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THE EFFECTS OF TRANSITION GROUP METAL IONS AND THEIR COMPOUNDS ON VARIOUS PLANT SPECIES

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Science of the University of London

by

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Bedford College, London

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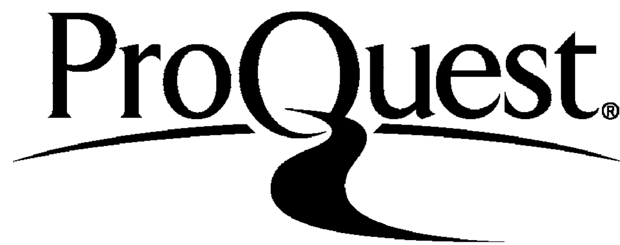

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ABSTRACT

Plants from metal rich sites were analysed and *Armeria maritima* from Dolfrwynog bog was selected for further study. Tolerance indices were established for tolerant and non-tolerant ecotypes. Copper was found accumulated mainly in the root. Anatomical, histochemical and electron microscopical studies indicated that the major accumulating area within the tolerant plant was the hypodermis. Phloem, endodermis, medullary sheath and tannin bearing cells also showed high concentrations.

Chemical extraction schemes were carried out on root material. 29.0% Of the metal was removed with low molecular weight water soluble materials, 11.8% was solubilized by the proteolytic enzyme pronase and 49.0% was associated with pectic and similar polysaccharidic material. Differential ultracentrifugation indicated that copper was mainly linked to cell wall material. Dialysis with tartaric acid resulted in the removal of 44.8% of the copper present in root material, suggesting only weak bonding to cell wall pectates.

Water extractable copper from roots was in the form of bis-prolinato copper(II). A high proportion of the free amino acid pool in tolerant *Armeria maritima* consists of proline, even when the plant is not under copper stress. Non-tolerant plants have much lower levels of proline. Copper is removed from sensitive sites by guttation from leaf salt glands. A multistage mechanism of tolerance is suggested.

The small fraction of the total copper in the bog available to plants is probably complexed to humic and fulvic acids. A complex(es) present in the bog extract had a mole ratio of complexing agents to copper of 1.15 and a log stability constant of 6.0.

For comparison the effects of platinum group metals on crop species were studied and plant metal levels presented. The relative order of toxicity was found to be: Pd(II) > Pt((IV) > Rh(III). Platinum was also applied in various complexed forms to corn, but no significant differences in their effects was observed.

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THE EFFECTS OF TRANSITION GROUP METAL IONS AND THEIR COMPOUNDS ON VARIOUS PLANT SPECIES

Foreword

Soils containing high levels of heavy metals have characteristic tolerant flora associated with them and the distribution of certain of these species has been used in geochemical prospecting. The means whereby plants tolerate and even accumulate large levels of toxic metals without showing serious toxicity symptoms are far from fully understood. Possible tolerance mechanisms are reviewed in the introduction. The present study is an attempt to ascertain the chemical forms and physical locations of copper accumulated by *Armeria maritima*. The nature of the copper available to the plant has also been investigated.

In the introduction a brief botanical background is given including sections on root morphology, mineral nutrition and mineral uptake mechanisms Further sections outline the rôles played by copper and other micronutrients in plant metabolism, describe copper and iron toxicity symptoms and survey metal chemical bonding in plants.

The major part of this thesis is concerned with the uptake of labile forms of copper. To act as a comparison with this, a chapter is devoted to an examination of the uptake of non-labile complexes of platinum group metals by crop species.

Particular attention is paid throughout to the various techniques which have been employed in this study.

CHAPTER I

INTRODUCTION

1.1 Cell Structure

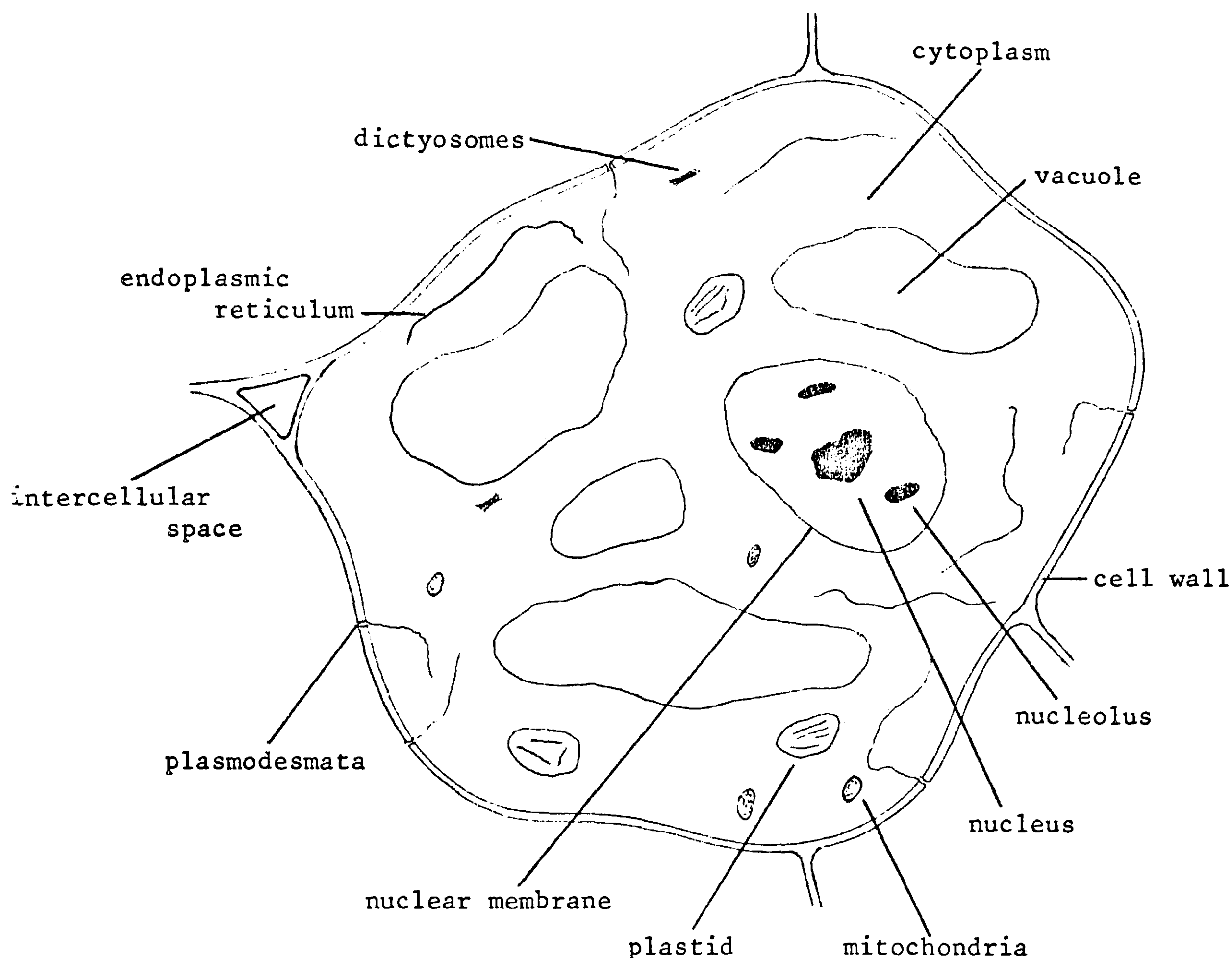


Figure 1. A schematic view of a generalised higher plant cell.

Although plant cells show considerable variability in their size, the great majority of them have diameters varying from 5 to 15 μm, and share a broadly similar structure. The typical young 'living' cell is bounded by a cell wall. Immediately within this wall lies a membrane, the plasmalemma, pressed against the cell wall by the turgor pressure of

the cell's contents. The plasmalemma effects a barrier between the living contents of the cell, called the protoplasm, and the external cell wall.

The cytoplasm is the least differentiated part of the protoplasm consisting of a viscous ground substance in which various sorts of organelles and granules are suspended. Cytoplasm consists of 85 to 90% of water by weight and contains dissolved salts and carbohydrates as well as proteins and fats in a colloidal state. Within the cytoplasm there may be one or more cavities called vacuoles containing watery cell sap. As cells mature these vacuoles grow and combine until there is generally a single large vacuole confining the cytoplasm to a thin layer lining the wall. The various substances present in the cell sap, inorganic salts, carbohydrates, proteins, amides, alkaloids, pigments, tannins etc., are either stored supplies for use in metabolic processes in the protoplasm or by-products of metabolism. The vacuoles are separated from the cytoplasm by the vacuolar membrane or tonoplast.

Of the numerous protoplasmic constituents the single largest body is the more or less spherical nucleus, which in meristematic cells attains as much as 75% of the volume of the cell. The nucleus is composed of gel-like sap or nucleoplasm in which are suspended chromosomes and one or more nucleoli. The chromosomes, which are the bearers of genes, consist of deoxyribonucleic acid (D.N.A.) and proteins. The nucleoli comprise ribonucleic acid (R.N.A.) and protein and are derivatives of chromosomes, playing a rôle in the control of protein synthesis within cells. The whole nucleus is separated from the surrounding cytoplasm by the nuclear membrane.

The various other protoplasmic inclusions shown in figure 1 are dictyosomes, plastids, mitochondria, ribosomes and endoplasmic reticulum. Ribosomes are small spherical granular organelles occuring on the outside

of endoplasmic reticulum and also in the nucleus and chloroplasts. They have diameters of about 1.5 μm and consist of R.N.A. and protein. They function as the sites of protein synthesis. The predominantly lipo-protein mitochondria are found universally in cells. They appear as short rods or thin filaments averaging from between 0.5 to 2 μm in length. The vessels are bounded by two distinct membranes. Interconversion of organic acids occurs within the inner membrane, whilst the processes of electron transfer, oxygen uptake and energy conservation take place in or on the inner membrane. A loose grouping of various organelles occuring in different kinds of plant cells are termed plastids. They are round, oval or disc shaped bodies that can be divided into two types: firstly the pigmented chloroplasts, which are the sites of photosynthesis, and chromoplasts, to which carrots and tomatoes owe their colour; and secondly the non-pigmented amyloplasts, which synthesise starch, and elaioplasts, which synthesise fats or oils. Although the cytoplasm appears as a structureless 'soup' an extensive membranous system of tubules and sacs, called endoplasmic reticulum, traverses it from the plasmalemma to the nuclear membrane, linking the various cytoplasmic bodies. So that when the cytoplasm as a whole moves, the endoplasmic reticulum moves too, maintaining an ordered structural integrity.

Root Morphology

All living plants need a supply of inorganic nutrient for survival. These are obtained at the required sites within the plant by a sophisticated ion transport system originating at the root. Since heavy metal toxicity generally results in severe root damage, then tolerant species must exclude these metals from sensitive sites within the root. Therefore their tolerance mechanism must reside partially at least in the root (figure 2).

The outer surface of the cortex is formed by the epidermis which consists of a layer of thin-walled cells. The root hairs are simply extensions of these cells extending out at right angles for as much as 10 mm in some plants. The cortex itself consists of elongated cells which on maturing become highly vacuolated. The arrangement of cortical cells is generally in successive concentric layers being usually many layers thick.

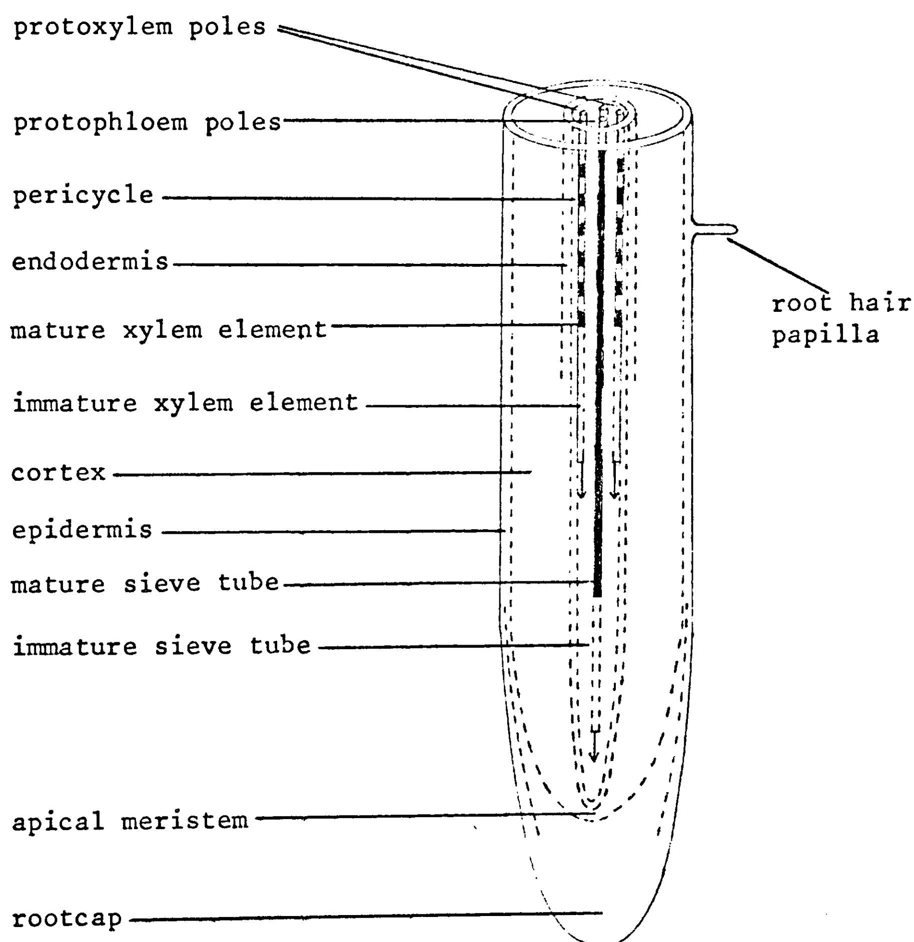


Figure 2. Diagrammatic structure of the root tip of *Nicotiana tabacum*, (after Esau, 1953).

The inner boundary of the cortex is the endodermis which comprises a single annular layer of cells. These cells are unusual in having a continuous layer of fatty suberin impregnating the radial walls of each cell. Suberin, which also occurs in barks and corks, has a similar structure to that of cutin which is found in the epidermis of most cell walls. This layer called the Casparian strip is attached to the plasmalemma, thus preventing the movement of solutes within the cell wall from the cortex to the stele (figure 3). The endodermis is semi-permeable, which prevents outward leakage of solute. The osmosis thus set up is responsible for 'root pressure'.

The innermost part of the root is called the stele. It consists of at least three types of adult tissues all derived from procambium: the pericycle, and two systems of connecting vessels, the xylem and the phloem. The phloem or food conducting tissues form part of the internal mechanism for carbohydrate and mineral transport within the plant.

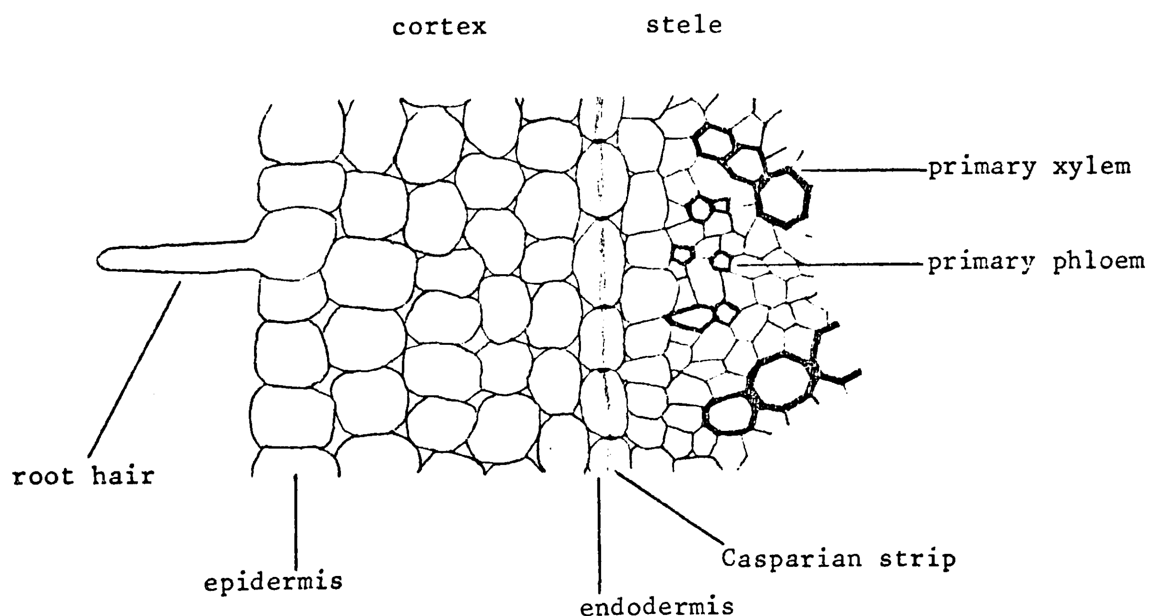


Figure 3. Root cross section.

Xylem vessels can be regarded as basically water and mineral carrying tubes, which are associated positionally with phloem elements. The xylem and phloem together form a continuous vascular system throughout all parts of the plant. Xylem elements are formed from elongated cells which are arranged in files. The walls between adjacent cells break down forming continuous vessels through which solute flow can take place unimpeded.

