After 5 min, 0.5 mL of 2% (w/v) α-napthol was added and the absorbance at 570 nm read after 10 min against glucose standards. Flat-bed paper electrophoresis was used to monitor the isolation of the Zn-binding fraction in the chromatographic step (Figure 5). Electrophoresis of preparations of Zn-binding fractions was performed in a water-cooled flat-bed chamber under a N_2 atmosphere, on a 23-cm length of Whatman 3MM paper for 2 h at 200 V; buffer conditions varied (15 mM NH_4HCO_3, pH 8.5; 10 mM sodium maleate, pH 5.6; and 10 mM NH_4COOH, pH 6.5 were normally used and gave similar results).

Elemental analyses (Zn, Cu, Fe, Ni, Mn, Co, Mg, Ca, Na, K, S, P, B, Se, Mo, Al, Cd, Hg, Pb, C) of chromatogram fractions were performed on a simultaneous ICP (ARL 34000), with a tunable spectrometer set at 247.86 nm to determine C directly. Zinc measurements were also obtained by assaying γ radiation from ^{65}Zn on a γ spectrometer. The ICP and γ radiation assays matched closely in both magnitude and profile; radioassay Zn values are presented in Figures 1, 2, and 4.

Chromatography of a sample of the Zn-binding fraction was performed on an amino acid analyzer (using a column of Chromosorb C3, and pH 3.33 sodium citrate buffer as eluant; protein amino acid mode) with UV detectors (monitoring at 254 and 210 nm) placed in the sample stream ahead of the mixing and reaction coils. Several chromatograms, with added standards and on hydrolysis products, were performed on the same column without the UV detectors.

High-performance liquid chromatography of samples of the Zn-binding fraction was performed on several types of column packing including reversed-phase, diol, propylamino-cyano (PAC), and Toyo Soda SW packings with gradients of acetonitrile/0.1 M NH_4COOH in proportions from 19:1 to 3:7 (by volume). This gradient gave well-resolved separations.

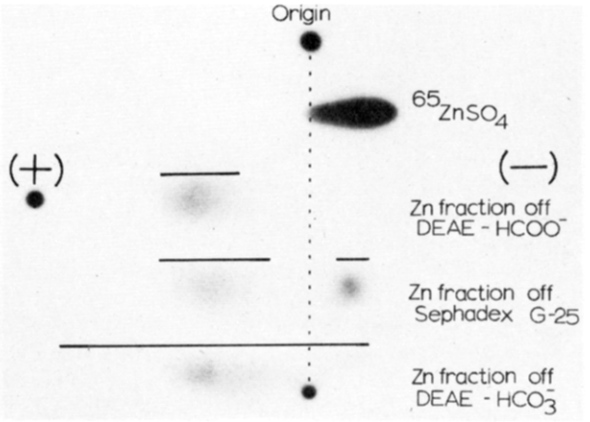

Figure 5. Autoradiogram of electrophoretogram of Zn-binding fractions from successive purification steps. Electrophoresis performed in 10 mM sodium maleate buffer. Three ^{65}Zn reference spots (dark circles) mark the origin line and anodic direction on the electrophoretogram. Solid lines indicate the extent of ninhydrin-reactive compounds in adjacent sample.

RESULTS

Some 58 ± 3% (mean ± standard deviation of the mean from 15 extractions) of total tissue Zn (tissue contained 8.5 μg of Zn g^{-1}) was solubilized in the initial extraction of freeze-dried lettuce leaf; ultrafiltration indicated that 73 ± 3% (n = 15) of this Zn was found in the nominal range of 1000–10 000 Da, 16 ± 3 below 1000 Da (n = 15), and 11 ± 4% above 10 000 Da (n = 15). Recovery of applied radiolabeled Zn was 70 ± 4% (n = 8) in chromatograms on weak anion-exchange media, DEAE-cellulose (Whatman DE-52; Figure 1), and gel filtration media (Sephadex G-25; Figure 2). Much of the lost ^{65}Zn could be recovered from the chromatography matrix by elution with a dilute mineral acid.

