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Plants Which Accumulate Metals. Part II. An Investigation of the Soluble Zinc Containing Extracts from Two Australian Species

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The zinc soluble in aqueous extracts of two Australian zinc accumulating plants has been investigated. In Crotalaria novae hollandiae *extracts zinc is in the form of the uncomplexed aquocation. The extracts from Polycarpaea glabra show that there are two soluble zinc complexes, one in the stem material and one in the flower heads. The stem complex is cationic at pH values of 6.9 and 7.9 and was hydrolysed to give D-galacturonic acid. Further extractions confirmed that soluble zinc in* Polycarpaea glabra *is associated with pectic carbohydrates and not with proteins.*

Introduction

Comparatively few soluble metal complexes from plants have been identified. Phenolic hydroxyl and carboxylic acid groups in plant tissues have been suggested as ligands [2]. Complexes which have been identified are: iron with citrate [3], malate and maleate [4]; chromium as the tris(oxalato)ion [5]; copper bound to proteins [6], polypeptides and amino acids; zinc as the aqua cation [8–10]; as amino acid compounds [10], and associated with pectates [11]. Pectic substances in general are known to bind cations and have ion exchange capacity [12].

The general method of investigation involves the extraction of plant material with water or aqueous ethanol (or the use of an exudate), followed by separation and examination by chromatographic and electrophoretic methods. In all the steps, chemical changes may occur which possibly lead to incorrect conclusions. If the complexes are kinetically labile, then those which are thermodynamically stable under intact cell conditions need not be those which are stable in the extracts. Complexes may not survive chromatographic treatment [13]. The initial results from such studies must be treated with some caution.

In this paper we present the results of studies of the soluble zinc containing extracts [1] from the Australian species *Polycarpaea glabra* and *Crotalaria novae hollandiae*.

Experimental and Results

Extraction Procedure

Dried plant material was ground using a Glen–Creston agate ball mill. Approximately 3 g of ground material was shaken for twelve hours with deionized water, or 80% aqueous ethanol. The mixture was centrifuged at 2000 r.p.m. for five hours. After decantation the material was re-extracted and centrifuged. The centrifugate was added to the original extract. The total extract was evaporated to 1 cm^3 using a rotary evaporator.

Purification

Initially the concentrated solutions were chromatographed by downward paper chromatography. The fraction containing the zinc was collected from the end of the paper. This procedure was very lengthy and a protein containing compound with the same Rf value in butanol pectic acid/water (12:3:10) as the zinc, led to the erroneous conclusion [14] that the zinc was a soluble protein. Finally Sephadex columns in conjunction with a desalter were used.

First set of experiments; Investigation of the ethanol extract of *Polycarpaea glabra* flowers and the aqueous extract of *Polycarpaea glabra* stem.

The ethanol extract of the flower heads has been shown [1] to contain 36% of their total zinc, while aqueous extract of stems has been shown to contain 47% of their total zinc [1]. The two extracts were purified by descending paper chromatography. Samples, together with aqueous zinc ions were chromatographed by ascending paper chromatography using Whatman 3MH paper and butanol/acetic acid/water (BAW) 12:3:10 by volume (upper layer). When the zinc was located with dithizone it was found that there were two distinct forms of zinc, one in each of the flowers and stems, neither corresponding to ionic zinc.

The extracts were chromatographed after having been passed through sephadex columns and the results are shown in Fig. 1. The Rf values of the zinc complexes in the stem and flower extracts are 10 and

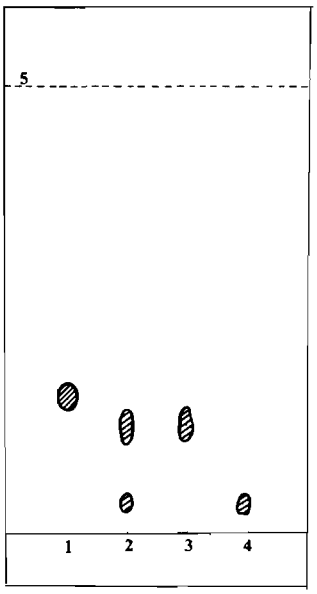


Figure 1. Chromatogram of extracts from stem and flower heads of *Polycarpaea glabra.* Solvent, BAW; paper, Whatman NO. 1. 1: ionic zinc; 2: mixed extracts; aqueous extract from stem and ethanolic extract from flower heads; 3: ethanolic extract from flower heads; 4: aqueous extract from stem; 5: solvent front.

23 respectively. The presence of two different complexes was confirmed by chromatographing a mixture of the two extracts, when two distinct spots could be seen. Further confirmation was obtained by running the samples on 90 cm long paper by descending chromatography. The well separated spots which resulted had similar Rf values (10 and 23) indicating that the different values are not caused by differential retardation at the origin, but by two distinct zinc containing species.