1.2 Uptake Mechanism

When plant roots are immersed in a salt solution, the solution quickly permeates a certain fraction of the root volume; and when the roots are further washed in water, nearly all of the salt is washed out (Epstein,1955). This result indicates that solutes can diffuse freely into part of the root. This part is called the free space or apoplasm and occurs in and between the cell walls of the cortex and epidermis and in the surface film of water on the root, comprising as much as 20% of some root volumes. Since the visible intercellular spaces are often much less than this in volume, a large fraction of the cell wall must therefore be in rapid equilibrium with the external solution.

Ions present in the free space are prevented from diffusing into cortical cells by the plasmalemma and from entry into the stele by the Casparian strip. So, in order that ions be taken up into useful metabolic sites within the plant, transport across the plasmalemma in cortical cells must first take place.

The mechanism by which active ion transport takes place is not fully understood. But the most widely accepted theory is that at the outer surface of the plasmalemma, specific binding agents or carriers

complex with an ion carrying it through the membrane and releasing it at the inner membrane surface. Specific ion binding proteins and cyclic compounds have been found.

Having gained access to the cell cytoplasm ions may be utilized by the cell. But the vast majority of them move, via cytoplasmic connections called plasmodesmata, from one cell to another towards the stele. This system is called the symplasm.

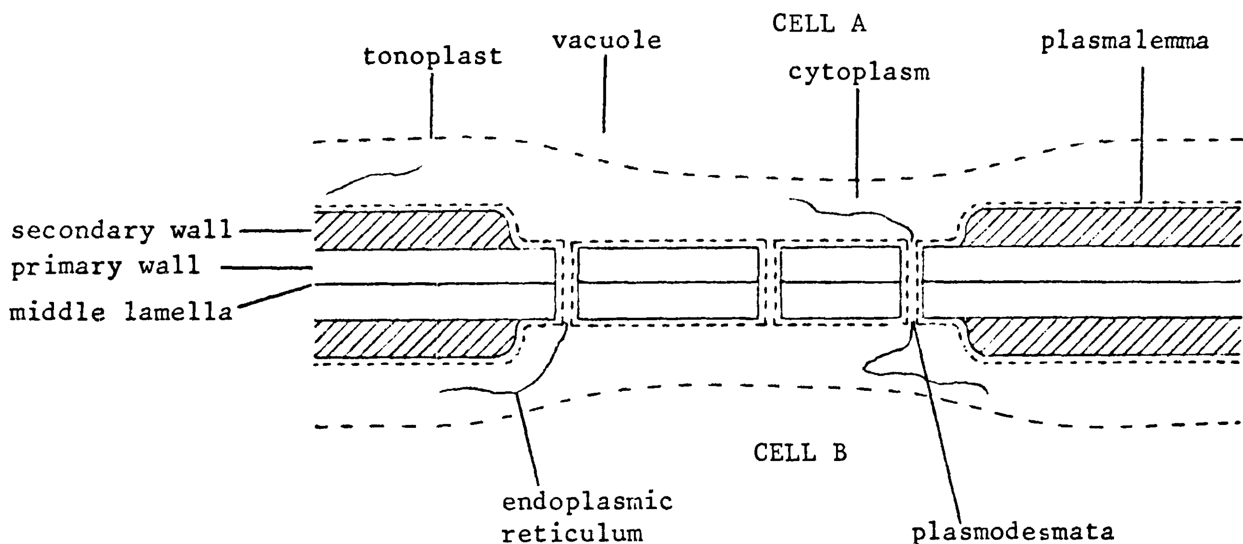


Figure 4. Diagram showing the cytoplasmic connections between cells.

Ions can travel greater or smaller distances in the apoplasm before transfer to the symplasm, for the all-important crossing of the endodermis. Clarkson (1974) showed that the majority of calcium moved in the apoplasm up to the endodermis. Once inside the stele, ions must somehow enter the non-living xylem vessels, for subsequent long distance translocation within

the plant. This step will involve the crossing, this time in the reverse direction, of stelar cell plasmalemmas. The latest theories on this subject suggest that another active transport carrier mechanism is involved (Laüchli, 1967, 1968) (Yu and Kramer, 1967). The ions thus released diffuse freely in the stelar apoplasm into the xylem vessels and hence, via the transpiration stream, to other plant cells, where once again active transport takes place.

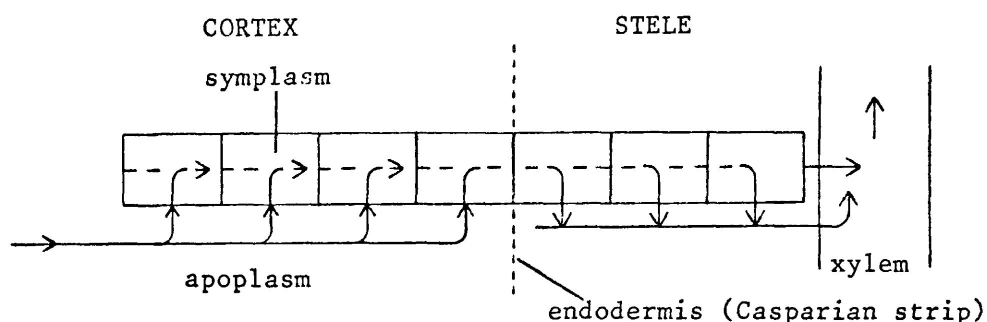


Figure 5. Ion flow pathways in a root (after Nye and Tinker, 1977).

Uptake kinetics

The active transport mechanism is highly selective and irreversible following Michaelis-Menten kinetics. This indicates that ion uptake is a function of the concentration of the ion, assuming no counterflow, i.e.

$$\text{Rate of uptake, } V = V_{max} \cdot \frac{[C]}{K_m + [C]}$$

where,

V_{max} = maximum rate of uptake

$[C]$ = ionic concentration

K_m = concentration of the ion giving half the maximum rate of absorption (Michaelis constant).

Numerous studies have been made in which the above kinetics is observed. These have been listed by Epstein (1972) and Nye and Tinker (1977). At relatively low ionic concentrations the change in the absorption rate decreases with increasing ion concentration until V_{max} is reached. At which point the carrier sites could be considered saturated. A substantial increase in ionic concentration at this point brings about a further increase in the absorption rate. Barber (1972) has suggested that uptake at higher concentrations is a passive process. It has been reported that two separate mechanisms probably exist (Epstein, 1972).

What has become clear is that mechanism 1 is an active process involving Michaelis-Menten kinetics, whilst mechanism 2 has no clear V_{max} or is actually a multiphase mechanism (Nissen,1971, 1974).

With two or more possible uptake mechanisms the question arises as to where they are located. Welch and Epstein (1968, 69) have shown that both mechanisms are sited at the plasmalemma, whilst Laties (1969) claims that the tonoplast is also implicated.

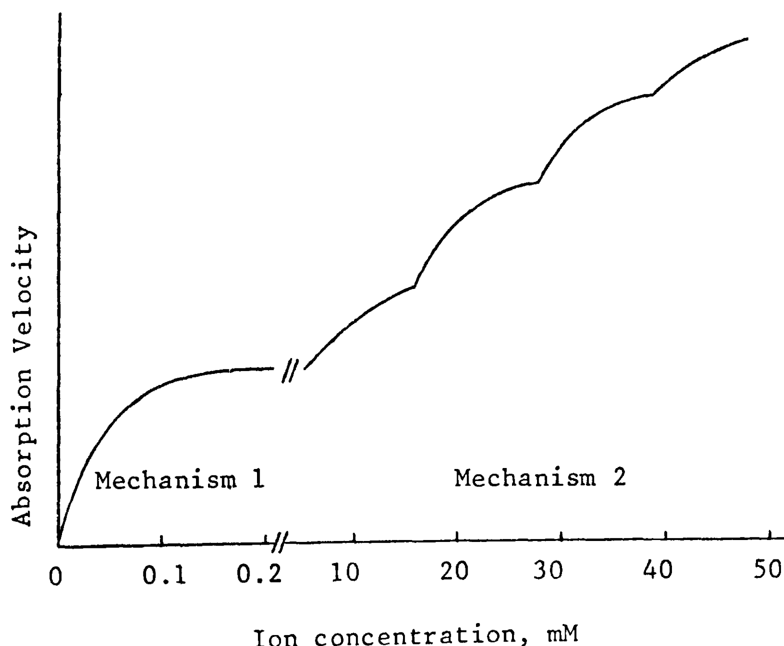


Figure 6. Generalised multiphase uptake isotherm for ions into roots (after Hodges, 1973).

There are three main factors involved in nutrient absorption: [C], V_{max} and K_m. Under controlled hydroponic conditions [C] is invariable, but the other factors vary according to root radius and length, temperature, antagonistic and synergistic effects of other ions, transpiration rate, oxygen availability and the quantity and quality of light.

1.3 The Mineral Composition of Higher Plants

When fresh plant material is dried to remove all traces of water, the dry material left comprises approximately 15% of the original mass, depending upon the species. Of this dried material about 90% consists of the elements carbon, hydrogen and oxygen in the molar ratio, 1:2:1, corresponding broadly to that found in polysaccharides. The remaining 10% is the mineral component of the plant. Although the quantity and number of elements assimilated depends heavily upon the soil-root interface conditions,a general pattern becomes clear. The elements nitrogen, potassium, phosphorus, calcium, magnesium, sulphur and silicon make up the major part of the total mineral content of the plant. The following elements found in much smaller quantities are known as trace elements: chlorine, aluminium, iron, boron, manganese, zinc, copper and molybdenum.

The mere presence of an element in a plant does not, however, demonstrate a need for that element. Whether a particular element is necessary or not has long been an important consideration for workers in plant nutrition. The elements can be introduced into a plant by any one of three media: atmosphere, water and soil. These media are enormously complex and samples of them may contain most, if not all, of the naturally occuring elements, albeit in some cases, in miniscule quantities.

Essentiality

For many centuries the need for mineral additions to heavily cultivated soils has been appreciated. But exact experimentation of the type familiar today was begun by Boussingault prior to 1840. He grew plants on inert media using nutrient solutions to supply the plants' mineral requirements.

Using Boussingault's methods, in 1869 Raulin showed that zinc was essential for the growth of *Aspergillus niger* (Raulin, 1869). Since that time numerous studies have brought to light other elements essential for plant growth.

During this century improved water purification techniques have meant that it has been possible to remove suspected essential elements almost totally from the nutrient solutions, and hence to establish their importance or otherwise to the plant. The classification of essential elements into those required in relatively large amounts, that is macronutrients, and those required in smaller quantities, that is micronutrients, is arbitrary. The following elements have been established as macronutrients: carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium and sulphur. The known micronutrients consist of chlorine, iron, copper, zinc, molybdenum, manganese and boron. These elements are universally required by all higher plants.

A comparison of the lists of the major elements in plants and the nutrients required by them shows two discrepancies, the absence of silicon and aluminium. Both of these elements are ubiquitous in soils and are best considered as apparently non-essential. Since even the purest solutions may contain levels of elements below current levels of detection, and a requirement may not have been established. Silicon, in fact, has been shown to be essential for the normal growth of *Equisetum arvense* (Chen and Lewin, 1969).

The story of the elevation of chlorine to essential status underlines the above point. No chlorine requirements had been found for any plant until purified air, as well as purified water, was used in culture experiments, there being enough chlorine in the atmosphere (present as chloride ion) to meet plant requirements. The work was carried out by Broyer et al. (1954) on tomato plants. Since then the chloride ion has been implicated in the photo-oxidation of water in isolated chloroplasts (Arnon, 1961).

Two other elements which appear in small quantities in many plants but are essential only to certain species are sodium and cobalt. Cobalt is known to be required for nitrogen fixation by legumes (Evans et al., 1965), but this is not in itself evidence for essentiality. More recently a legume, *Trifolium subterraneum*, grown with a full nutrient supply, including adequate nitrogen, but excluding cobalt, showed deficiency signs (Wilson and Nicholas, 1967). Brownell and Wood (1957) showed that the halophyte species *Atriplex vesicaria* has a definite sodium requirement.

Definitions of essentiality have changed since criteria were first suggested by Arnon and Stout (1939). It is now considered sufficient to require that an element simply be involved in normal plant metabolism.

Micronutrient functions

Of the known micronutrients all but two, chlorine and boron, are transition metals. They are more commonly referred to as heavy metals, that is metals with a relative density larger than five. Transition metals have similar electronic structures and hence a number of physical and chemical properties in common. The metals readily form complexes, show variable oxidation states and catalytic capabilities.

There are four main ways in which essential elements can be utilized by plants:

(i) by forming part of complex organic molecules;

(ii) by involvement in ionic balance;

(iii) by participating in the working of enzymes, usually by binding the enzyme and substrate together;

(iv) by creating a redox system, necessary for many basic metabolic functions.

Heavy metal micronutrients are largely involved in the latter two modes of action. Since this thesis is concerned mainly with copper tolerance, the functional rôle of the element will be considered briefly here. In addition, because there are numerous reported synergistic and antagonistic effects between the element and iron, the latter will also be mentioned.

Copper. - Oxidases are a group of enzymes which catalyse the reduction of molecular oxygen to water. This group comprises various copper containing members: cytochrome c oxidase, ascorbate oxidase, laccase, phenol oxidase, tyrosinase (Beinert and Palmer, 1965). Plastocyanin is a copper containing protein found in chloroplasts and is a member of the photosynthetic electron transfer chain (Katoh et al., 1962). Copper also has ancillary functions in non-copper containing enzymes.

Iron. - Iron functions in forming an iron-porphyrin complex which can react with magnesium to produce chlorophyll. Various other iron-porphyrin compounds, the cytochromes, are respiratory electron carriers. Chloroplasts contain certain cytochromes and in addition non-iron-porphyrin compounds, ferrodoxins, both of which are involved in light induced electron transfer (Buchanan and Arnon, 1970). In addition many enzymes based on the iron-porphyrin structure have been isolated, they include nitrite and sulphite reductases, catalase and peroxidases. Iron can also act as a cofactor in certain enzyme systems.

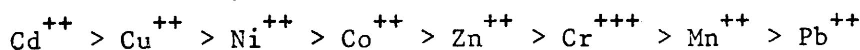
Mineral balance

For normal growth an exact balance of the micronutrient supply to the plant root is essential. Abnormal concentrations of micronutrients and indeed other elements will often result in the production of deleterious effects on the growth of plants. Heavy metals are present in soils, either bound to humic materials, clays or hydrated oxides of iron, manganese and aluminium, or less often as inorganic compounds.

Due to this immobilisation of heavy metals by soils only zinc, copper and nickel toxicities have been observed frequently. Another group, lead, cobalt, beryllium, arsenic and cadmium have been infrequently observed as toxic elements. Of these only lead and cadmium are of much interest since in recent years increasingly large levels have been entering the food chain (Foy et al., 1978).

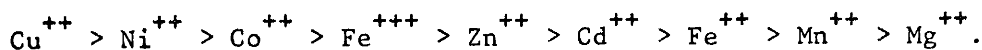
Orders of activities of the heavy metals in inducing toxicity symptoms in plant species are dependent on the species involved. But a fairly clear pattern has emerged over many years of careful study (Hewitt, 1951, 1953),(Hunter and Vergano, 1953),(De Kock, 1956), (Davis and Beckett, 1978).

The order of toxicity of some of the transition metal cations is as follows:



$$Cd^{++} > Cu^{++} > Ni^{++} > Co^{++} > Zn^{++} > Cr^{+++} > Mn^{++} > Pb^{++}$$

This order closely matches the order of stability of certain metallo-organic complexes chosen by Mellor and Maley (1948), which is:



$$Cu^{++} > Ni^{++} > Co^{++} > Fe^{+++} > Zn^{++} > Cd^{++} > Fe^{++} > Mn^{++} > Mg^{++}.$$

It has, however, been stressed by Krauss (1956) that localised modification of chelating effects can occur. Magnesium, for instance, is not readily replaced by copper or iron in chlorophyll.

Most iron present in leaves is found in the chloroplasts (Price, 1968). But more specifically a correlation between iron and chlorophyll content was noted by Jacobson and Oertli (1956). Deficiency of iron, therefore, causes general chlorosis of new leaves and the disruption of energy relations caused by the absence of cytochrome production. De Kock (1956) stated that the iron in chloroplasts was attached to phosphoproteins with little free iron in the ferrous state and physiologically active. He further suggested that iron controls phosphoprotein levels and thus the concentration of other heavy metals associated with the phosphoproteins. Evidence that iron is involved in the production of chloroplastic protein would seem to support this theory (Sideris and Young, 1956).

Iron toxicity is difficult to identify by plant symptoms. A bronzing or speckling of the leaf is usually observed. De Kock and Strmecki (1954) found that phosphorus-iron ratios in healthy leaves had narrow limits, chlorotic leaves having both high and low values.

The external indications of copper deficiency are chlorotic or deep blue/green leaves often with rolled margins. Die-back of shoots also occurs accompanied by multiple bud formation and seedlings do not develop. Insufficient copper has multifarious metabolic consequences; interfering with protein synthesis, thus causing an increase in soluble nitrogen compounds and adversely affecting the normal cellular levels of reducing sugars and acids (Brown et al ., 1958). Brown and Holmes (1955) also noted that iron accumulated at the nodes of corn plants under such conditions.