Associated peaks of several transition metals were found when the 1000–10 000 Da ultrafiltration fraction was chromatogrammed on DEAE-cellulose (Whatman DE-52; Figure 1), with Zn and Cu peaking in fraction 56, Zn and Mn in fractions 77–79, and Fe and Mn in fractions 91–92. Trace amounts of other transition metals were also observed in the ICP chromatogram analyses, but the data are not shown in Figure 1. Cadmium was associated with both of the Mn peaks; Ni closely followed the Cu profile, and a Pb peak was present at fraction 73. Further association of certain metal peaks with S and P peaks was observed (Figure 1), notably the association of S with the Zn/Cu/Ni peak at fraction 56 and the Fe/Mn/Cd peak at fractions 91–92 and of P with the Zn/Mn/Cd peak at fractions 77–79. Trace amounts of Ca and Mg were also present in these transition-metal peaks.

The transition metals collectively behaved differently when compared to the alkali and alkaline-earth metals. Almost all of the latter elements eluted near the void volume of the column (Mg and Ca at fraction 25, K and Na at fraction 27), whereas the transition metals were all retained to some extent. A peak of C, presumably neutral or positively charged organic compounds, also eluted at the column void volume (fraction 29). The HCO_3^- gradient (nominally from 0 to 0.3 M NH_4HCO_3) may be followed in the latter half of the trace for the chromatogram in Figure 1. Boron peaked in fraction 64. The major S peak

at fraction 81 had a corresponding peak in the UV absorbance at 254 nm, while the Fe peak at fraction 60 also had a corresponding UV peak. Apart from these, there was little correlation of the elemental peaks with the UV absorbance peaks.

In the chromatogram of the major Zn-binding peak (from Figure 1) on Sephadex G-25 (Figure 2), the eluted Zn-binding fraction corresponded to a shoulder in the major UV-absorbing peak. A scan of the UV spectrum of the Zn peak (fraction 91) showed a distinct shoulder at 251 nm that was absent in the later UV peaks but lacked any further spectral maxima. The profiles of Zn, Cu, Ni, and Co were very similar. All showed a peak at fraction 91 and a shoulder peak at fraction 95, and these were also present in the S profile (Figure 2). The P profile differed from that of the other elements, although some P was present in the Zn peak. Quantitatively, the majority of the trace metals present was Zn; Zn and S were present in a mole ratio of 1:3.1, while Zn and P were in a mole ratio of 1:0.6.

Chromatography of a sample of the Zn-binding fraction from the DEAE-HCO_3^- chromatogram (Figure 1) on a calibrated column of Sephadex G-25 indicated that the Zn fraction had a molecular weight of around 1250 (Figure 3). The molecular weight was not influenced by treatment with mercaptoethanol prior to chromatography. However, mercaptoethanol treatment resulted in an inorganic $ZnSO_4$ standard eluting at a molecular weight of 900, possibly due to the formation of a S-bridged polynuclear aggregate.

The chromatography of the combined Zn peaks from the Sephadex G-25 chromatogram (Figure 2) on DEAE-cellulose (Whatman DE-51) also indicated the presence of two similar Zn-binding forms (Figure 4). These were closely associated with other metals with similar profiles (Cu, Fe, Mn, Ni, Co, Ca, Mg); moreover, S (Figure 4) and P (not shown) both had similar profiles to Zn. The mole ratios of Zn to S and Zn to P were 1:1.8 and 1:0.25, respectively. Both B and C showed single, symmetrical peaks eluting with the second "shoulder" seen in the Zn profile (Figure 4). The UV absorbance detector signal was at over 2 AU between fractions 20 and 25. The levels of α-napthol-reactive compounds (normally reducing sugars) were found to closely match the profile of the Zn-binding compounds (Figure 4). The mole ratio of Zn to reducing sugars (as glucose) was found to be around 1:16. The UV–visible spectrum of the color reaction seen in this assay was identical with that of the glucose standard, indicating that it was almost certainly due to a furfural-producing reducing sugar; other color reactions seen at high levels of transition metals gave different colors that could be easily distinguished from that of the standard glucose and Zn-binding fractions.