Electrophoretic Experiments

The electrophoretic behaviour of the two zinc complexes was investigated using a Shandon–Southern low voltage apparatus and Whatman number 1 paper. Buffers were pyridine/acetic acid at pH values of 6.9 and 7.9. The stem complex moves toward the negative electrode at a rate half that of zinc ions. The flower complex is immobile at both pH values.

Chromatographic Identification of Ligands in the Zinc Complex from Polycarpaea Glabra *stems.* A variety of solvent systems, paper and thin layer plates was used to study the complex, the majority of which caused the complex at least partially to dissociate. The following methods were finally used: the extract of *Polycarpaea glabra* stems was passed through a Sephadex 25 column. The zinc-containing fraction was located by spotting the eluent on to filter paper impregnated with dithizone. The complex was intermediate in mobility and a molecular weight of under 1000 is indicated. The complex was hydrolysed by heating for 30 minutes with hydrochloric acid (2*M*). The complex solution and the hydrolysed complex solution were both chromatographed as follows:

(i) TLC plates, butanol/acetic acid/water (12:3:5). The complex was not ionic zinc (Rf complex = 11, Rf ionic zinc = 21). The chromatographs of the complex and the hydrolysis product gave no colours with ninhydrin, and no positive results for acids or phenols.

(ii) The hydrolysed complex was chromatographed on silica gel plates and developed with benzene/acetic acid/methanol (1:1:3). Orthophosphoric acid–aminobiphenyl reagent revealed a clear yellow–brown spot, indicating the presence of carbohydrate (Rf = 35). Under these conditions this Rf value is close to that for D-galacturonic acid [15] and few other carbohydrates have a value of less than 50 with this system. An identical chromatogram sprayed with anisidine–phthalic acid gave a distinct brown colour, again indicating uronic acids.

(iii) The hydrolysed complex was chromatographed using silica gel plates and methylethylketone/acetic acid/methanol (3:1:1). Galacturonic acid was again indicated by a brown spot (Rf = 10) with orthophosphoric acid–aminobiphenyl reagent. The unhydrolysed extract did not show the presence of galacturonic acid with the zinc spot, but the presence of a carbohydrate is indicated with anisidine–phthalic acid. The zinc complex in the flowers has not been identified because of lack of material. Zinc in the extracts of *Crotalaria* leaves and stems when chromatographed by any of the above methods behaved always as ionic zinc.

Preparation of Zinc in the Soluble Protein of Polycarpaea Glabra *by the Levitt method [16]*

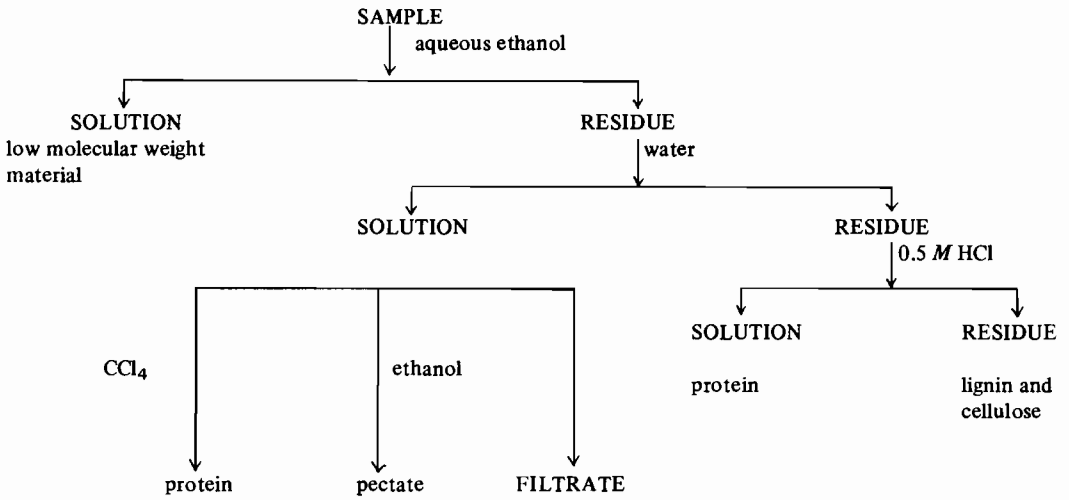
In duplicate 4 g of freeze dried material was extracted with ether, aqueous ethanol and then washed with buffer solution at pH 7.0. After the solid had been filtered off ammonium hydroxide–ammonium sulphate mixture was added to precipitate the protein. The protein was filtered off, suspended in water and dialysed for two days. It was evaporated to dryness and finally wet-ashed for zinc by atomic absorption spectrophotometry. The filtrate remaining from the protein precipitation was divided. One part was analysed for zinc, the other was treated with ethanol, the precipitated pectates were removed and the filtrate analysed for zinc. The results are shown in Table I.