By far the most arresting symptom of copper toxicity in plants is chlorosis. Most of the heavy metals, in fact, induce chlorotic symptoms, superficially similar to those produced by iron deficiency. These ill effects can to some extent by alleviated by applying iron to the plant, usually as a chelate. Apart from leaf chlorosis, stunted roots and shoots

also characteristic of iron deficiency are observed. Both conditions show premature differentiation of xylem close to the promeristem in the root. Cell necrosis in the leaves is similar but more severe in the case of copper toxicity. Commonly observed also are blunt, brown enlarged root tips and a 'barbing' effect due to the formation of lateral roots near the tip (Mullen, 1980). Microscopic analysis indicates fewer cells in the root tip meristem and much cellular disruption (Daniels et al.,1973).

In a study into the effects of increasing the concentration of copper on the enzyme activity of lettuce seedlings, Mukherji and Das Gupta (1972) showed that catalase, peroxidase and I.A.A. oxidase activity all showed increases directly proportional to the copper concentration. They suggested that growth inhibition was caused by increased auxin breakdown. An attendant increase in soluble protein was observed.

A.& C. Reilly (1973) have found that in the labiate *Becium homblei* uptake of iron is not affected by high levels of copper at the roots. Accumulation of iron in the chloroplasts does not occur, thus leading to a fall in chlorophyll production.

The primary site of metal toxicity is in the root. De Kock (1956) suggested that the toxic metals must displace iron from an iron-protein chelate in the root, thus removing iron from metabolic sites.

1.4 Mineral Levels in Plants

Although plants will absorb all minerals that are present in the soil solution in available form, they show some selectivity and species variation. Typical values of mineral concentrations have been suggested, based on numerous separate analyses. Table 1 below presents typical elemental levels in plant foliage together with cellular levels and the sort of nutrient solution concentration that would be expected to give rise to these levels.

Table 1. Typical levels of mineral elements in foliage of plants. (After Hewitt and Smith, 1975).

Element	p.p.m. in dry matter	mM in cell sap	Nutrient mM
N	15000 - 35000	150 - 350	15
P	1500 - 3000	7 - 14	1
S	1000 - 3000	4.5 - 140	1.5
Ca	10000 - 50000	35 - 175	5
Mg	2500 - 10000	15 - 60	1.5
K	15000 - 50000	55 - 180	5
Na	200 - 2000	1 - 12	1
Fe	50 - 300	0.15 - 0.75	0.1
Mn	25 - 250	0.06 - 0.6	0.01
Cu	5 - 15	0.01 - 0.03	0.011
Zn	15 - 75	0.03 - 0.15	0.002
Co	0.2 - 29	0.005 - 0.05	0.0002
B	15 - 100	0.2 - 1.3	0.05
Mo	0.5 - 5	0.004 - 0.075	0.0005
Cl	100 - 1000	0.4 - 4	0.1

Critical levels

The effect of elemental levels on plant growth are best observed simply by plotting a graph of dry weight yield against the concentration of the element in the plant tissue (fig. 7).

The curve presented is the type obtained for essential elements. The deficiency zone represents a situation in which the level of nutrient solution is insufficient to provide the plant with the quantity of the element required for maximal growth. The critical concentration is

defined as that tissue level which produces a 10% drop in yield. Tissue concentrations of the element above the level at which maximal growth is observed represent luxury consumption of the element.

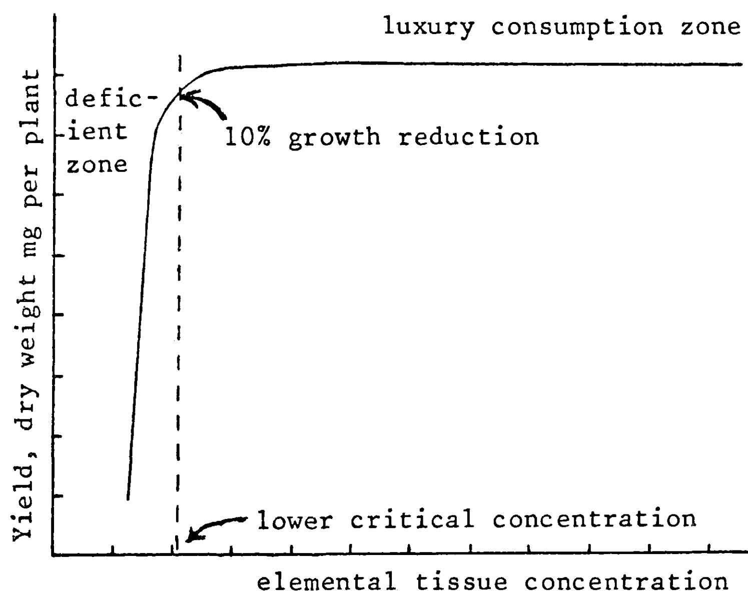


Figure 7. Graph showing variation of dry weight yield of a plant with tissue concentration.

In practice it is often difficult to obtain such curves, particularly for the micronutrient elements. This is because even in rigorously purified culture solutions enough of the element may be present to preclude deficiency symptoms. Even if the element has been totally eliminated from the solution air-borne contamination is still possible. Finally in some small species there may be enough of the element in the seed alone to allow the plant to grow to maturity without ill effect.

At higher levels of nutrition a drop-off in yield attributable to toxic effects is observed. The yield of dry matter from a given solution is found to vary from sample to sample for any one species, but the lowest

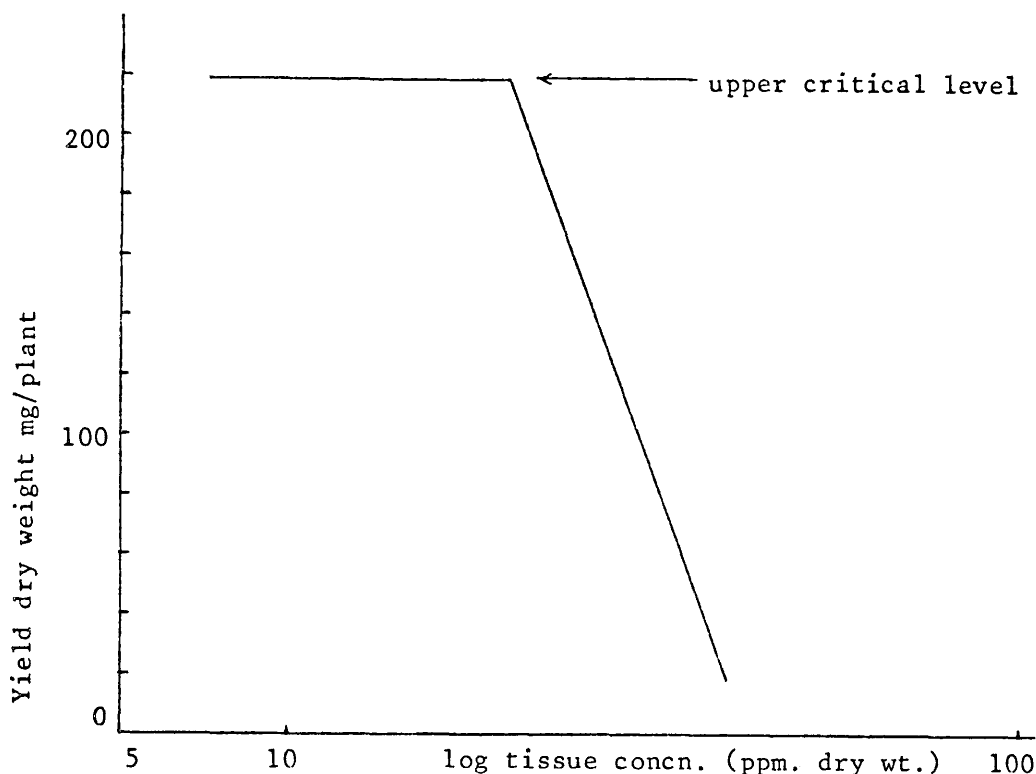


Figure 8. Graph showing the dependence of yield on the log of the tissue concentration of copper in young barley, after (Beckett and Davis, 1977).

level of the element in the plant tissue necessary to cause a drop in yield was quite independent of growth conditions (Beckett and Davis, 1977).

Beckett and Davies found that by plotting the logarithms of the tissue metal concentration against the yield, their data reduced to two straight lines whose intersection represented the upper critical concentration of the element, fig. 8.

Upper critical levels show surprisingly little inter-species variation for the more toxic elements. The copper values for barley, rye grass, lettuce, rape and wheat being: 19, 21, 21, 16 and 18 p.p.m. dry weight respectively (Davis and Beckett, 1978). Although the above work is concerned with the assessment of the relative hazard of various growing environments, a knowledge of upper critical levels of a metal for a certain species allows one to distinguish between normal ecotypes and those which either tolerate or accumulate toxic levels within themselves.

1.5 Tolerance

Any plant community is greatly affected not only by the obvious micro- and macroclimatic conditions prevailing but also by the soil atmosphere it experiences. The mineral status of a soil has two major ways of affecting the composition of a particular community. Firstly insufficient, sufficient or toxic quantities of mineral nutrients will have a direct effect on the plants present. Secondly there will also be their influence on the pH of the soil solution. The pH itself has been shown to affect the availability of elements by Ignatieff (1952).

Characteristic floras

The best documented example of minerals affecting the ecology of an area is that of soils derived from calcium bearing rocks.

Table 2. A comparison of characteristics of calcareous and acid soils(after Epstein, 1972).

Calcareous soils	Acidic soils
High calcium content	Low calcium content
High pH	Low pH
Nutrient rich	Low in available nutrients
Solubility of heavy metal ions low	Solubility of heavy metal ions high
Bacterial activity high	Bacterial activity low
Well drained	Poorly drained

The above table clearly indicates the multifarious factors that can dictate the make-up of these and other characteristic floras.

The nature of these calcareous and acidic soils has had an important effect upon the evolution of calciphile and calcifuge floras. All plants

thriving on calcareous soils are calciphilous, those with a specific requirement for calcium being calcicolous. The species not found on these soils are calcifuge and require low pH's to thrive.

In addition to the above, other important characteristic floras are the halophytic, selenium, serpentine and galmei communities. Only careful solution culture studies of these flora will indicate which soil properties are affecting the plants' viability.

'Heavy metal species'

Unique floras characteristic of soils containing high levels of heavy metals consist of plants associated with serpentine and galmei floras. Such communities can occur on natural or man-made sites, resulting from deposition of slagheaps or tailings, usually with low concentrations of nutrient elements present. The factors influencing the colonization of the sites are the toxicity of the heavy metals present, the paucity of other essential minerals, the exposed nature of these sites and over efficient drainage.

Ability to colonize such unfavourable sites must certainly have arisen genetically. Since there is such a wide genetic variability in all species, isolated individuals with the necessary adaptations will start to colonize such sites. Once established, albeit poorly, the added benefit of decreased competition would enhance the consolidation of the new community. These tolerant varieties have been labelled as heavy metal tolerant ecotypes and are not considered to be distinct species since they are morphologically identical with the non-tolerant variety. Nevertheless, certain heavy metal tolerant ecotypes show morphological differences from the non-tolerant variety and are classified as separate sub-species.

Indicator plants

Some tolerant species have distributions which enable mineral anomalies to be located. Such plants are indicator plants and many of them have been used commercially for prospecting. They can be divided into two classes. The first class are universal indicators which only grow on mineralized substrates. Examples of these include *Viscaria alpina* (Vogt, 1942) and *Becium homblei* (Horizon, 1959) for copper; *Thalspi calaminare* (Dorn, 1937) and *Viola lutea* (Schwickerath, 1931) for zinc and *Astragalus* species for selenium and uranium (Cannon, 1957). The second and more common class are local indicators, which are species adapted to mineralized substrates but grow elsewhere also. *Polycarpaea spirostylis* (Skertchly, 1897), *Elschscholtzia mexicana* (Cannon, 1960) and *Armeria maritima* (Henwood, 1857) all indicate copper.

Indicator plants are typically herbaceous members of the Labiaceae, Caryophyllaceae or bryophytes and are usually most effective for copper. They all have very high elemental contents in their ash, at least ten times greater than values in unmineralized soils. Lists of known indicators have been published (Cannon, 1960), (Brooks, 1972).

The use of biogeochemical methods in mineral prospecting has become standard practice particularly in the U.S.S.R., where important contributors in the field have been Malyuga (1964), Chikishev (1965) and Viktorov et.al. (1964). Important studies have also been made by workers in Canada, Australia, South Africa and the United States of America (Cannon, 1960), (Cole, 1965), (Nicolls et al., 1965), (Wild, 1968).

Tolerance mechanisms

Numerous theories concerning the possible mechanisms for heavy metal tolerance have been presented since Bradshaw (1952) first reported heavy metal tolerance in ecotypes of *Agrostis tenuis*. These mechanisms

have been reviewed notably by Antonovics et.al. (1971), Wainwright and Woolhouse (1975), and Ernst (1976). External tolerance mechanisms will not be considered here since they are not concerned directly with the active metabolic processes of the plant, dealing rather with specific soil conditions.

Internal tolerance mechanisms can be classified as follows:

(i) The deposition of the toxic metal at non-crucial sites, e.g. in the cell wall or vacuole;

(ii) The excretion of the toxic metal, e.g. by leaf abscission, via salt glands or by guttation;

(iii) Complexing of the metal within the cell, as high stability complexes;

(iv) Drastic metabolic alterations, e.g. increase in the levels of affected enzymes, subtle changes in enzyme structure or even changes in the protoplasm to allow enzymes to operate.

Studies involving heavy metal uptake in simple organisms have provided some elegant mechanisms by which tolerance is achieved. The techniques and information generated have been of importance in investigating the tolerance problem in higher plants.

Laboratory produced strains of the copper tolerant yeast *Saccharomyces cerevisiae* deposit excess copper as copper sulphide at and in the cell walls (Ashida, 1965)(Kikuchi, 1965). This is apparently due to a change in the normal sulphur metabolic pathways resulting in the production of hydrogen sulphide and thus copper sulphide. However, some copper tolerant strains have been shown to produce little or no hydrogen sulphide, and copper complexation in the cell has been suggested to explain this (Naiki et al., 1976). Work with *Aspergillus niger* has provided further

evidence of complexation. Ashworth and Amin (1964) showed that mercury tolerance in *A. niger* was due to non-protein sulphydryl groups protecting sensitive sites within the cell.

Contrasting results have been obtained recently with different species of copper tolerant algae. *Chlorella* has been shown to actually exclude copper (Foster, 1977), whereas tolerant *Scenedesmus* incorporate copper into their nuclei presumably in the form of a metallo-protein complex (Silverberg et al., 1976).

Tolerance mechanisms in higher plants

Deposition

Several papers, all relating to studies on the roots of metal tolerant clones of *Agrostis* species, by Turner, Bradshaw, Peterson, Gregory and Marshall have implicated the cell wall as the main deposition site for toxic metals. The degree of deposition of toxic metal at the cell wall was in fact found to be related to the degree of tolerance of the plant to that metal. The evolution of tolerance appears in most cases to be specific for the metal or metals present in toxic quantities in the substrate. No correlation could be found between heavy metal accumulation and sulphur metabolism.

A few other species have been found to accumulate metals at the cell wall. Gambi (1967) used histochemical techniques in finding that the nickel accumulator *Alyssum bertolonii* concentrated nickel in epidermal and sclerenchymal cell walls. Another nickel accumulator, *Hybanthus floribundus*, was also shown to deposit large quantities of nickel mainly at the epidermal cell walls (Farago et al., 1975).

Certain other experiments whilst not dealing specifically with heavy metal tolerant ecotypes have pointed to the cell wall as the major accumulation site. Studies by Hammett (1928) and more recently by

Malone et al., (1974) have shown lead deposits at cell wall sites. The latter study using high power microscopic techniques indicated that dictyosomes actually transferred lead from within the cytoplasm through the plasmalemma and thus into the cell wall. Since lead toxicity is rarely observed in nature, due to strong sorption of lead by soils, studies with lead have not been carried out under strictly physiological conditions and therefore conclusions about tolerance mechanisms should not be drawn. Cartwright (1966) working with *Trifolium subterraneum* found 64% of the copper present was in cell walls and Diez-Altares and co-workers showed germinating *Zea mays* tissues to accumulate significant levels of zinc in the cell walls under normal nutrient conditions.

Another obvious possible method of excluding toxic metals from metabolic sites in the cytoplasm would be for the plant to compartmentalize the metal in the vacuole. This theory has been suggested and supported by several studies carried out on European zinc accumulator species by Ernst (1977) and Mathys (1973).

Running in conjunction with the selective cellular distribution of metals by tolerant plants is the possibility of varying the metal distribution between plant parts. Reasonably clear patterns have emerged from the literature over the years. In general herbaceous plants accumulate larger levels of toxic metal in their leaves than in their roots whilst grasses and certain other small species accumulate in the opposite manner (A& C. Reilly, 1973), (Ernst, 1972), (this thesis). Since deciduous plants shed their leaves annually this will result in a regular method for heavy metal removal. Alternatively grasses and other small species, in regularly renewing their roots by the process of vegetative reproduction, minimise build-up of toxic metals. Dykeman and De Souza (1966) in describing the uptake and distribution of copper within trees growing in a copper swamp found significant levels in both roots and leaves or needles. The majority of the above studies were carried out on samples

collected from heavy metal rich soils and thus the possibility of surface contamination of root samples cannot be discounted.