Measurements of sulfhydryls in this chromatogram (Figure 4) gave almost undetectable levels under standard assay conditions. Upon standing, the color reaction developed slowly and a sulfhydryl peak was observed in fraction 20 (data not shown). This profile did not match total S as determined by ICP measurement (Figure 4), and it is possible that a difference in sulfhydryl content exists between the two S peaks. The reaction of sulfhydryl groups may have been inhibited by being complexed with trace metals.

Paper electrophoresis of ^{65}Zn-labeled, Zn-binding components obtained from the sequence of chromatograms performed in Figures 1, 2, and 4 showed increasing proportions of anionic Zn migration with successive purification steps (Figure 5). Further, the areas of detectable ninhydrin-positive compounds became limited to that region associated with the Zn-binding complex, indicating that other amino-containing components had been successfully removed. Conventional polyacryamide gel electrophoresis of a sample containing 80 μg of Zn did not show any detectable protein staining with Coomassie blue.

A major difficulty in the amino acid analyzer and HPLC chromatograms was the loss of the radiolabeled Zn from the column effluent; the Zn was retained on the column packing.

Chromatography of a sample of the Zn-binding ligand on an amino acid analyzer initially showed two major ninhydrin-reactive compounds eluting 17.9 and 22.5 min after injection (data not shown). After further work, it became apparent that the preparation was unstable as a further peak, eluting at 16.5 min, became larger, despite the precaution of retaining samples frozen at −20 °C. Of the three major peaks eluted in the chromatogram, the first cochromatographed with a tag of aspartate and the third with a tag of glutamate but the second did not cochromatogram with threonine (the closest amino acid). However, although the three compounds were stable to $NaBH_4$ reduction, they were not stable to acid hydrolysis (in 6 N HCl, 90 °C for 12 h).

Samples of each compound were obtained by preparative chromatography on the amino acid analyzer. Acid hydrolysis was performed on these, and all compounds gave similar patterns. Tentatively identified amino acids were aspartate, threonine, glutamate, glycine, isoleucine, and γ-aminobutyrate with traces of leucine, serine, valine, and cysteine. Further work is needed to verify the identity of these hydrolysis products, since the analyzer was only operated on a protein amino acid regime.

Chromatography on an amino acid analyzer with the two UV detectors inserted in the effluent stream ahead of the reaction coil indicated the presence of other compounds passing through the analyzer column at 8.8 min (data not shown). Measurements of α-napthol-reducing activity throughout the chromatogram established that almost all of the reducing sugars eluted in this peak. A trace amount of α-napthol reducing activity was also present in the compound eluting at 17.9 min.

When the major Zn-binding fraction from the second ion-exchange chromatograph (Figure 4) was fractionated by HPLC chromatography on a PAC column, approximately 10 peaks were observed (data not shown). All major peaks had detectable levels of α-napthol-reducing activity, this being greatest in the fractions eluting between 20 and 30 min. The conditions of the PAC chromatogram were such that the most polar compounds eluted last.

DISCUSSION

The extraction of a large proportion of the total Zn in lettuce leaf as low molecular weight complexes is comparable to similar proportions extracted from soybean seed (i.e., 65%; Walker and Welch, 1981), of Cu compounds found in subterranean clover (Walker, 1980; Walker and Webb, 1981), and of Ni compounds found in soybean leaf (Cataldo et al., 1978). In contrast, a relatively small portion of plant Fe was present in low molecular weight forms in this (Figure 1) and other studies (12% in soybean seed; Walker and Welch, 1981). Phytoferritin and enzymes appear to comprise the major portions of soluble Fe in leaf tissue (Tiffin, 1972; Hewitt, 1979) and the existence of Fe phytate complexes aggregated in the aleurone globoids of seed has been indicated (May et al., 1980).