TABLE I. Percentages of Total Zinc in *Polycarpaea Glabra* Stems Extracted and Precipitated by Various Solvents.

Ether	Ethanol	Plant Residue	Protein	Solution after Protein Precipitation	Solution after Pectate Precipitation
2	13	34	1	49	6

Determination of Zinc in Soluble Protein and Pectate in Polycarpaea Glabra *Stems by the Sevag Method [17]*

A sample of *Polycarpacea glabra* was taken through the following sequence: (results in Table II)



Discussion

The chromatographic results have established that there are two types of soluble zinc complex in *Polycarpaea glabra,* one in the stem material and one in the flower heads. Neither of these complexes is ionic zinc, however the soluble zinc in the *Crotalaria* species appeared to be cationic aquo-zinc ions.

The stem complex of *Polycarpaea glabra* is comparatively stable with respect to the complex from the flower heads. It has low mobility in butanol/acetic acid/water, and dissociates in many other solvent systems. The extraction studies (Tables I and II) show that most of the soluble zinc is associated with the pectate fraction and not with proteins, thus the suggestion that zinc is associated with proteins was in error [14].

More than half the zinc in the two species studied in this work is soluble [1] and this is in agreement with results for corn leaves [18] and for *Agrostis tenuis* shoots [11]. The zinc complex in the last species was anionic but was not stable enough to be identified [11]. The cell wall as a major site of zinc accumulation has been demonstrated in *Agrostis species* [11, 18–20]. Peterson [11] using *Agrostis tenuis* gave ^{65}Zn to both zinc tolerant and copper tolerant plants. Subsequently, he found that the zinc tolerant plants contained more ^{65}Zn in the pectate

TABLE II. Percentages of Total Zinc Extracted from *Polycarpaea Glabra* Stem Material Using the Sevag Method [17].

Aqueous Ethanol	Water			0.5 *M* HCl	Residue
11	42			46	1.5
	Protein	Pectate	Remainder		
	4	88	8		

extract of root cell wall than either the copper tolerant or non-tolerant plants. In a further study [20] of this species Turner and Marshall suggested that the tolerance mechanism is associated with an altered carbohydrate content of the cell wall coupled with metabolic adaptations.

Thus a possible mechanism [11] of zinc tolerance in *Agrostis tenuis* is accumulation by a passive process not requiring metabolic energy [21], and storage in the root cell wall cation binding sites. Peterson [11] suggests that in this way large amounts of zinc would be inactivated in the root cell walls.

With *Polycarpaea glabra* and *Crotalaria novae hollandiae* the zinc is transported to the aerial parts of the plant. In *Polycarpaea* the soluble zinc is obtained as zinc galacturonate after hydrolysis and 88% of the water soluble zinc (37% of total) is associated with the pectates. Since galacturonic acid is the main structural component of pectic polymers, zinc is therefore established as being associated with this type of carbohydrate in the aerial parts of the plant. Similarly it has been shown that soluble nickel in *Hybanthus floribundus* leaves is associated with pectic carbohydrates [13]. The suggestion that calcium is associated with cell wall pectins was made some years ago; it seems likely that calcium forms pectate bridges, a process which limits cell wall plasticity. Calcium ions thus link together chains of polygalacturonic acids, by means of ionised carboxyl groups. It has also been shown that the polygalacturonic acids from pectin will collect other metal ions from water [23]. If the ions Cu^{2+}, Ca^{2+}, Zn^{2+} and Ni^{2+} are present in the same solution then the collection decreases in the order Cu>Ca>Zn>Ni. It seems unlikely that pectic acids have a high selectivity toward zinc and nickel compared with calcium. It has been shown, however [24], that in the case of alginate, an increase in the L-guluronic content leads to an increased affinity for calcium ions and a higher gel strength of the resultant calcium alginate [25]. The stereochemical modification of units in a polysaccharide chain appears to be widespread [26] and at least one epimerase has shown to depend on the presence of calcium. Recent work on cell wall pectic polymers [27–30] indicates a limited number of polymeric structural components. It therefore appears possible that in plants such as *Polycarpaea* or *Hybanthus* modification of the cell wall polymers to give increased affinity for certain metals may take place.

However, the alternative mechanism, that in which active metabolic processes are involved in the transport and storage of zinc in the aerial parts and where the carbohydrates are non specific cation storage sites, is attractive. The specificity of tolerance and accumulation would then reside with specific carriers. These would transport the metal through the cell wall to the storage sites.

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