Clearly any other method of excretion could be a means of ridding a plant of unwanted products. Guttation of heavy metals has been observed in various european species (Ernst, 1969), (this thesis). Finally a novel method of immobilizing metal is employed by a New Caledonian nickel accumulator *Sebertia acuminata* which accumulates up to 25% dry weight of nickel in its latex.

Metabolic changes

Considerable evidence has been presented to show that there is a fundamental difference between the metabolism of tolerant and non-tolerant ecotypes. Woolhouse (1970) found that certain enzymes in roots of lead tolerant *Agrostis tenuis* were tolerant of high lead levels, whilst the same enzymes in non-tolerant *Agrcstis tenuis* were adversely affected at the same levels. More recent studies both *in vivo* and *in vitro* have shown that *in vitro* there appears to be no difference between enzymes from tolerant and non-tolerant sources. Enzymes within intact cells of tolerant plants were indeed more tolerant of higher metal levels than their counterparts. Evidently some active enzyme protection mechanism is operating within the living cell (Mathys, 1975) (Ernst, 1977). Studies by Cox (1976) have revealed that cell wall acid phosphatases of zinc tolerant and non-tolerant clones of *Anthoxanthum odoratum* were different, yet Cox et al. (1976) were unable to distinguish between soluble acid phosphatases from similar sources. Similarly a copper tolerant cell wall acid phosphatase in *Agrostis tenuis* has been established (Wainwright and Woolhouse, 1975).

Complexation

The concept of inert complexation has received a lot of attention but few definite complexes have been isolated. Much of the work is open to the criticism that artifacts may have resulted during the various extraction procedures. Nevertheless Reilly has reported the presence of copper-amino acid or copper-peptide complexes in the cell sap of *Becium homblei* (Reilly et al., 1970) (Reilly, 1972) and Farago and Mullen (1979) have found a copper-proline complex in tolerant *Armeria maritima*. In attempts to elucidate the means by which metals travel through the plant, xylem exudates have been analyzed and various organic acid-metal complexes have been identified (Tiffin, 1966, 1967). Mathys (1977) proposed that mustard oils and oxalate ions act as complexing agents within the vacuoles of zinc tolerant *Silene cucubalis* and *Thlaspe alpestra*. Pitt (1977) has shown pectin to be the complexing agent for zinc in tolerant *Polycarpaea glabra*.

1.6 The Structure and Chemical Composition of Cell Walls

The cell wall has repeatedly been shown to act as a heavy metal accumulation site. Thus a knowledge of the chemical composition of cell wall material is a prerequisite to an understanding of the possible mechanisms of complexation. Cell walls, although varying greatly in size and thickness, are generally composed of three layers: the intercellular substance, or middle lamella, the primary wall and the secondary wall. All plant cells are surrounded by a thin primary cell wall usually from 1 - 3 μm thick and fairly elastic. The middle lamella is a layer joining primary walls of separate cells. Secondary walls, not present in all cells, are laid down within the primary wall by the cell protoplast. They are relatively thick, 5 - 10 μm, and rigid giving strength to the cell.

Primary cell walls are composed basically of highly structured cellulose microfibrils wound together to form strands. These strands are

also laid in an ordered fashion determining the basic shape of the cell. Between the strands are capillaries containing various complex polysaccharidic molecules, lignin and water all responsible for the characteristic properties of the cell wall. The microfibrils are composed of 1 - 4 linked β-glucopyranose units with a degree of polymerization (D.P.) of 2000 - 14000.

The polysaccharides make up by far the largest non-cellulosic component of the cell wall. Historically they have been classified in terms of their solubility characteristics (Northcote, 1963). More recently, however, detailed procedures have enabled the elucidation of the structures of these often highly complex polymers.

The non-cellulosic polysaccharides can be crudely separated into three groups:

(i) polysaccharides or neutral hemicellulose,

(ii) polyuronides or acid hemicellulose,

(iii) pectic substances.

Essentially pectic acids are composed of 1,4 linked anhydro-α-D-galacturonic acid units but also contain L-arabinose, and D-galactose, though other sugars may be present (Aspinall, 1970). The basic polyuronide back-bone has side chains of neutral sugars and may be more or less methyl esterified at its carbonyl groups, D.P. 400 - 2000. The middle lamella is particularly rich in calcium and magnesium salts of pectic acids.

There is a steady gradation from pectic substances, consisting largely of acid sugars, through acid hemicelluloses, consisting of large proportions of both non-acidic and acidic sugar units, to the pure polysaccharides (neutral hemicellulose).

Acid hemicellulose is composed of varying amounts of xylose, arabinose, rhamnose, glucuronic acid, o-methyl glucuronic acid and galacturonic acid,

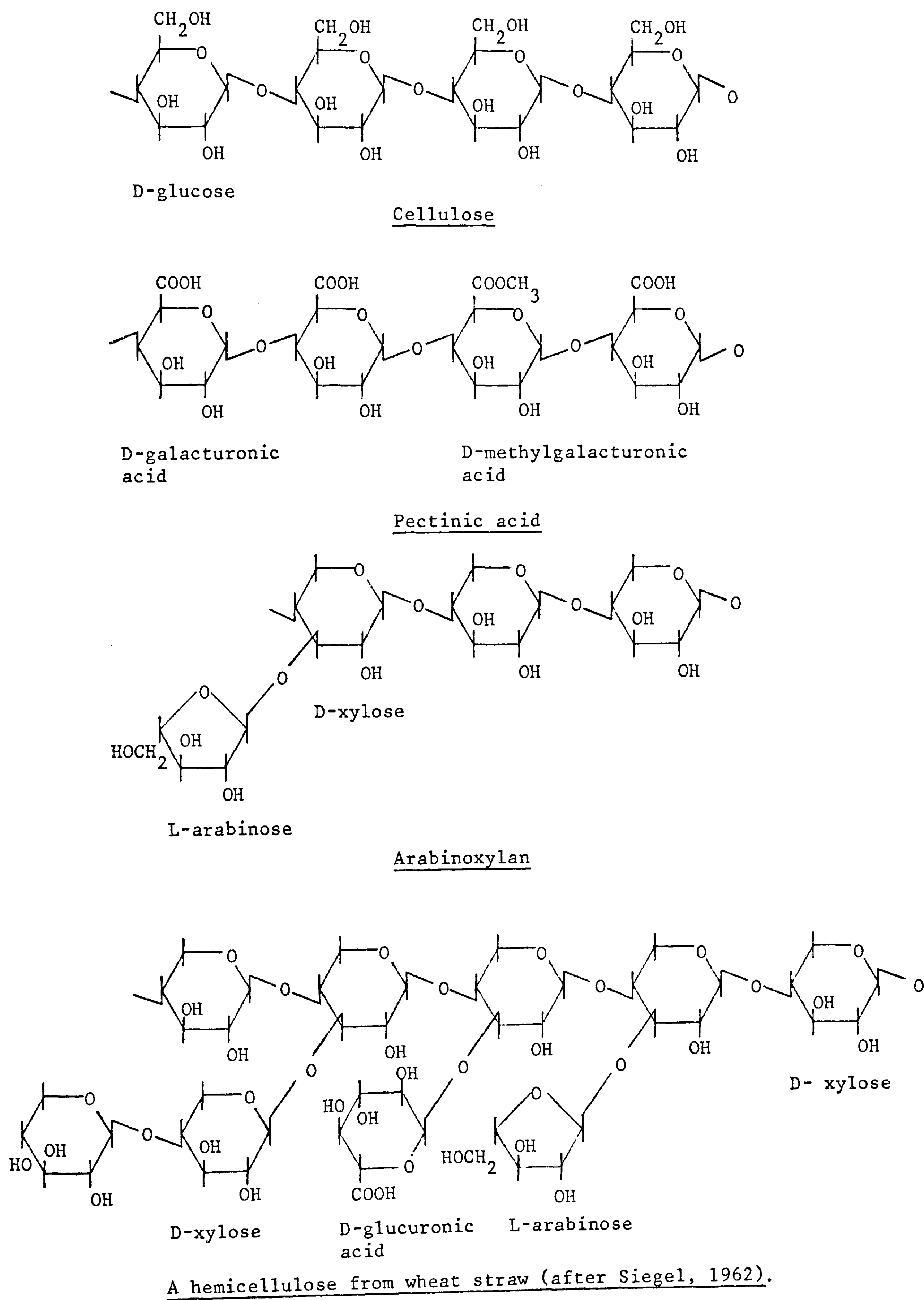


Figure 9. Units of cell-wall polysaccharides.

the uronic acids always occuring as end or side groups. By comparison with cellulose and pectic material the molecular sizes are relatively small, D.P. 40 - 200.

The pure polysaccharides, commonly called glycans, can be homopolymeric, e.g. arabans, galactans, xylans or mannans, or copolymeric e.g. glucomannans, galactomannans, arabinogalactans, or arabinoxylans. They have similar degrees of polymerization and structures to the acid hemicelluloses. The xylans are thought to form long strands parallel to the microfibrils.

All of the polysaccharides that compose the non-cellulosic matrix of the cell wall affect the hydrophobic or hydrophilic nature of the matrix and thus partially determine the physical properties of the cell wall.

Lignin is often a major constituent of secondary cell walls. They are complex, aromatic, high molecular weight polymers based on coniferyl, sinapyl and p-hydroxycinnamyl alcohols, acting as a cross-linking filler with the fibrillar structure producing the great rigidity typical of secondary thickening.

Finally young cell walls have been shown to contain small amounts of protein, rich in hydroxyproline. The protein is linked by an O-glycosidic bond to carbohydrate (Lamport and Northcote, 1960). The glycoprotein probably consists of a polysaccharide backbone with small protein side chains.

1.7 Cell Wall Metal Accumulation

The cell wall has often been claimed to be the major site of heavy metal accumulation in tolerant plants, and most especially in members of the *Agrostis* family. Since heavy metals usually accumulate in roots many workers have restricted their search for the mechanism of heavy metal tolerance to the root. In a series of studies, following a report by Bradshaw (1952) on the resistance of *Agrostis tenuis* to lead and zinc poisoning, groups of workers at the University College of North Wales at Bangor have systematically studied the means by which members of the *Agrostis* family avoid the deleterious effects of abnormally high levels of toxic metals in the soil (Gregory, 1964) (Turner, 1967, 1970) (Turner and Gregory, 1967) (Turner and Marshall, 1971, 1972).

Turner (1970), using an ultracentrifugation technique on homogenized *Agrostis tenuis* root material from various sources, showed clearly that cell wall material from metal tolerant clones grown at normal nutrient levels contained more of the metal to which the plant was tolerant than did cell wall material from non-tolerant clones. At high levels of the metal again the tolerant material was accumulating larger quantities than the non-tolerant. His concomitant observation, that the tolerance of one metal did not mean that the plant was tolerant to other metals present at toxic levels in the soil , was in accord with the findings of Jowett (1958), Gregory and Bradshaw (1965) and Bradshaw et al. (1965). In an earlier fractionation study Gregory (1964) had found cell wall material to be the major accumulation site in copper tolerant *Agrostis tenuis* roots.

Studies of metal localization in other species are not always easy to interpret since often non-tolerant ones have been used and the levels of nutrition are extremely variable. Cartwright (1966) found that 64% of

the copper in the nodules of *Trifolium subterraneum* was in the cell walls. Diez-Altares and Bournemisza (1967) showed that as much as 43.6% of the total zinc was present in the cell walls of germinating *Zea mays* grown at normal nutritional levels of zinc.

Ernst (1972) has carried out ecophysiological studies on several African heavy metal tolerant species and concluded that metal binding was metal, species and tissue dependent. He did observe, however, that large proportions of nickel, lead, zinc and copper were not removed from cortex material by sequential extraction. After organic solvent, water and dilute acid extraction of dried plant matter the material remaining is thought to be largely cell wall material, so that residual metal after sequential extraction is usually considered to be largely bound to cell walls.

Reilly and co-workers have studied the herb *Becium homblei* and other copper tolerant species from the Zambian copper belt: Reilly (1969), (Reilly et al., 1970) (Reilly and Stone, 1970) (Drew and Reilly, 1972). A leaf cell wall preparation of *Becium homblei* contained approximately 17% of the total copper present. The preparation, however, removed smaller carbohydrate molecules and the total leaf copper levels were not particularly high (Reilly et al., 1970). Drew and Reilly (1972) found that between 20 and 50% of the copper accumulated in roots of *Chloridion cameronii* and *Tracypogon spicatus* was bound in a relatively insoluble form, but little of the insoluble copper actually corresponded with their cell wall preparations of these grasses.

Electron microscopes with attached X-ray microprobes have been employed in the search for metal deposition sites at the cellular level. Waisel et al. (1970) have found aluminium to be located largely in the lumen of bean and barley roots. An even distribution of copper throughout bean leaves was observed by Daniels et al.(1973), whereas Malone et al.(1974),

without the use of the microprobe, showed that lead is deposited in the roots of corn.

The chemical fractionation study chosen by Peterson (1969) was designed to show not only sites of deposition but also the differences in the ways that non-tolerant and tolerant materials cope with toxic conditions. At high and low levels of zinc nutrition *Agrostis* spp. tolerant to zinc have it present largely in an insoluble form whereas non-tolerant material or material tolerant only to copper comprised mainly soluble zinc forms. Further fractionation showed that zinc was associated with insoluble protein and to a greater extent with pectic substances. Thus zinc tolerant plants contained five to six times more zinc in the pectate extract than did non-tolerant plants. Diez-Altares and Bournemisza (1967) employing a similar method found that most of the zinc present in the cell walls of germinating corn tissue was associated with protopectins and hemicelluloses. Further evidence for the complexation of heavy metals by cell wall carbohydrates comes from Pitt (1977) who showed that most of the zinc present in *Polycarpaea glabra* stems was firmly held in the pectate fraction.

Pectic substances have considerable cation exchange capacity and bind cations in the Donnan Free Space of cell walls (Dainty and Hope, 1959) (Mattson et al., 1949). Knight et al. (1961) found that cation exchange capacity was proportional to the uronic acid content of the plant material. The functional groups present in pectic substances responsible for cationic bonding are the carboxy and hydroxy groups of the uronic acid units. Several workers have presented evidence of heavy metals binding to these and other groups (Timberlake, 1959) (Ennis, 1962) (Broda, 1965). Accumulation of zinc by isolated

barley roots was found to be a passive process consistent with the above cation exchange mechanism (Findenegg and Broda, 1965). It seems certain, therefore, that part of the tolerance mechanism of some plants depends upon their ability to bind large quantities of toxic metal at pectic cation sites in the cell wall. If accumulation is purely a passive cation exchange process this does not then explain the absolute specificity of tolerance observed by Bröker (1963), Bradshaw et al. (1965), Gregory and Bradshaw (1965) and Turner (1970). It would seem that the binding of heavy metals to cell wall pectates is an important part of the overall picture of tolerance but absolute specificity must be sought elsewhere.

Copper has been reported as forming complexes of considerable stability with proteins and various other plant products (Mills, 1954). This great affinity of proteins for copper and other metals was studied by Gurd and Wilcox (1956) and in addition to this a great specificity was reported by Bayer (1964). Several reports of metal-protein links have been presented. Gilbert (1951) showed that copper-protein complexes were formed in tung and associations between heavy metals and crude protein have been observed in potatoes (Levitt and Todd, 1952). Bremner and Knight (1970) found that a considerable amount of the zinc present in rye grass was removed by the peptic digestion of water insoluble material. Smith (1953) found the copper accumulated by dried and powdered citrus roots to be directly related to the non-replaceable protein nitrogen of the root. A similar relationship between protein nitrogen and copper was claimed by Rasheed and Seeley (1966) for several different species. These studies point clearly towards links between heavy metals and proteins, yet none of them concern tolerant species so that conclusions about a tolerance mechanism cannot be drawn.

Reilly (1969, 1972) presented evidence which suggested the existence of copper-protein complexes in the leaves of copper tolerant *Becium homblei*. Contrasting sharply with this Turner (1967), using proteolytic enzymes, found that proteins were not involved in heavy metal tolerance in *Agrostis tenuis* roots. He later tried to correlate the nitrogen content of the cell wall fraction with zinc tolerance, but without success (Turner and Marshall, 1972). More recent studies on other living organisms have confirmed the undoubted biological links between copper and proteins. Naiki and Yamaguta (1976) found copper binding proteins in a copper resistant yeast and Silverberg et al. (1976) discovered intranuclear metal binding proteins in a copper tolerant algae.

Turner and Marshall (1971, 1972) found that the uptake of zinc by cell wall preparations from root homogenates of *Agrostis tenuis* was directly related to the tolerance of the intact plant and that this was a non-metabolic process. They concluded that tolerance was associated with an altered carbohydrate composition of the cell wall, even though carbohydrates have not been reported as being specific metal binding agents. The importance of the increased capacity of tolerant plant cell walls should not be overemphasised according to Wainwright and Woolhouse (1977). They pointed out that in their experiments observed differences in tolerance and differential absorption capacities of cell walls were not necessarily linked, since the absorption capacities of the cell walls were in any case exceeded.