ICP analyses revealed that all of the biologically essential trace metals determined had anionic complexes in the ultrafiltration isolate from lettuce leaf (Figure 1). Some specificity of different trace metals for particular ligands is apparent. For example, Cu is not associated quantita-

tively with the second, smaller Zn-binding peak, and similarly Mn is present in the second Zn species and in the second Fe species. Although trace-metal elements have been previously demonstrated to occur in low molecular weight anionic forms in plants (Tiffin, 1977; Van Goor and Wiesma, 1976; Wiesma and Van Goor, 1979; Cataldo et al., 1978; Bremner and Knight, 1970; White et al., 1981; Grill et al., 1985), we are not aware of other studies making comprehensive comparative observations such as those above, apart from our earlier work on soybean seed (Walker and Welch, 1981).

The formation of stable complexes of trace metals with S- and P-containing compounds is well-known. The mole ratio of Zn to S remained above unity (i.e., 1:1.8) during purification of the Zn-binding fraction while the mole ratio of Zn to P decreased (i.e., 1:0.25). Thus, the presence of S within the Zn ligands seems likely, while P may be a contaminant.

An association of B with sugars, resulting from the formation of complexes of borate with cis hydroxyl groups, has been described (Lewis, 1980). Possibly, the trace amounts of B found in the chromatograms of the current study are involved in such associations. However, a further association in trace-metal complexes cannot be discounted without further experimentation.

Ion-exchange and gel filtration chromatography with volatile buffers provided a convenient preparative procedure for the preliminary isolation of the low molecular weight trace-metal complexes. We recommend it over the preparative paper electrophoresis that has also been used (Tiffin, 1972). However, quantitative recovery of complete complexes in such fractionations was not possible, probably because of the instability of the complexes themselves. The chromatography of the complexes on silica-based PAC media, the other HPLC media tested, and on the amino acid analyzer cation-exchange resin led to complete removal of the Zn from the chromatographic fractions.

Although a technique for chromatography of labile Zn complexes involving saturation of the media with Zn and addition of Zn to the mobile phase has been advanced (Evans et al., 1979), we have not used such a procedure because of the possible formation of further artifactual Zn complexes that this technique may create. Liquid–liquid countercurrent chromatography (Ito and Conway, 1984), in which no solid matrix with ion adsorption sites is present, may be useful in this respect.

Flat-bed electrophoresis and autoradiography were used to monitor the isolation of the anionic Zn-binding fraction in earlier chromatographic steps (Figure 5). Several attempts to recombine HPLC chromatogram fractions with radioactive Zn failed to form anionic Zn-binding compounds. It is not clear whether this was a methodological problem or an indication that (1) ligand(s) may lose activity in becoming individual free ligand(s), (2) essential components of the initial complex(es) were also retained on the column packing, or (3) the metal-binding groups of the complexes are unstable and undergo reactions to forms that cannot bind transition metals (e.g., autoxidation of sulfhydryl groups).

Possibly, nicotianamine is present in the isolated Zn-binding fraction. There are three considerations that support this contention. First, nicotianamine, the Fe ligand known to be present in many higher plants, does elute close to the position of the largest ninhydrin-positive peak found in our amino acid analyzer chromatogram (Rudolph and Scholz, 1972). Second, its complex has a UV spectrum similar to that of the complex under study (Scholz, 1970). Third, acid hydrolysis yielded further amino acid constituents, as if the unknown was a peptide. However, the recent review of Sugiura and Nomoto (1984) revealed that the acid hydrolysis products obtained from mugineic acid, a phytosiderophore related to nicotianamine, included homoserine and γ-amino-α-hydroxybutyrate. Further analysis of the complex mixture of compounds present in the Zn-binding fraction may yield more ligands, since no reported nicotianamine type compound contains S or, to our knowledge, has a α-napthol-reducing function (Figure 4).

These latter features of natural low molecular weight complexes of Zn have not been reported before. Rauser (1984) described a low molecular weight Cu complex (1700 Da) from the roots of *Agrostis gigantea* in which a small amount of Zn was present. This complex contained cysteine and had UV spectra similar to the complex isolated in the current study. The isolation technique used a heat denaturation procedure similar to that described for nicotianamine isolation (Stephan and Rudolph, 1984).