The vast majority of the *in vivo* and *in vitro* experiments mentioned here have been carried out using aquated metal ions. However, naturally occuring tolerant ecotypes will generally experience soil solutions consisting of much lower levels of soluble toxic metal, mostly in complexed form. Stiff (1971) has proved that soluble copper in river waters is comprised almost entirely of complexed forms and Hodgson *et al.*(1965, 1966),

studying soil solutions, found that as much as 99% of the total copper present was complexed. It seems likely, therefore, that a high absorption capacity within its cell walls may be important to a tolerant plant.

Wainwright and Woolhouse (1975), in reviewing the literature on physiological mechanisms of heavy metal tolerance, cast serious doubts upon Turner and Marshall's (1972) conclusion, that proteins were not responsible for the cell wall binding of zinc in tolerant *Agrostis tenuis*. They indicated that there was more than enough nitrogen present in *Agrostis tenuis* root cell walls to account for all the observed metal binding. So, therefore, a direct relationship between nitrogen and zinc uptake would not be expected. In addition they stated that digestion with proteolytic enzymes is often only partially effective in solubilizing proteins and consequently metal-protein complexes may remain in cell walls even after such treatment. Indeed cell wall proteins are notoriously difficult to release (Lamport, 1965). Wyn Jones et al. (1971), using a cellulase digestion, released 66% of zinc from *Agrostis tenuis* root cell walls as a complex containing both amino acid and sugar residues. A noteworthy contradiction to the theory of cell wall accumulation is found in the work of Rathore et al. (1972). They found little zinc taken up by cell walls and little difference between zinc tolerant and non-tolerant bean cultivars in their zinc uptake patterns.

Nevins et al. (1967) have shown that plant cell wall composition is genetically controlled and Wainwright and Woolhouse (1975) have found tolerant cell wall enzymes in plants. It, therefore, seems possible that tolerant species could have developed specific binding sites within their cell walls to cope with the particular metal stress to which they are subjected. Since heavy metal tolerant species have not been shown

to have an increased requirement for the metal to which they are tolerant, at very low levels of nutrition (Allen and Sheppard, 1971) (McNeilly and Bradshaw, 1968), entry of the metal into the tolerant plant must not be restricted. Therefore any metal specific binding sites in the cell wall must not be so strong that they produce deficiencies within the plant.

If binding of heavy metals at cell walls is non-specific then the mechanism responsible for specific ion accumulation must be sited at the physiological barrier between the apoplasm and the cytoplasm, that is the plasmalemma. Rathore et al. (1972) found a decreased zinc uptake rate in tolerant *Phaseolus vulgaris* over a non-tolerant variety. This decrease may be due to a slowing of transport across the plasmalemma by interference with the carrier mechanism and or the existence of an efflux carrier. This sort of relatively low uptake rate for a toxic metal might, depending on nutritional levels, result in a relative build up in the outer free spaces.

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CHAPTER II

PRACTICAL TECHNIQUES

2.1 Metal Determinations

Within the wide areas of study which include plant nutrition, plant mineralization and metallic functions in plants the single most important feature is the need for the unequivocal determination of one or more of the metals present. This is almost always achieved by ashing the sample to remove organic material and subsequently or at the same time effecting solution of the analyte. Analysis of dry plant material using atomic absorption by flameless emission or by spectrographic means would clearly have many advantages, but both techniques have not yet been developed sufficiently. Having obtained the metal in a soluble form it may be determined colorimetrically, gravimetrically, polarographically, by anodic stripping or by one of the spectroscopic methods currently in vogue.

Many of the non-spectrophotometric methods are insensitive and require concentration steps before accurate determinations can be made. The major problem, however, is interference from other elements in solution. To eliminate such interferences elaborate removal and masking methods are necessarily employed. Despite the difficulties in determining low levels of trace metals, non-spectrophotometric methods, notably colorimetry, were those recommended by official analytical bodies until recently. Now, however, due largely to rapid advances in instrument design, spectroscopic methods, and especially atomic absorption spectrophotometry, have almost completely replaced other methods for the routine estimation of elements.

2.2 Atomic Absorption Spectrophotometry

Of all the methods available for regular accurate quantitative analysis of metals in a wide variety of materials atomic absorption spectrophotometry has enjoyed the greatest popularity in recent years. Since Walsh (1955) laid down the basic principles relating atomic absorption to metal concentration, enormous advances have been made in improving the reliability and sensitivity of the technique. Massman (1974, 1976) has critically reviewed the state of development of the technique in recent years. The technique consists of the following steps: sample solution nebulization, atomization, atomic absorption and detection.

A sample solution is drawn into a nebulizer by an air stream, converting the solution into a fine spray. The spray passes through an expansion chamber, in which as much as 90% of the droplets condense. A fuel gas is also introduced into the expansion chamber. The combined air-fuel and suspended solution then passes through a narrow slot and is ignited. The purpose of the ignition process is to convert as large a percentage as possible of metal ions in the sample solution into atoms in their ground state. This is generally accomplished using an air-acetylene flame at an approximate temperature of 2300 ^{o}C (Willis, 1965).

A light source, producing light of the correct wavelength required to raise atoms of the metal in the ground state into an excited state, is passed through the flame and observed by a monochromator-detector system. The amount of absorption can be related to the analyte concentration by comparing the unknown sample with known standards. The most common types of light source consists of a hollow cup cathode made of the metal being determined, with a tungsten anode and an inert, low

pressure, carrier gas. Any source variation will necessarily bring about a variation in the detected signal. This problem is removed by splitting the beam in two, passing one through the flame and the other through a reference channel, recording the beam and analyzing the ratio of the intensities of both beams to find the actual absorption.

Interferences

Although atomic absorption spectrophotometry is specific for the element under investigation a number of different types of interference can affect both the sensitivity and accuracy of the technique. Spectral interferences have largely been eliminated by improvements in machine design and have been reported by Lovett et al. (1975).

Ionization interference occurs when ions of the element are found in the flame. The ions will not absorb energy at the same wavelength as atoms in their ground state and in addition these ions may emit at the detecting wavelength. This problem, mainly encountered in the analysis of group I and II metals, is largely overcome by the addition of large excesses of a group I metal salt to both the samples and standards.

A far more serious problem is that of chemical interference which occurs when the analyte takes the form of a refractory compound in the flame. Anions such as phosphate, silicate and aluminate form stable compounds with elements like calcium and magnesium. These compounds are not fully dissociated in the flame so that absorption is depressed. The simplest solution is to add a 'releasing agent' like strontium or lanthanum in excess (Willis, 1961). The releasing agent forms more stable compounds with the interfering anions thus releasing the analyte fully for atomic absorption.

Matrix effects constitute a further source of interference and are related to the chemical and physical nature of the solution. High levels of solid particles in the flame can cause both scattering and molecular absorption and thus erroneous results (Koirtyohann and Pickett, 1966). Many modern instruments are designed to correct this problem automatically by the use of a deuterium lamp background corrector (Koirtyohann and Pickett, 1965). The absorbance of the sample at a nearby wavelength will indicate the level of non-atomic absorbance and compensation can be made.

Differences in the viscosity and density of the sample and standard solutions mean variations in the populations of ground state atoms in the flame. This often presents the biggest problem to the analyst and emphasises the need for careful matching of sample and standards. Where it proves difficult to match the analyte solution with standards it is possible to make sequential additions of small volumes of a concentrated standard each time taking absorbance readings. The results can be used to produce a graph of absorbance against metal added. Extrapolation to zero absorbance yields the original level of metal in the sample.

Flameless Atomization

Where atomic absorption by flame atomization proves too insensitive or where sample quantities are severely limited a useful adaptation of the above technique has been developed (L'vov, 1961). This involves the use of an electrically heated graphite rod, such as that described by Massman (1968), to bring about atomization of the analyte. The graphite tube is water cooled and bathed in an atmosphere of argon to prevent oxidation.

Analysis of a sample involves a four-step temperature-time cycle. The first is a short low temperature drying procedure, followed by a longer higher temperature stage designed to remove the sample matrix. The third

step is a very short high temperature stage in which the analyte is fully atomized. A final burn off stage is employed to remove any lingering traces of analyte. Great care must be taken to ensure that absorption at stage three is entirely atomic. To this end specific programmes should be developed not only for each different element but also for the matrix in which it occurs.

2.3 Preparation of Plant Material for Analysis by Atomic Absorption

Having taken every possible precaution to obtain uncontaminated dry powdered plant material for elemental analysis the problem of getting the material into solution for atomic absorption spectrophotometry must be solved. Destruction of organic material must be achieved to obtain solution and this can be brought about by either of two oxidative processes known as wet and dry-ashing.

Dry ashing is typically accomplished by heating the sample in a silica crucible to between 400 ^{o}C and 700 ^{o}C in a muffle furnace. This method allows for the handling of large numbers of samples but is not without drawbacks. The more volatile metals, lead, mercury and cadmium may be partially lost even at low temperature whilst other metals such as copper and lead may become retained on the crucible (Gorsuch, 1959). Wet ashing involves the destructive oxidation of the organic material by heating with nitric acid with or without other acids added. The process can be carried out at much lower temperatures thus reducing losses by volatilization and absorption. A recent collaborative study reported by Isaac and Johnson (1975) indicated similarity between results using both techniques.

Wet ashing methods

Most ashing mixtures contain various proportions of nitric and perchloric acids with or without sulphuric acid added (David, 1958) (David, 1959) (Gorsuch, 1959) (Allan, 1961). In fact the 3:1:1, nitric, perchloric, sulphuric acid mixture has lately become virtually a standard

method (Kirkbright and Sargent, 1974) (Christian and Fieldman, 1970).

Addition of sulphuric acid to the ashing mixture reduces the risk of the sample drying and exploding. When all three acids are used, after ashing the non-volatile elements will remain in a sulphuric acid solution. Sulphuric acid has been shown to produce a loss of sensitivity in the determination of certain elements as well as toxic vapours in the flame (Allen and Parkinson, 1969).

Middleton and Stuckey's method (1954) involves the repeated use of nitric acid and avoids the problems with perchloric and sulphuric. Gorsuch (1959) in assessing these methods found good reproducibility between most of them. More recently Dokiya et al., (1975) have compared techniques finding considerable variability between methods. Ashing, however, was only carried out for twelve hours and in some cases was clearly not complete.

Although modern instruments are sufficiently sensitive for most routine procedures increased sensitivity can be brought about by extracting and thereby concentrating elements with immiscible organic solvents or by the addition of miscible solvents to the sample solution (Allan, 1961b) (Boline and Schrenk, 1977).

Numerous suggestions have been made for saving time in routine analysis. Premi and Cornfield (1968) found that several heavy metals were quantitatively extracted from plant material by boiling for fifteen minutes with 6M hydrochloric acid. The use of 50% hydrogen peroxide with sulphuric acid has also been successful as a rapid ashing method (Johnson, 1967). Certainly the most esoteric method yet reported is the development by Gleit and Holland (1962) of a low temperature radio frequency induced oxygen plasma which efficiently destroys organic matter.

Throughout this thesis the method employed for metal analysis is wet ashing with nitric acid, following an approach similar to that of Middleton and Stuckey (1954) and subsequent atomic absorption spectrophotometry.

Generally with a flame to effect atomization but sometimes using the flameless adaptation.

Contamination

Closely linked to the question of metal determination is that of contamination. This unfortunately takes on many varied forms and particularly in the investigation of trace elements in plants is a formidable problem. Any potential form of unwanted metal must necessarily be eliminated, these include analytical compounds, apparatus, water, soil, dust and airborne sources. Some types of contamination can be prevented by careful experimental technique and simple precautions, but water contamination is insidious and is minimized and constantly monitored rather than ever being completely removed.

Soil contamination will affect all plant roots and splashing may also affect the aerial parts of those with low growing habits. The enormous quantities of heavy metals found in mineralized soils are such that even microscopic particles embedded in the plant material may give entirely anomalous results on analysis. Meticulous washing regimes are routinely employed on field plant samples, but how much of the metal within the plant is also removed is rather uncertain.

Airborne contaminants consist not only of dust particles and falling debris, but also of mineral salts. Laboratories for trace metal studies ideally should be lined with stainless steel and have a filtered air supply, whilst water supply and waste systems should be entirely plastic. Unfortunately the present work was largely conducted in a laboratory with rusting iron girders and partially oxidized brass fittings and taps. To combat this, apparatus was always cleaned directly before use; vessels were never left open to the atmosphere, even during use; and paper sheets were left over

unused apparatus. The use of blank extracts and blank ashings were other means of monitoring airborne contamination. In growth experiments it is difficult to completely exclude dust, though air is filtered before being pumped into the solutions. Metal levels in solution are checked occasionally, throughout the experiment, on a random basis.

Chemical reagents used in analysis are a certain, albeit easily controlled, source of inaccuracy. Top grade analytical materials are used as a matter of course for all purposes.

All the equipment used throughout the course of these studies, which include not only standard glassware, but items such as muslin, sand, pure nickel spatulas, polythene tubing etc., were all carefully washed and acid washed before use.

Water is used at all stages of analysis and is of course the medium for the hydroponically grown plants. Normal laboratory sources of water, either tap or distilled, are completely unsuitable for the work presented here. A criteria of acceptable water purity was arrived at by considering what error would be allowable in the most extended of the extraction studies. In extraction schemes the total volume of water used may be very large. Therefore, in order to achieve an error of less than 2% in the extraction of dried leaf material, water containing less than 0.005 p.p.m. copper was required.

Water produced by a metal still had up to 0.10 p.p.m. copper (the still had a brass tap) in water drawn initially after a rest period. This water, after deionization using a mixed resin exchange column, was found to contain no more than 0.0022 p.p.m. copper. A constant reading of the conductance of the water coming off the column provided adequate control over water quality. In addition to this a further all-glass distillation

was employed, the final copper level being too low to detect confidently. Water, therefore, used at all stages throughout the present work, even for rinsing purposes, was distilled, deionized and then glass distilled.

Losses

Losses of analyte can be overcome by extreme care in sample handling and thus are largely avoidable. By employing gentle wet ashing conditions, particularly at the beginning of the procedure when vigorous frothing generally occurs, and also by the use of replicates, difficulties are minimized. Spitting and vapour losses from the wet ashing mixture are circumvented by the use of short-neck glass funnels placed in the necks of the test tubes or round-bottomed flasks generally used for ashing purposes.

Care was taken that samples for atomic absorption analysis with low metal levels were stored for as short a period as was possible; and if a sample was kept for longer periods storage in polyethene was found to be preferable to glass. It follows also that low concentration standards should be made up daily from concentrated stock. All ashed solutions were kept slightly acid to prevent metal losses by precipitation or by algal or bacterial growth. In nutrient solutions bacterial growth did occur even at refrigerator temperatures so that regular millipore filtering was carried out to remove bacteria.

2.4 Extraction Schemes

The plant analyses presented in Chapter III indicate relatively high copper levels, but this information gives no idea of how the metal is distributed chemically within the plant tissues. Total copper within the plant represents the sum of all the copper containing compounds,

e.g. proteins, co-enzymes, simple complexes (for translocation), etc.. More detailed information could be derived from a series of extraction schemes designed to remove discreet types of cellular components with as little overlap as possible. Extraction studies involve the sequential treatment of powdered or macerated plant material with aqueous and non-aqueous solvents.

Divers schemes have been presented, most, unfortunately, with slightly differing aims. Thimann and Bonner (1933) analysed the cell walls of *Avena sativa* coleoptiles. They obtained pure cell wall material by repeatedly extracting ground samples with hot and cold water and by washing on a coarse filter to remove colloidal material. An initial ether extract produced no loss in dry weight of the sample. They extracted pectic substances by the method of Nanji and Norman (1928) using hot 0.5% ammonium oxalate solution. Further heating with 2% sulphuric acid removed protein and hemicellulose, then heating with 2% potassium hydroxide removed the remaining polysaccharides leaving pure α-cellulose as the residue.

In a similar study by Christiansen and Thimann (1950), ether was shown to extract waxes and fats from the cell wall. The weakness of both of these methods is that dilute acid extracts cell wall proteins as well as hemicelluloses, so that a micro-kjeldahl protein determination had also to be made to find what proportion of the extract was polysaccharidic.

In 1953, in an adaption of his earlier scheme, Bonner devised a more complex procedure (Boroughs and Bonner, 1953). This involved dividing the *Avena* coleoptile cell-wall residue into two portions and treating each part separately. One portion was extracted with 0.2% ammonium oxalate to remove pectates followed by treatment with 0.05 M hydrochloric acid at 85°C

to obtain protopectins. The other sample was treated with cold 4% sodium hydroxide to solubilize polyuronide hemicelluloses and further with cold 17.5% sodium hydroxide to extract polysaccharides; again leaving α-cellulose as the final residue. The authors also remarked that schemes of this nature do not result in quantitative separation of the components into neat chemical compartments.

The first fractionation study which was actually designed to systematically investigate the chemical status of metals in plants was that of Bowen et al. (1962), based loosely on the method of Hewitt and Notton (1960). The study involved the feeding of radioactive isotopes to tomato plants prior to harvesting to facilitate subsequent analysis. Initially an ethanol extraction was made, followed by a 0.2 M hydrochloric acid treatment. To the acid fraction an equal volume of acetone was added to precipitate proteins and pectates. A further hot 0.5 M perchloric acid extract was likewise treated to precipitate nucleic acid. A final boiling with 2 M sodium hydroxide for ten minutes dissolved most of the remaining proteins and polysaccharides, leaving a residue composed of lignin and cellulose.

Diez-Altares and Bornemisza (1967) presented an extensive procedure concentrating on the fractionation of proteins as well as polysaccharides. In common with Boroughs and Bonner (1953) they adopted a dual scheme designed to separate pectic materials. Residual material after protein extraction was divided into two and one portion treated with 2% ammonium oxalate and further by 0.05 M hydrochloric acid to remove pectates and protopectates respectively. The other portion was treated sequentially with 0.1 M sodium hydroxide, 0.44 M sodium hydroxide and boiling 1.5 M potassium hydroxide to obtain polyuronide hemicellulose, polysaccharides

and lignin, residual material being α-cellulose. In the light of earlier schemes the alkaline extracts appear rather mild, so that a certain amount of overlap between fractions would be likely to occur.

In a study into the form of selenium in *Spirodela oligorrhiza* Butler and Peterson (1967) reported the successful use of a broad spectrum protease (pronase) to solubilize plant proteins. Peterson (1969) later combined the use of pronase with part of the procedure of Diez-Altares and Bornemisza (1967), producing a neat and elaborate if lengthy process.

Bremner and Knight (1970) used an extraction scheme for examining zinc in rye grass. After an initial 80% ethanol extraction followed by a water extract, they used various enzymes in an attempt to solubilize proteins, hemicellulose and cellulose. Reilly (1969), studying the binding of copper by *Becium homblei* (De Wild) Duvign and Plancke, sequentially extracted material in a soxhlet apparatus with dioxan, butanol, methanol, water, followed by shaking with 1 M sulphuric acid for five hours. This scheme had a number of apparent drawbacks. Ernst (1972) noted that both dioxan and methanol were removing water extractable copper compounds. In addition to this there is a large proportion of copper unaccounted for in the final residue. Ernst also attempted to solubilize heavy metals from accumulator plants (1969, 1972), by using solvents of increasing cation exchange capacity, viz. butanol, water, sodium chloride, citric acid and hydrochloric acid. In one plant, however, as much as 57% of the metal was left in the residue.

Most standard methods for isolating cell wall components involve initial removal of pectic substances by boiling water or aqueous ammonium oxalate solution, followed by delignification with chlorous acid at 70°C. The next step is a bulk removal of polysaccharides by extraction with 2 M potassium hydroxide solution, leaving an α-cellulose residue. The extract

is neutralized to precipitate mainly neutral hemicelluloses and then treated with ethanol to precipitate acid hemicellulose (Whistler and Feather, 1965). This and similar procedures must be rejected for the present work. Since the bulk solubilization of polysaccharides, whilst almost certainly releasing all of the copper present, will form a 'soup' of no value for further study.

Before a final choice could be made as to the best type of extraction procedure to be employed, it was decided to use a simple extraction procedure first. Information from this study would enable certain classes of compounds to be more carefully investigated.

2.5 Chromatography of Plant Extracts

As a result of studying the metal levels in extracts produced by the above schemes it became clear that soluble complexes were present in plants studied. Further knowledge of these complexes was achieved by their purification, isolation and identification using various forms of adsorption chromatography. Chromatography as a technique lends itself particularly well to the separation of large numbers of chemically similar plant products.

Both ascending and descending paper chromatography was employed with Whatman 1 and 3MM papers and thin layer chromatography with cellulose MN300 polythene thin layer sheets. Several solvent systems were tried including phenol-water, 4:1, w/v., and butanol-ethanoic acid-water, 12:3:10, v/v/v (upper layer).

2.6 Location of Metal Sites

The actual site of metal deposition is of crucial importance to an understanding of how a plant tolerates the toxic metal. Crude separation

of a plant into its constituent parts and subsequent metal analysis will give initial ideas on where to search. However, a far clearer picture is obtained by the techniques of differential ultracentrifugation, histochemistry and electron probe microanalysis.

Histochemistry

Histochemistry has been of enormous value in the study of the chemical composition of living material. Plant material is first killed, then fixed and sectioned for microscopic study. The sections are stained with one or more chemicals to provide visible evidence of compounds that may be of potential interest.

As a rule stains giving vivid colours are chosen. This allows easy detection even when small quantities of the analyte are present, as is often the case with toxic metals in plants. Care must be taken to find an unambiguous stain which is not easy since the great majority of reagents for heavy metals tend to be non-specific. The stain used in this work formed soluble compounds, so that photography was used not only to produce a permanent record of the section but also to allow the accurate observation of the metal accumulation sites.

Differential ultracentrifugation

This technique provides a means whereby various cell organelles can be separated from one another firstly by breaking the cell walls and then by using a centrifuge to isolate particles having different densities. The earliest studies were in the main concerned with the isolation of chloroplasts, nuclei etc., and carried out using relatively slow centrifuges with relative centrifugal forces up to 20 000 xg (McClendon, 1952) (Jagendorf and Wildman, 1954) (Granick, 1938). More recently developed high speed ultracentrifuges (500 000 xg) can be used to sediment small viruses and ribosomes.

There are two means of homogenizing plant cells, either by crushing and grinding in a type of pestle and mortar or by chopping in a blender. Either method can cause considerable damage to cell organelles and for quantitative study the best means of homogenization must be determined. Another important consideration, absolutely necessary for this type of work, is the need to work at temperatures just above freezing in order to slow down a host of oxidation reactions, which, whilst not occurring when the cell is intact, take place rapidly when the cell is disrupted. Large changes in pH can occur on homogenization and some organelles can suffer osmotic shock, the medium in which they exist suddenly becoming diluted. Both of these difficulties are solved by the use of a sucrose buffer solution as the homogenizing medium.

The rate of sedimentation of a particle depends on its own density, size and shape and the external factors of medium and centrifugal force. Differential precipitation of cell components is brought about by varying the centrifugal force and the time. A typical fractionation is presented below:

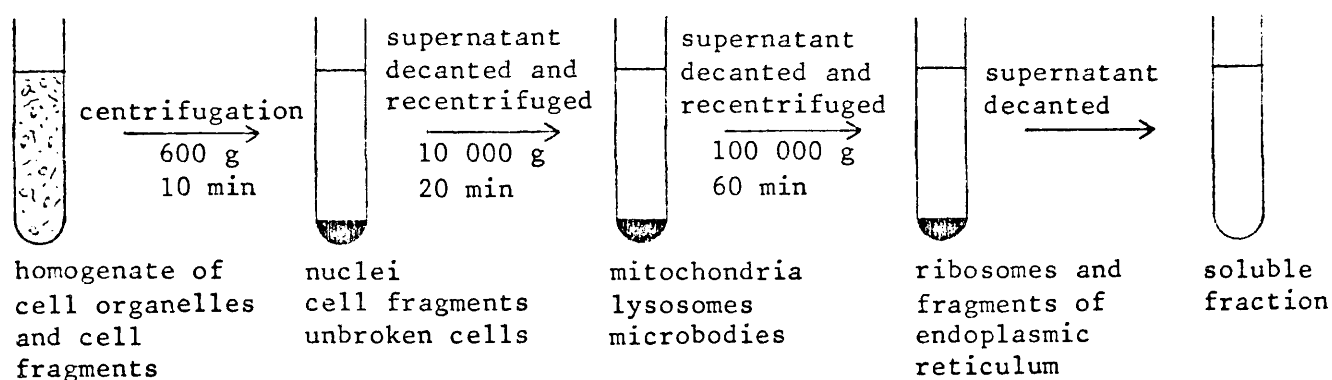

Figure 10. A typical fractionation scheme.

Unfortunately using different machines at the same 'g' values different sedimentation rates may be achieved. Sedimentation rates must be calculated for the machine being used and the rate is generally expressed as the sedimentation coefficient, s, or in Svedburg units of S where $S = 10^{13}s$ (De Duve and Berthet, 1953). S values generally increase with molecular mass but not proportionally.

Electron microscope microanalysis

Electron microscope microanalysis is a more recent adaption of the technique of electron microscopy which was put forward as long ago as 1935 by Knoll (1935) and has since developed into the single most important tool of the cytologist. The scanning electron microscope operates by passing a fine electron beam to and fro over a specimen causing the material to emit various electron types, X-ray and cathodoluminescence; thus various means of observing the surface are available. Generally, low energy secondary electrons are collected from the sample, amplified and displayed on a cathode ray tube screen. The variations in brightness producing topographical detail.

Most scanning electron microscopes now produced are readily adapted to collect the X-ray beams produced by electron impact on the specimen. The wavelength and intensity of the X-ray emissions will be dependent on the elements present and their concentration in the sample. By setting the X-ray detector to receive only the wavelength characteristic of a certain element a picture of the distribution throughout the plant, or across a section, can be formed. The technique at present is unreliable for elements lighter than sodium, but fortunately this does not include any of the known inorganic nutrient elements except boron.

The technique must be carried out under high vacuum therefore living specimens must first be killed and dried. The classical chemical methods of

fixing, dehydrating and wax embedding are unsuitable as they are certain to cause some translocation of the elements present. Originally, dried samples were produced simply by air drying from water, ethanol or pentanone impregnated samples but now cryostatic techniques of freeze drying and freeze substitution with or without resin infiltration have replaced them (Appleton, 1972)(Weavers, 1972)(Marshall, 1972)(Laüchli et al., 1970).

The dried material is sectioned providing a specimen 3 - 4 mm thick, though this is not critical, which is further glued to a conducting base plate. The specimen is then coated with a thin layer of carbon followed by gold to reduce charging effects in the beam.

It is perhaps surprising that scanning electron microscopy and microanalysis were not applied to plant materials until recently in a series of studies on metal distribution in plants by Laüchli and co-workers (Laüchli and Schwarder, 1966)(Laüchli, 1967)(Laüchli et al., 1971). Although the minimum level of an element that may be detected is dependent on the matrix and the element itself, for heavy metals in plants the detection limit is currently 0.1% or 1000 p.p.m. This means that micronutrients are well beyond the limits of detection of the microanalyser thus literature studies have been restricted to the observation of sodium, potassium, calcium, magnesium and phosphorus.

2.7. Experimental Methods for Plant Growth Studies

Introduction

Water culture or hydroponics has grown as a research technique and a commercial method of crop production from numerous experiments concerned initially with the nature of vegetation then later on with which elements were essential for plant growth. The first of these experiments was carried out by Woodward (1699) who successfully grew different plant species in water from various sources. The first deliberate additions of mineral salts were made by de Sassure (1804) in experiments which showed the necessity

of various ions including nitrate.

The real foundations of modern hydroponic technique were laid down by Knop and Sachs working independently in the years following 1860. They both suggested the use of standard solutions containing the now familiar macronutrients. Knop (1865) published a nutrient formula which,with a few additions,is similar to those used today. Many of the modern nutrient solutions are based on those proposed by Hoagland and Arnon (1950) or developed at research stations such as Rothamstead or Long Ashton. These are all presented in the standard text on solution culture by Hewitt (1966).

In the majority of cases the experimenter requires normal plant growth for optimal levels of nutrient supplied. Since the macronutrients consist of three cations; potassium, magnesium and calcium and three anions; nitrate, sulphate and phosphate, they can all be supplied by three salts. This has two drawbacks, firstly there is little scope for varying ionic concentrations and secondly that nitrate uptake from solution results in an increase in pH. By introducing ammonium ions pH stability is increased, as uptake of ammonium nitrogen results in a lowering of pH, and more flexibility is enjoyed. These four salts are made up into separate concentrated stock solutions. Micronutrients, minus iron, are added together to form a single stock solution and iron as an ethylenediaminetetraacetic acid complex completes the list. Culture solutions are made up freshly from stock solutions immediately before use.

Inadequate aeration of roots will result in abnormal root growth and ultimately in plant death. Although it has been shown that shallow containers allow enough exchange of oxygen between the atmosphere and the solution for normal root growth, it is nevertheless common practice to supply filtered compressed air to solutions.

Contamination

The single greatest problem facing those engaged in hydroponic work is that of mineral contamination. Even if the work is not directly related to micronutrient study a complete check and, if possible, control must be kept on the nature and sources of contamination. These sources can be as follows: water, containers, rooting media, nutrient salts, seeds, atmosphere.

Clear polystyrene food boxes with lids were found to be mineral free, cheap and convenient to handle. Plant supports were made from black polystyrene foam which was also found to be mineral free. Both sand and vermiculite were used for seed germination and acid washed rigorously to to remove contaminants. In an effort to minimize inevitable trace impurities in mineral salts 'Analar' reagent grades were always used. Although some species pass on significant amounts of certain elements to their progeny, via their seeds, these considerations are not relevant here. Contamination via air borne salts certainly occurs but is not generally serious unless oxidized metal fitments are present in the growth room. Aluminium, iron or copper contamination is particularly likely to happen in this way.

Regular renewal of solutions is desirable and the period between changes depends on the nature, size and number of plants per unit solution volume. Solutions were generally changed weekly, but water was added more regularly to maintain the original solution volume. The purity of the water used is considered elsewhere.

Growth conditions

Light intensity and quality must be closely controlled and even throughout the experimental growth area. A balance between fluorescent ultra-violet and tungsten lighting should be struck in order to obtain as near a natural light as possible. In addition automatic time controlling of

light and dark periods is desirable. Day and night temperatures and humidity must also be carefully controlled in order to achieve normal healthy growth.

If a particular growth experiment is not related to a certain species a choice of plant must be made. It is usual to choose a familiar crop species, such as *Phaseolus vulgaris*, *Zea mays*, *Lycopersicon aesculentum* or *Hordeum vulgare*, so that abnormalities can be identified if necessary by reference to the literature. It must be noted that individual plants grown under indentical conditions can exhibit enormous variability. These differences in growth are due to genetic variation, seed weight variation, and rate of germination. Genetic variation can be reduced to a large extent by using F_1 hybrid seeds wherever possible.

Elaborate methods for ensuring that all the individualsin an experiment have exactly similar growth conditions have been reported. Shive (1915) introduced a revolving table whilst others have favoured the random movement of culture vessels. In an environment controlled growth room the only serious problem is likely to be the dropping off of light intensity towards the edges of the room. This is resolved by simply carrying out experimental work towards the centre of the light banks.

Controlled environment growth rooms are now commonly used for these kinds of experiments and provide the best possible conditions for carrying out reproducible physiological and nutritional work. The data obtained from these studies, however, should only be reported with reference to the exact experimental conditions prevailing. Clearly nutrient solutions can never exactly replicate natural soil conditions, but the systematic alteration of one nutrient variable cannot be done in any other way, owing to the complexity of the interrelating factors in the soil atmosphere.

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CHAPTER III

COLLECTION AND SAMPLING OF HEAVY METAL TOLERANT SPECIES

Introduction

A general discussion on heavy metal tolerance has been undertaken elsewhere. It was decided to use copper tolerant species for study as copper lends itself well to analytical investigation. Being one of the most commonly occuring heavy metals, tolerant species are particularly well characterized. In addition to this numerous mineralized sites in the British Isle have been listed (Gadgil, 1969)(Henwood, 1857).

Three sites were selected for detailed study; two well documented sites plus a third selected for control purposes. Numerous visits were made to all three sites, only the most important of which are listed below. As with all botanical fieldwork of this nature, sampling techniques and more especially plant cleaning should be adapted according to the prevailing conditions. Details are included wherever relevant. Replicates have similar sample reference numbers with A or B added as suffixes.

3.1 Sites Investigated:

Bunmahon Cove   Bunmahon, Co. Waterford, Eire

There are various old mine workings in the area with numerous open mine shafts. Sample numbers I1 and I2 were taken from the cliff face beside a drainage way above a copper stained man-made rock harbour wall. The soil was a very thin loam. Sample number I3 was taken from above the cliff from a deeper loam soil and was thought to be a garden escape.

Collection was carried out during wet weather and no washing facilities were available on site. The samples were transferred into plastic bags with attendant soil and the plants were brought back to the laboratory for analysis one week later. Analysis consisted of washing

and drying the plants, weighing, grinding and finally wet ashing followed by atomic absorption spectrophotometry.

A second trip was made to Bunmahon Cove. This time with the specific intention of collecting *A. maritima*, a well documented local indicator species, which grows abundantly in the area on non-mineralized ground. The plant samples were packed dry and processed as before. More thorough washing procedures were employed to ensure that no soil contamination of the samples was occuring.