Reducing sugars have also been found in anionic Cr complexes isolated from a variety of plant sources (Huffman and Allaway, 1973; Blincoe, 1974; Starich and Blincoe, 1982). This complex (molecular weight 1700–3000 depending on the plant source) chromatographed with fluorescamine-positive amines that could be removed without apparent disturbance of the structure (Starich and Blincoe, 1982). Its stability to elevated temperatures was examined in some detail; the complex could be identified after boiling for 30 min or charring of the tissue prior to extraction.

Spiro and Saltman (1969) reported that a wide range of polynuclear complexes of Fe are formed with sugars (fructose, glucose, sucrose, pentoses, tetroses, dextrans) and organic acids such as citrate, by simple hydroxylation reactions, and through sulfur-bridged complexes. The chemistry of the Fe fructose complex has been studied as a model system, and a molecular weight of 2000 was reported.

The need for abundant levels of low molecular weight Zn complexes in plants may be manifold.

First, study of the mobile forms of trace metals present in the vascular systems of plants has implicated low molecular weight species in the transport process (Tiffin, 1977). Such species are frequently anionic (White et al., 1981), as in the current study. Thus, they may be readily translocated, whereas free ionic cation movement is limited by the exchange capacity of negatively charged groups in the cell walls of xylem elements (Van de Geign et al., 1979), particularly for those elements forming very stable complexes such as the transition metals.

Second, such low molecular weight forms are presumably easily degraded, and thus accessible for transfer to macromolecular sites. The pool of low molecular weight anionic trace-metal complexes might thus be termed "physiologically available" in the same sense that trace minerals present in enzymes are "physiologically active" (Olsen, 1972). Low molecular weight forms of Cu were reported to be most abundant in the youngest leaf tissue of subterranean clover, wherein enzyme (and tissue) synthesis may be considered to proceed fastest (Walker, 1980). These first two arguments apply equally well to all trace metals.

Third, the provision of abundant levels of a Zn complex formed with a low molecular weight ligand may provide a buffer system to absorb other trace metals. It is widely recognized that many low molecular weight transition-metal species have catalytic activities, e.g. decarboxylation by Cu (Mottola et al., 1968) and amide hydrolysis by Cu

and Zn (Groves and Dias, 1979). Such catalytic roles may have been contributed by low molecular weight complexes in the origin of life (Österberg, 1974). However, it seems likely that the complexity of higher plant metabolism is such that low molecular weight metal species would not have sufficient specificity or activity to serve efficiently as enzymes. Therefore, organisms may suppress such activities by synthesizing certain metal-binding ligands. A Zn complex with a low molecular weight ligand may provide a buffer system to absorb other more toxic transition-metal ions, Zn being low on the Irving–Williams series with such ligands (e.g., nicotiamiamine; Benes et al., 1983) and thus readily displaced from its complex. Indeed, estimates of the relative stabilities of the trace metal ligand complexes and the number of binding sites each provide may be obtained from chromatograms, such as Figure 1, by making two assumptions: that they are all in equilibrium and that they all follow the Irving–Williams series.

The abundance of low molecular weight complexes in plant tissues grown with high concentrations of trace metals lends support to a role of low molecular weight metal complexes in detoxification. Phytochelatins (Grill et al., 1985; Jackson et al., 1985) have been compared to the plant metallothioneins isolated by Rauser (1984) and others and were thought to be derived from glutathione in vivo. Glutathione is considered to be ubiquitous in nature, although only occasionally reported in plants (Fahey and Newton, 1983; Meister and Anderson, 1983). It has been recognized that glutathione is involved in other areas of detoxification metabolism, forming thiol-linked adducts with many organic compounds (Fahey and Newton, 1983). There is little evidence of a similar role for glutathione metabolites in heavy-metal detoxification; however, Tong and Rao (1983) reported that a γ-glutamyl transferase is activated by mercury vapor exposure in the rat, and the high stability of Hg–S ligands is well-known. Although no other γ-glutamyl peptides have been identified as playing a role in metal binding in plant metabolism (around 70 are known, including many containing S or Se; Kasai and Larsen, 1980), the possibility that such compounds are present in our isolate cannot be excluded.