Examination of the results presented in Fig.12 would seem to indicate that the greatest uptake of copper was experienced by those plants in or near to the drainage ways; their roots being bathed in a dilute solution of copper ions and copper chelates. A certain amount of contamination,

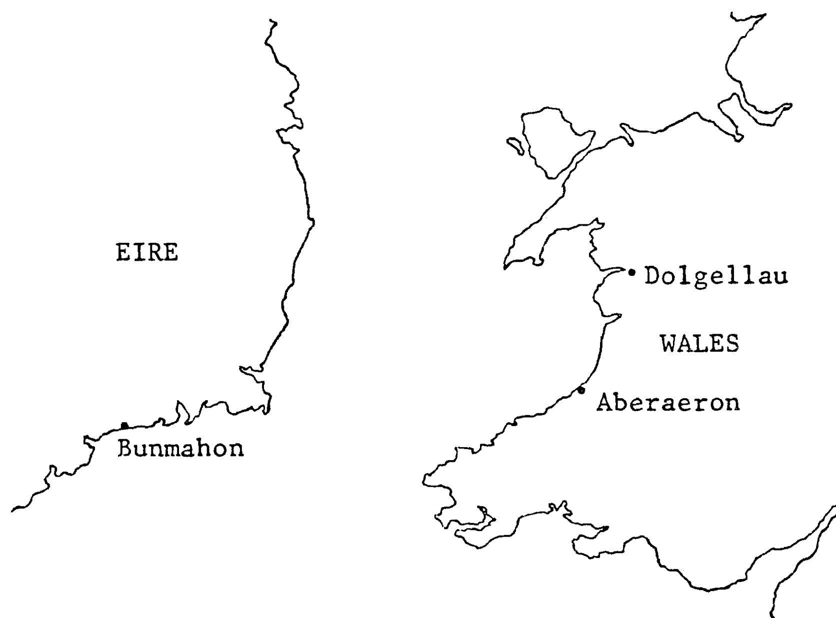


Figure 11. Location of sample sites.

however, was suspected. The more detailed analyses of *A. maritima* are more interesting since contamination was most unlikely and the sectioning of plant samples gave more information about the transport of copper within the plant.

Figure 12. Analysis of samples from Bunmahon Cove, Eire.

Collection Date	Plant	Sample Dry Weight	Sample Details	Ref No.	Cu ppm(dry)	Zn ppm (dry)	Fe ppm (dry)
28.12.73	*Plantago coronopus*	0.9854	leaf	I1l	76	71	254
"	"	0.6801	root	I1r	470	235	2206
"	*Cerastium sps.*	1.7703	leaf	I2l	475	119	203
"	"	1.1243	stem	I2s	1157	116	240
"	*Veronica hebe*	1.7456	flower	I3f	29	58	206
"	"	5.7734	leaf	I3l	19	78	93
15.9.74	*Armeria maritima*	1.2699	lower root stock	AM 1004a	150		
"	"	0.8354	"	AM 1004b	144		
"	"	0.5500	upper root stock	AM 1005a	27		
"	"	0.8629	"	AM1005b	29		
"	"	2.1365	intermediate root stock	AM1006a	37		
"	"	2.4828	"	AM1006b	36		
"	"	0.5401	leaf	AM1007a	30		
"	"	0.6392	"	AM1007b	31		

The samples AM 1004-7 were parts taken from a number of plants collected from one area on the cliff-side and show levels of copper uptake by their roots ten times that found for non-mineralized grounds. By far the greatest copper deposition was in the oldest part of the woody rootstock.

Dolfrwynog Bog   Coed-y-Brenin Forest, nr. Dolgellau, Gwynedd, Wales.
Grid Reference SH 742 256

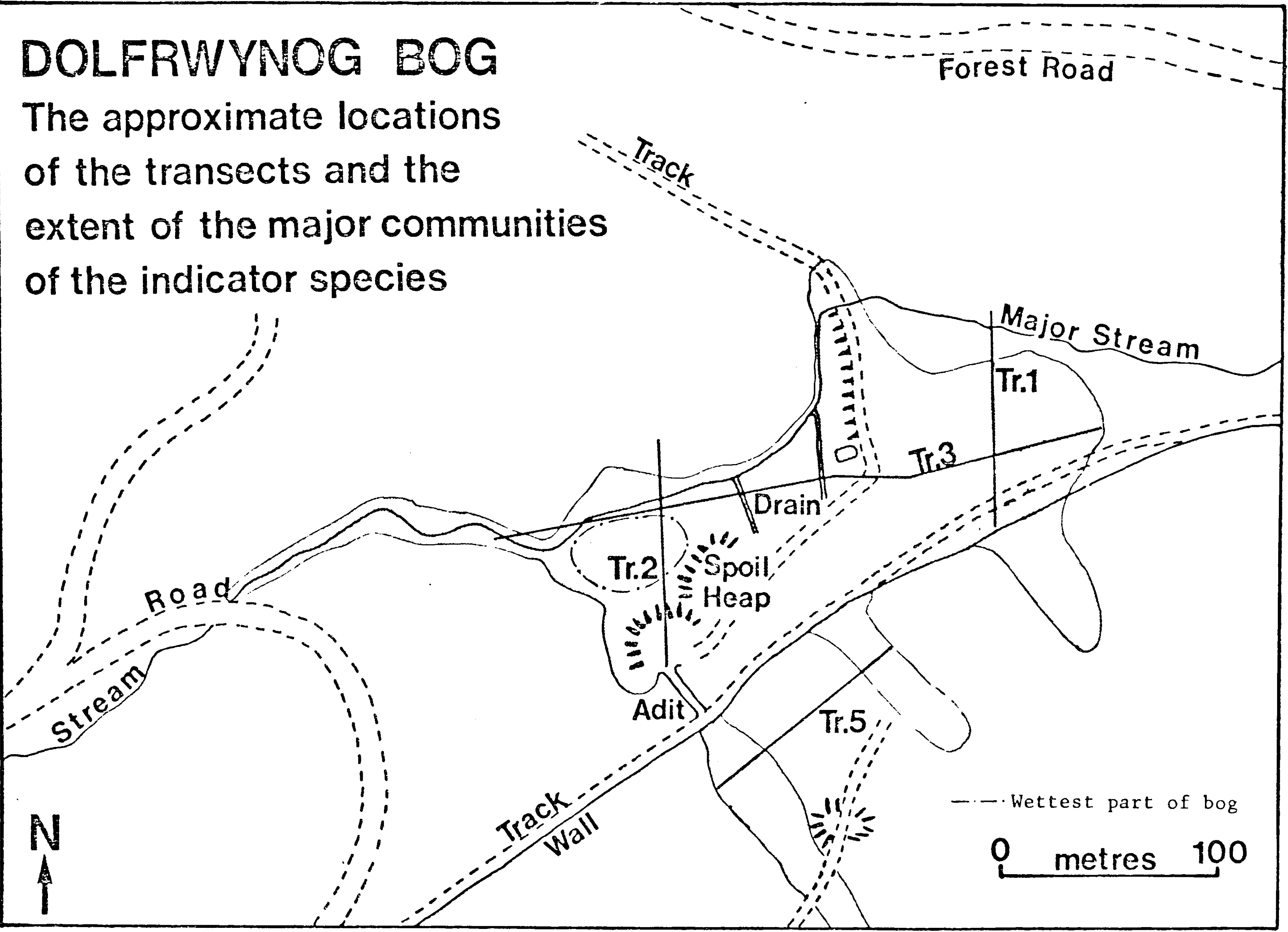
Armeria maritima (Mill.) Willd. has long been known as a metal indicator species. Henwood (1857) was the first to report its presence in the Dolfrwynog area in Wales.

Copper mineralization in the area is associated with the Coed-y-Brenin porphyry deposit. This is located on the Eastern flank of the Harlech Dome where middle and upper Cambrian sediments, dipping steeply eastwards, have been intruded by diorite sills, slocks and minor dykes. The copper occurs as veinlets of chalcopyrite in both the lightly fractured older diorites and the sediments (Rice and Sharp, 1976). The deposit occurs in upland plateau country of moderate relief, which has been deeply dissected by the Afon Mawddach, and its tributary the Afon Wen, which flow into the sea, at Barmouth, 15 km away.

Henwood on his visit to the area in 1856 described an extensive deposit of copper impregnated peat of which as much as 70 acres had been worked in 1816. The bog area in Henwood's day consisted of an upper peat bed in places up to 2 feet thick consisting of decomposing grass with rotten oak and hazelwood. Below this was a few inches of stones and beneath that a further, less copper rich, peat deposit.

Depending on copper content the turves had been cut and either ashed on site or sent directly to Swansea for smelting. In one year 2000 tons of ash were sold, giving some idea of the extent of the workings.

Figure 13



DOLFRWYNOG BOG
The approximate locations of the transects and the extent of the major communities of the indicator species

In the intervening years mining and forestry activity has drastically changed the nature of the area, the extent of the bog now being some 10 acres only. The deciduous forest consisting mainly of sessile oak, *Quercus petraea* (Mattuschka) Liebl., has been almost completely replaced by conifers. What has not changed, however, is the predominance and luxuriance with which *A. maritima* grows in the area. This undoubtedly gave rise to the popular belief that the plant indicated the presence of metal in the soil.

Recently Lefèbvre (1972) has shown copper tolerant *A. maritima* at Dolfrwynog to be morphologically indistinct from their nearest neighbours, the normal maritime populations of *A. maritima*. Ernst (1969) has indicated by pollen analysis that the indicator species date from at least the 12th century A.D.

A systematic geobotanical survey of the area by Smith (1979) has proved an excellent insight into the relationship between the soil copper levels and the type of ground cover. In addition to this plant metal levels of the more abundant species are also presented (Smith, 1979b).

Transect 2, Fig. 13 crosses the wettest area of the bog most densely populated with indicator species and is therefore of greatest interest. Geobotanical and geochemical data obtained from transect 2 are presented in Fig. 14 .

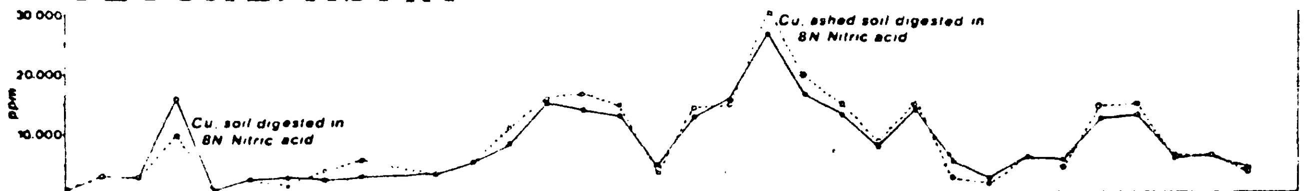
On the relatively well drained spoil heap from 0 to 30 m *Minuartia verna* (L) Hiern is the dominant species. Between 35 and 60 m the ground is permanently waterlogged. At the time of the survey the area between 50 and 55 m was 2 cm below water level. Apart from the stream at 75 m there were areas of water movement at 45 m and 55 m which were characterized by high copper levels in both soil and plant samples. At 40 m *M. verna* grows partly submerged in relatively fast moving water. *A. maritima* appears unable to compete for this type of site owing to its low growing habit. From

GEOBOTANY

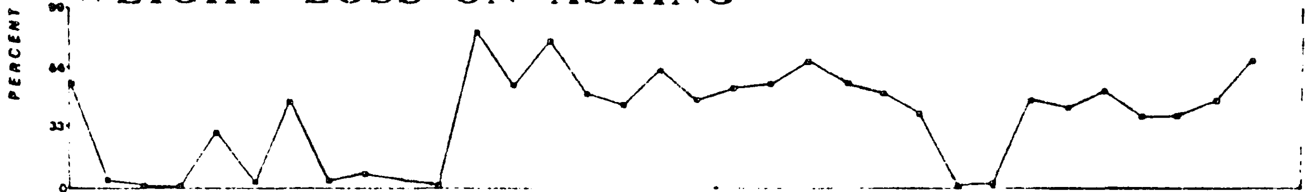
PERCENTAGE COVER

Lichens
Mosses
Sphagnum spp
Dryopteris filix-mas
Rumex acetosa
Drosera rotundifolia
Ranunculus acris
Pinguicula vulgaris
Potentilla erecta
Galium saxatile
Betula seedlings
Pinus seedlings
Quercus seedlings
Carex echinata
Juncus effusus
Juncus kochii
Eriophorum vaginatum
Vaccinnium myrtillus
Erica tetralix (dead)
Erica tetralix
Calluna vulgaris (dead)
Calluna vulgaris
Deschampsia caespitosa
Agrostis tenuis
Holcus lanatus
Molinia caerulea
Armeria maritima
Minuartia verna
Bare Ground

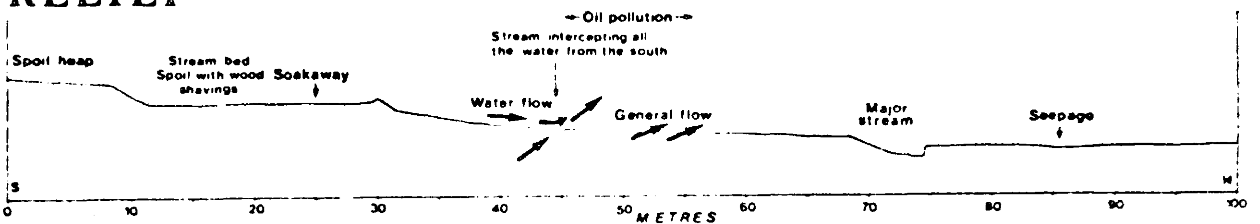
GEOCHEMISTRY



WEIGHT LOSS ON ASHING

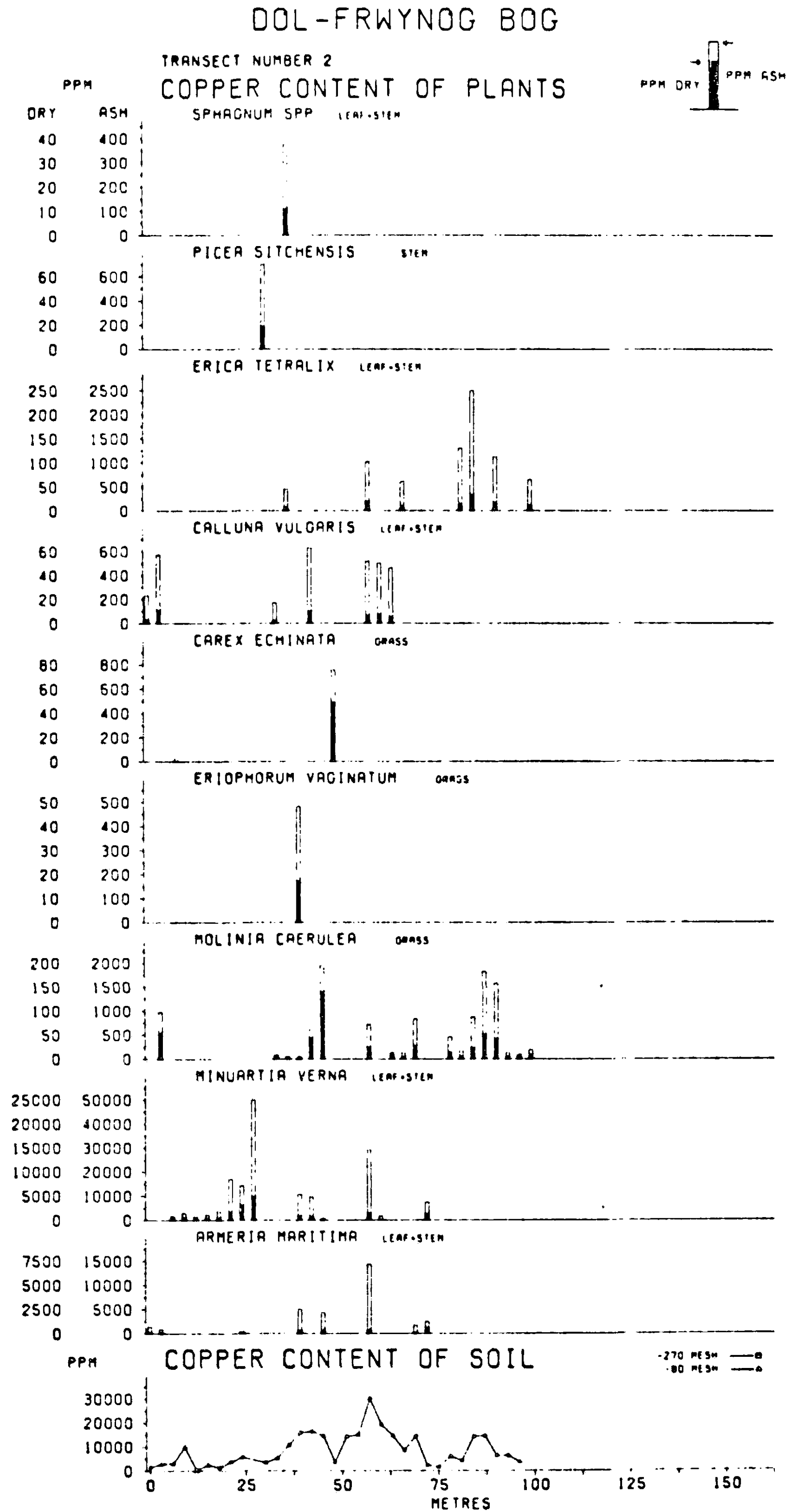


RELIEF



DOL-FRWYNOG BOG TRANSECT 2

Figure 15



45 to 60 m , the area of highest soil copper, *A. maritima* almost totally dominates. At 85 m *A. maritima* occurs without competition from *M. verna* whilst in ground with comparable levels of copper, from 40 to 60 m, they occur together. The pH at 85 m was 4.3 whereas that between 40 and 60 m was 5.5. *M. verna* occurs commonly over limestone in the Pennines and may be less tolerant of acid conditions. Another possibility is that *A. maritima* may be genuinely more tolerant of higher available levels of copper at low pH (Smith, 1979). From the levels of copper in the aerial parts of plants occuring on transect 2, it is clear that *A. maritima* and *M. verna* are accumulating by far the largest amounts of copper (Fig.15).