Indeed, our major Zn-binding fraction (see Figures 1, 2, and 4) possessed many characteristics in common with the phytochelatins reported by Grill et al. (1985): For example, Cu and Ni were also bound by this fraction; S (most likely a sulfhydryl group) was associated with this metal-binding fraction; the UV spectrum was similar to that reported by Grill et al. (1985) with an UV absorption shoulder occurring at 251 nm; it had a molecular weight similar to that of some of the phytochelatins (i.e., 1250; see Figure 3); the Zn to S ratio remained above unity throughout all of the isolation procedures (i.e., about 1:2); acid hydrolysis yielded glutamate, glycine, and a trace of cysteine along with other amino acids.

However, there were some characteristics that our major Zn-binding fraction did not share in common with the phytochelatins. Primarily, our fraction contained reducing sugar activity which, to our knowledge, would not occur with phytochelatins.

The profusion and diversity of sugars, sugar phosphates, organic acids, amino acids, peptides, and other possible trace-metal ligands in plant tissue make the current situation (i.e., a partly defined, complex containing at least two trace metals in abundance—Zn and Cu) understandable. The associated ligands in the current study appear to include reducing sugars and amino acids and contain sulfur. Isolation of a Zn-binding compound in a plant extract does not necessarily imply a Zn-binding role in vivo, since dissociation and redistribution of Zn among ligands during isolation is possible. Further evidence of a specific ability to influence Zn metabolism (e.g., the ability of nicotianamine to normalize Fe translocation in the tomato mutant Chloronerva may indicate that nicotianamine is an Fe carrier in plants; Ripperger and Schreiber, 1982; Rudolph and Scholz, 1972) is required before implicating any low molecular weight ligand in Zn binding in vivo.

LITERATURE CITED

Akers, H. A.; Abrego, V. A.; Garland, E. *J. Bacteriol.* **1980**, *141*, 164–168.

Benes, I.; Schreiber, K.; Ripperger, H.; Kircheiss, A. *Experientia* **1983**, *39*, 261–262.

Blincoe, C. *J. Sci. Food Agric.* **1974**, *25*, 973–979.

Bremner, I.; Knight, A. H. *Br. J. Nutr.* **1970**, *24*, 279–289.

Budesinsky, M.; Budzikiewicz, H.; Prochazka, Z.; Ripperger, H.; Romer, A.; Scholz, G.; Schreiber, K. *Phytochemistry* **1980**, *19*, 2295–2297.

Cataldo, D. A.; Garland, T. R.; Wildung, R. E.; Drucker, H. *Plant Physiol.* **1978**, *62*, 566–570.

Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–72.

Evans, G. W.; Johnson, P. E.; Brushmiller, J. G.; Ames, R. W. *Anal. Chem.* **1979**, *51*, 839–843.

Fahey, R. C.; Newton, G. L. In *Functions of Glutathione. Physiological, Toxicological, and Clinical Aspects*; Larsson, A., Orrenius, S., Holmgren, A., Monnerik, B., Eds.; Raven: New York, 1983; pp 251–260.

Fushiya, S.; Sato, Y.; Nozoe, S.; Nomoto, K.; Takemoto, T.; Takagi, S. *Tetrahedron Lett.* **1980**, *21*, 3071–3072.

Gomah, A. M.; Davies, R. I. *Plant Soil* **1974**, *40*, 1–19.

Grill, E.; Winnacker, E.; Zenk, M. H. *Science (Washington, D.C.)* **1985**, *230*, 674–676.

Groves, J. T.; Dias, R. M. *J. Am. Chem. Soc.* **1979**, *101*, 1033–1035.

Hewitt, E. J. *Spec. Publ.—Chem. Soc.* **1979**, *No. 36*, 91–127.

Huffman, E. W. D.; Allaway, W. H. *J. Agric. Food Chem.* **1973**, *21*, 982–986.

Ito, Y.; Conway, W. D. *Anal. Chem.* **1984**, *56*, 534A–554A.

Jackson, P. J.; Barton, K.; Navanjo, C. M.; Sillerud, L. O.; Trewhalla, J.; Watt, K.; Robinson, M. J. In *Proceedings of the First International Congress on Plant Molecular Biology*; Galau, G. A., Ed.; University of Georgia Center for Continuing Education: Athens, GA, 1985; p 35.

Johnson, C. M.; Stout, P. R.; Broyer, T. C.; Carlton, A. B. *Plant Soil* **1957**, *8*, 337–353.

Kasai, T.; Larsen, P. O. *Fortschr. Chem. Org. Naturst.* **1980**, *39*, 173–185.

Lewis, D. H. *New Phytol.* **1980**, *84*, 209–229.

May, L.; Morris, E. R.; Ellis, R. *J. Agric. Food Chem.* **1980**, *28*, 1004–1006.

Meister, A.; Anderson, M. E. *Annu. Rev. Biochem.* **1983**, *52*, 711–760.

Mottola, H. A.; Haro, M. S.; Freiser, H. *Anal. Chem.* **1968**, *40*, 1263–1266.

Noma, M.; Naguchi, M. *Phytochemistry* **1976**, *15*, 1701–1702.

Olsen, S. R. In *Micronutrients in Agriculture*; Mortvedt, J. J., Giordano, P. M., Lindsay, W. L., Eds.; Soil Science Society of America: Madison, WI, 1972; pp 243–264.

Österberg, R. *Nature (London)* **1974**, *249*, 382–383.

Price, C. A.; Clark, H. E.; Funkhouser, E. A. In *Micronutrients in Agriculture*; Mortvedt, J. J., Giordano, P. M., Lindsay, W. L., Eds.; Soil Science Society of America: Madison, WI, 1972; pp 231–242.

Rauser, W. E. *J. Plant Physiol.* **1984**, *115*, 143–152.

Ripperger, H.; Schreiber, K. *Heterocycles* **1982**, *17*, 447–461.

Rudolph, A.; Scholz, G. *Biochem. Physiol. Pflanz.* **1972**, *163*, 156–168.

Scholz, G. *Biochem. Physiol. Pflanz.* **1970**, *161*, 358–367.

Spiro, T. G.; Saltman, P. *Struct. Bonding (Berlin)* **1969**, *6*, 115–156.

Starich, G. H.; Blincoe, C. *J. Agric. Food Chem.* **1982**, *30*, 458–462.

Stephan, U. W.; Rudolph, A. *Biochem. Physiol. Pflanz.* **1984**, *179*, 517–523.

Sugiura, Y.; Nomoto, K. *Struct. Bonding (Berlin)* **1984**, *58*, 107–135.

Sugiura, Y.; Tanaka, H.; Mino, Y.; Ishida, T.; Ota, N.; Nomoto, K.; Yoshioka, H.; Takemoto, T. *J. Am. Chem. Soc.* **1981**, *103*, 6979–6982.
Thompson, J. F.; Tiffin, L. O. *Plant Physiol. (Suppl.)* **1974**, *53*, Abstract 125.
Tiffin, L. O. *Plant Physiol.* **1970**, *45*, 280–283.
Tiffin, L. O. In *Micronutrients in Agriculture*; Mortvedt, J. J., Giordano, P. M., Lindsay, W. L., Eds.; Soil Science Society of America: Madison, WI, 1972; pp 199–229.
Tiffin, L. O. In *15th Annual Hanford Life Science Symposium*; ERDA: Oak Ridge, 1977; pp 315–334.
Tinker, P. B. *Philos. Trans. R. Soc. London, B* **1981**, *294*, 41–55.
Tong, Y. S.; Rao, G. S. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **1983**, *42*, 626.
Van de Geign, S. C.; Petit, C. M.; Roelofsen, H. *Commun. Soil Sci. Plant Anal.* **1979**, *10*, 225–236.
Van Goor, B. J.; Wiesma, D. *Physiol. Plant.* **1976**, *36*, 213–216.
Walker, C. D. Unpublished Thesis, Murdoch University, Perth Australia 1980.
Walker, C. D.; Webb, J. In *Copper in Soils and Plants*; Loneragan, J. F., Robson, A. D., Graham, R. D., Eds.; Academic: Sydney, 1981; pp 189–212.
Walker, C. D.; Welch, R. M. In *Trace Element Metabolism in Man and Animals*; McCHowell, J., Gawthorne, J. M., White, C. L., Eds.; Australian Academy of Science: Canberra, 1981; pp 155–157.
Welch, R. M. *J. Plant Nutr.* **1981**, *3*, 345–356.
White, M. C.; Chaney, R. L.; Decker, A. M. *Plant Physiol.* **1981**, *67*, 311–315.
Wiesma, D.; Van Goor, B. J. *Physiol. Plant.* **1979**, *45*, 440–442.

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Determination of Tin in Wines and Sugar Beets by Means of Atomic Absorption Spectroscopy with Hydride Generation

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Hydride generation followed by atomic absorption spectroscopy has been used to determine the concentration of tin in 9.6% sulfuric acid solutions. These solutions were obtained by wet oxidation, with nitric and sulfuric acids, of biological samples in a Buchi 445 digestor. The effects of reaction time, hydrochloric acid presence, and pH values of the analyzed solution are outlined. The presence of hydrochloric acid is mandatory; minimum reaction times of 25 s and pH values between 0.9 and 1.25 are recommended. The four buffers potassium acetate, potassium phthalate, potassium chloride, and sodium hydrogen tartrate were compared. The first three are recommended for analysis with different sensitivities. The method allows for the determination of up to 3.3 ppb of total tin and 0.33 ppb of organic tin in the samples.

Over the past few years, quantitative determination of tin in foodstuff has attained considerable interest because of the ever-growing presence of tin both into cans on the market and in the active ingredients of pesticides, such as fentin hydroxide, fentin acetate, fenbutatin, and cyhexatin. These are all tin-based organic compounds. In many countries the law limits the presence of tin in foodstuffs.

The organic fraction of residues has greater toxicological relevance than the inorganic fraction. In fact, a concentration of 250 mg of inorganic tin/kg of food is generally considered a permissible limit in canned food (WHO, 1973), but concentrations as low as 0.15–0.03 mg of Sn/kg of food are the suggested limits for organic tin derivatives (FAO, 1979).

The total amount of tin in foodstuff is generally determined after sample mineralization. To detect organic tin, it is extracted with a slightly polar organic solvent (i.e., trichloromethane, dichloromethane, carbon tetrachloride, benzene, and toluene); the extract is then mineralized after removal of the solvent (Getzendaner and Corbin, 1973). In order to destroy the organic material, either wet oxidation (Corbin, 1970) or dry ashing (Gorsuch, 1970) techniques are available (FAO, 1979; Crosby, 1977).

The most used methods are gravimetric, titrimetric, polarographic, and spectrophotometric techniques (Crosby, 1977; FAO, 1979; AOAC, 1984).

Gravimetric and titrimetric techniques are based on the oxidation of tin, the precipitation of tin(IV) sulfide, and the conversion into oxide (for gravimetry) and into tin(II) chloride (for titrimetry). The oxide produced is weighed while the chloride is titrated by oxidation with iodine. Both methods of analysis are time consuming, demand skillful execution, and provide low sensitivity: 25 mg of total tin/sample is the minimum detectable amount (AOAC, 1984).

Polarographic methods are selective and offer good sensitivity (0.02 μg/mL). The detection limit is 0.1 μg/mL of tin, but anions, phosphate, severely interfere (Bourbon et al., 1982; Guinon and Garcia-Anton, 1985).

Spectrometric techniques are used, such as UV, visible and atomic absorption, with or without tin hydride generation (Crosby, 1977). Colorimetric techniques feature higher sensitivity (1–150 μg of tin) and are much easier to carry out. However, the interference by other metals is greater and the tin must be separated from the matrix. The most used chromogens are dithiol, pyrocathecol violet, phenylfluorone, and quercetin (Crosby, 1977; FAO, 1979). Flame atomic absorption spectrophotometry (AA) provide low sensitivity (1.5 μg/mL of tin in the final solution); heavy metals and anions, namely sulfate, severely interfere (FAO, 1979).

Other studies have described the combination of hydride generation technique with AA spectrophotometry—a technique that allows for lower detection limits (3 ng/mL of tin in the final solution) and more selectivity (Fernan-

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