Samples were taken from the wettest parts of the bog, (see Fig. 13), where *A. maritima* grows in bog material consisting mainly of the decomposed plant itself plus a small proportion of dark loam. The plants were growing with all but the leaf rosettes of the plants submerged and were removed with roots intact. The nearby stream provided adequate initial washing facilities, thus well-cleaned samples were placed in plastic bags prior to analysis.

The first visit provided a few samples for initial analytical work, these were enough to indicate exceptionally high levels of copper present in all but the youngest parts of the plant. All the specimens collected on 21st August, 1974 were bagged together and separated subsequently. The results of the metal analyses of these samples presented in Fig. 16 serve to highlight some of the main problems encountered with soil contamination. Sample numbers AM1001-3 have copper levels which appear completely inconsistent with numbers AM1001B-E. These apparent discrepancies can be understood by a knowledge of the way in which the plant grows in the bog.

The transition from living plant to bog material is very poorly marked in the case of *A. maritima*. As the plant ages the older leaves die but are not readily abscissed. Mature specimens therefore tend to have attendant

Figure 16. Analysis of samples from Dolfrwynog Bog, Dolgellau, Wales.

Collection Date	Plant	Sample Dry Weight	Sample Details	Ref. No.	Cu ppm (dry)
21.8.74	*Armeria maritima*	0.5210	root stock + leaf	AM 1001	15350
"	"	1.7558	leaf	AM 1002	17090
"	"	1.8488	root stock	AM 1003	10140
"	"	0.8508	upper root stock (re-washed)	AM 1001 B	8345
"	"	0.2794	young floret leaves (unsoiled)	AM 1001 C	250
"	"	0.1490	older leaf	AM 1001 D	4228
"	"	0.1694	older leaf	AM 1001 E	2893

rotting leaves on the older parts of the root-stock. Rotting material would provide suitable sites for copper uptake. Sample numbers AM1001-3 included quantities of dead leaf material, whilst in the case of the latter samples all the dead leaf material had been carefully removed. This also involved brushing the rootstock with a nylon brush where necessary.

A second visit was made to the site a year later on 19.7.1975. Samples of *A. maritima* were collected from the same site in the wettest part of the bog and similar washing procedures were adopted. Standing water in the area and the main stream were pH tested. Using Merck Neutralit pH 5-10 and Acilit pH 0-6 papers consistent values of pH 5.3 were found for both sites. Bog mud samples were collected for soil studies. Seeds were collected from ripe flower heads of *M. verna, A. maritima* and *Silene maritima* Withering for growth studies. *S. maritima* is another local indicator for copper found growing mainly alongside the track.

Although transects give a clear indication of the relationships between soil copper and plant species cover, they do not give an overall view of the floral composition of the bog itself. A visual assessment of the dominant species in the area outlined in Fig.13 is given below:

Habitat	Species
Wet sites	*A. maritima, M. verna, Calluna vulgaris* (L) Hull, *Eriophorum vaginatum* L., *Narthecium ossifragum* (L)Hudson, *Molinia caerulea* (L) Moench, *Droscera rotundifolia* L., *Agrostis tenuis* Sibthorp, *Erica tetralix* L., *Carex echinata* Murray.
Drier sites	*Holcus lanatus* L., *Vaccinium myrtilis* L., *Potentilla erecta* (L) Ränschel.
Stream side (cuts Tr.2 at 40 m)	*M. verna, Pinguicula vulgaris* L.
Better drained ground (around bog perimeter)	*C. echinata, Juncus effusus* L., *A. tenuis*.

In addition to those listed above lichens and mosses had a scattered distribution throughout the area. All the plants listed above are typically found in or around acid bogs with the exception of *A. maritima* and *M. verna.*

The results of analyses of samples collected on this visit are presented in Fig. 17 . Sample numbers 2001A, 3001B and 4001C were all taken from the same dense growing mat of material. Results of these analyses prove conclusively that old and dead leaves accumulate large levels of copper. The remaining analyses on *A. maritima* served to indicate metal distribution throughout a young plant. The results presented below indicate that copper is largely precluded from the younger rapidly growing parts of the plant.

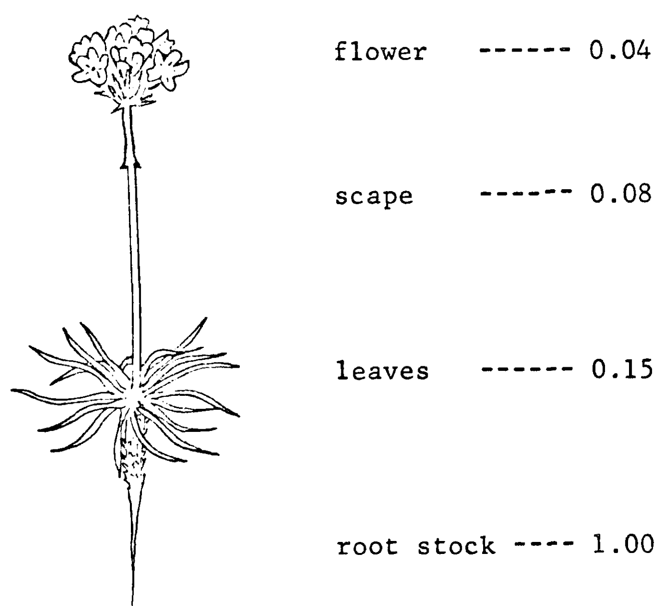


Figure 18.
The fraction of copper in various parts of a young *A. maritima* plant as related to the level in the root.

Two other species, *P. vulgaris* and *M. verna* found in the bog were analysed and the results presented in Fig. 17 .

Figure 17. Analysis of samples from Dolfrwynog Bog, Dolgellau, Wales.

Collection Date	Plant	Sample Dry Weight	Sample Details	Ref No.	Cu ppm(dry)	Fe ppm(dry)	Ca ppm(dry)	Zn ppm(dry)
19.7.75	*Armeria maritima*	1.5798	Whole plant (green)	2001 A	747			114
"	"	2.2496	Whole plant (minus dead material)	3001 B	1134			115
"	"	2.3582	Whole plant	4001 C	2460			149
"	"	0.2075	Green leaf (unsoiled)	2001-8/L1A	169	195	1012	
"	"	0.2023	"	2001-8/L1B	173	198	1063	
"	"	0.1256	Root stock (young)	2001-8/R1A	1170	231	1911	
"	"	0.1355	"	2001-8/R1B	1180	214	2030	
"	"	0.2769	Flower debris	M2A	54	298	957	
"	"	0.2847	"	M2B	51	320	878	
"	"	0.4271	Scapes unsoiled	2001-8/SC1	91	199		
"	--------	0.8014	Soil from roots of 2001 A	2001-S	5428 79*			
"	*Pinquicula vulgaris*	0.2916	Whole plant	M1	571			
"	*Minuartia verna*	1.6286	Aerial roots	M4	54			

* 1 M Ammonium acetate extractable copper

Aberaeron Bay   Aberaeron, Dyfed, Wales.
Grid Reference SN 462 635

A. maritima plants were required to ascertain the normal levels of copper in the species and for growth experiment control purposes. So a maritime non-mineralized site reasonably close to Dolgellau was chosen. The plants collected were growing just above a shingle beach beyond the normal high water level in a fairly thin sandy soil. The analyses undertaken are presented in Fig. 19 and served to confirm the presence of only normal soil levels of copper. Seed heads were collected for growth experiments.

3.2 Practical Atomic Absorption Analysis

Wet ashing and sample preparation

The method described is basically that proposed by Middleton and Stuckey (1954). Two similar methods for ashing were used; dried powdered plant material or soil was ashed in pyrex boiling tubes whilst dried extracts were ashed in pyrex round bottomed flasks. The various stages of sample treatment are outlined.

After collection or growth and washing (preferably *in situ*), materials were stored at low temperatures in polyethene bags until analysed. Further thorough cleansing, rinsing, inspection for surface contamination and freeze or oven drying were carried out. Powdered material was arrived at by the use of a Glen Creston agate ball mill and was sieved through a 320 μm stainless steel sieve before further low temperature ($40^{o}C$) oven drying to constant weight. Samples were carefully weighed into 25 cm^3 pyrex boiling tubes and ashing was effected by heating with 10 cm^3 of Ultra grade nitric acid.

Ashing tubes were fitted into an aluminium block and after initial frothing had ceased the temperature of the block was increased steadily

Figure 19. Analysis of samples from Aberaeron Beach, Aberaeron, Wales.

Collection Date	Plant	Sample Dry Weight	Sample Details	Ref No.	Cu ppm (dry)
8.4.76	*Armeria maritima*	1.5572	root stock	ABER 1	14
"	"	1.4592	leaf + flower	ABER 2	9
"	-----	0.9045	soil from roots of ABER 1	ABER 1-S	23

during the first few hours of ashing until gentle refluxing was achieved at a block temperature of 140°C. The ashing of small samples, 0.0 - 0.2 g, was usually completed in twelve hours. However, further additions of nitric acid were made and longer times employed where necessary. Ashing was judged to be complete when the solution was clear and colourless and any precipitate present was white. Samples were taken to near dryness, nitric or hyrdochloric acid was added and the resultant solution transferred with washings to volumetric flasks giving final solutions of 1% acid. The volume of acid required for ashing and the final volume of the solution to be analysed depends upon the weight of plant material used and the concentration of elements therein, which are found by initial experiment.

Extracts were similarly ashed with nitric acid in round bottomed flasks in sand baths on heating blocks. Generally samples were designed to contain convenient levels of the least plentiful elements for analysis e.g. copper, platinum etc., so that determination of the macronutrients magnesium and calcium were achieved by further dilution and addition of the necessary releasing agents.

3.3. Spectral Interferences

Flame atomic absorption

Since the earliest days of atomic absorption spectrophotometry it was noted that first period transition metals were significantly affected by the presence of other ions only when these are present in a large excess. The formation of refractory compounds is also less of a problem with these elements than others.

For copper no interferences were found with a twenty-fold excess of most cations and anions or with 5% nitric acid, although 5% of other mineral acids did interfere (Ramakrishna et al., 1967). They reported also

that only HPO_4^{2-}, SiO_3^{2-}, $B_4O_7^{2-}$ interfere with zinc at one hundred-fold excess and that acids did not interfere at the 5% level. Maruta et al. (1970) found that the flame position had an influence on the effect of nitric acid on copper, a depression of the signal being produced in the lower region of the flame. Sachdev et al. (1967) showed that none of a wide range of ions at fifty-fold excess interfered with iron absorption in an air-acetylene flame. Analysis of both calcium and magnesium is severely affected by the presence of various ions especially PO_4^{3-}, SiO_3^{2-} etc.. Willis (1969) reported the use of 1% strontium or lanthanum solutions which effectively removed these chemical interferences.

The platinum group metals platinum, rhodium, and palladium were first analysed by Lockyer and Hames (1959) and no inter-element effects were noted. However, in a later study Strasheim and Wessels (1963) noted numerous interferences caused by mineral acids at high concentrations, sodium ions and most especially for platinum and rhodium by other noble and base metals. Schnepfe and Grimaldi (1969) reported the removal of inter-element effects on rhodium by the addition of 1% lanthanum. A similar addition was used by van Loon (1969) to reduce interferences in the analysis of platinum and palladium.

Flameless Atomic Absorption

The low levels of platinum, rhodium and palladium translocated to the plant tops in experiments on crop species were often near or below atomic absorption flame detection limits, so that the flameless adaptation was employed. Everett (1976) reported numerous inter-element interferences from precious metals on the above. Whilst in the present work only one precious metal was fed to plants during any one experiment. It is interesting to note that in a repeat of Everett's work (carried out on a Varian Techtron AA-5 machine with a Model 63 carbon rod atomizer) by Towndrow (1979) using

a Pye Unicam SP1900 with a Beckmann-Massman furnace, dramatically different interferences were observed. Seemingly inter-element effects are highly dependent on the type of machine employed and the operating conditions chosen. In addition to this Towndrow studied the effects of other elements; copper, zinc, iron, nickel and sodium, on the platinum group metals. In the context of the present study only iron at the fifty-fold excess level may cause signal enhancement in the analysis of rhodium and palladium. The presence of several interferences using the flameless technique is highlighted. Fortunately it is possible with platinum, palladium and rhodium to use rather harsh ashing conditions without significant loss of analyte. The effects of mineral acids were also investigated by Towndrow (1979). Up to 5% hydrochloric acid was found to have no effect on signal sensitivity. Another study of the effects of mineral acids on the absorption signals of iridium, platinum and rhodium emphasised the unsuitability of nitric acid as a medium for flameless atomic absorption analysis (Adriaenssens and Knoop, 1974).

The absorbance signal obtained during atomization consisted of a double peak. The first being due to the initial removal of residual matrix material and the second, generally larger, peak due to the analyte. Results were only considered reliable where zero absorbance was achieved between peaks. Accuracy was ensured by using replicates of each sample, regularly running blanks and re-running standards. Whilst standard additions of possible interfering elements to standard would be impossible an adequate check on results was achieved by the dilution of flame analysed samples and the subsequent re-analysis using the carbon furnace; close correlations were obtained.

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CHAPTER IV

HYDROPONIC GROWTH EXPERIMENTS

Introduction

A discussion of experimental growth methods has been undertaken elsewhere in this thesis (p. 68). The present work was carried out in a controlled environment growth chamber manufactured by Prestcold Limited. The room had a concrete floor measuring approximately 14 m^2, a perspex ceiling and aluminium walls. The lighting system consisted of eight banks of 5 x 125 watt ultra-violet fluorescent strip lights and two banks of 4 x 60 watt tungsten filament strip lights; each bank being separately controlled. The maximum possible light intensity was 1000 ft-candles at bench height (1 m). Relative humidity was maintained automatically at 65%. Day and night lengths and temperature were also automatically controllable and maintained throughout all the experiments at a 14 hour day period with day and night temperatures of 20 ± 1°C and 15 ± 1°C respectively.

The growth experiments undertaken with tolerant and non-tolerant populations of *A. maritima* had a number of different purposes. Primarily they would serve to indicate degrees of tolerance and whether or not tolerance is a genetic response to copper stress. Further to this yield measurement and plant metal level analysis might be expected to throw some light upon the tolerance mechanism. In addition a significant amount of salt secretion was noted and considered worthy of investigation.

4.1. Assessment of Heavy Metal Tolerance

Agrostis species are much studied species found growing on mineralized soils and mine tailings extensively throughout the United Kingdom. Bradshaw (1952) observed that the root growth of *Agrostis tenuis* grown on tip material from a lead mine was suppressed whereas plants normally found there had normal root systems. This suggested that certain *Agrostis tenuis* plants

were more tolerant of high lead and zinc concentrations in soils than others.

Wilkins (1957) devised a method for determining the level of tolerance shown by *Festuca ovina* to lead. Plants of *Festuca ovina* were tillered and rooted in calcium nitrate (1 g per litre) and when a constant rate of growth had been obtained, lead nitrate was added to the solution. The amount of growth was measured four days later. The rate of growth of the roots in the lead solution as a percentage of growth in calcium nitrate alone gave an index of tolerance. The only fundamentally different adaptation of this method was that of Jowett (1958, 1964) who grew tillers of *Agrostis tenuis* and *Agrostis stolonifera* in solutions containing metal salts with calcium nitrate and controls in solutions containing only calcium nitrate under similar conditions.

4.2. Comparison of Methods

The method chosen for any particular case depends on the species being studied. The methods described above were designed specifically for grasses that spread by rooting tillers, although the methods are readily adaptable for other freely rooting dicotyledons (Allen and Sheppard, 1971) . Testing for seed tolerance to heavy metals and particularly using small seeded species like *Armeria maritima* presents problems in the handling of the young seedlings.

Three methods of germination were compared to see which provided the best material for solution culture. Firstly seeds were germinated on moist hard filter paper Whatman No.541 on a tray covered with black polythene sheeting. Secondly seeds were germinated in a tray of acid washed sand covered with clear polythene and finally fine grain "Perlite" was used instead of sand. The first method produced seedlings with elongated hypocotyls. Both of the other methods were satisfactory, the sand method

being preferred only because the sand particles were more easily removed from the root hairs.

The need for an intermediate culture vessel was indicated by the facts that root lengths could not be observed in sand and germination times were widely spread. The vessel would allow roots with similar root lengths to be collected.

4.3. Assessment of Tolerance to Copper of Tolerant *Armeria maritima*

Experimental

Fully mature seedheads of *Armeria maritima* ssp maritima collected from Dolfrwynog bog (SH 742249) were thoroughly air dried and stored in polythene bags in a dry place. For the experiment seeds were removed by hand from their calyces, which was necessary since only about 30-40% fertilization of the fruiting heads is achieved.

The seeds were checked for uniformity of size and approximately 250 were soaked in 400 ml of demineralized water for five hours, the water being continually aerated and changed three times. Subsequently the seeds were placed in rows on a 355 x 217 mm seed tray of acid washed sand and covered lightly with a further 2 mm layer. The tray was then placed in a trough of demineralized water for a matter of seconds until the sand was moistened. A layer of cotton material lining the tray prevented the sand from washing away. After any excess moisture had been allowed to drain away the seed tray was transferred to an environmentally controlled growth room with a 14 hour day, 1000 ft-candles of illumination and the temperature maintained at 20°C during the day and 15°C at night. The tray was covered with polythene sheeting to prevent loss of moisture during the germination period.

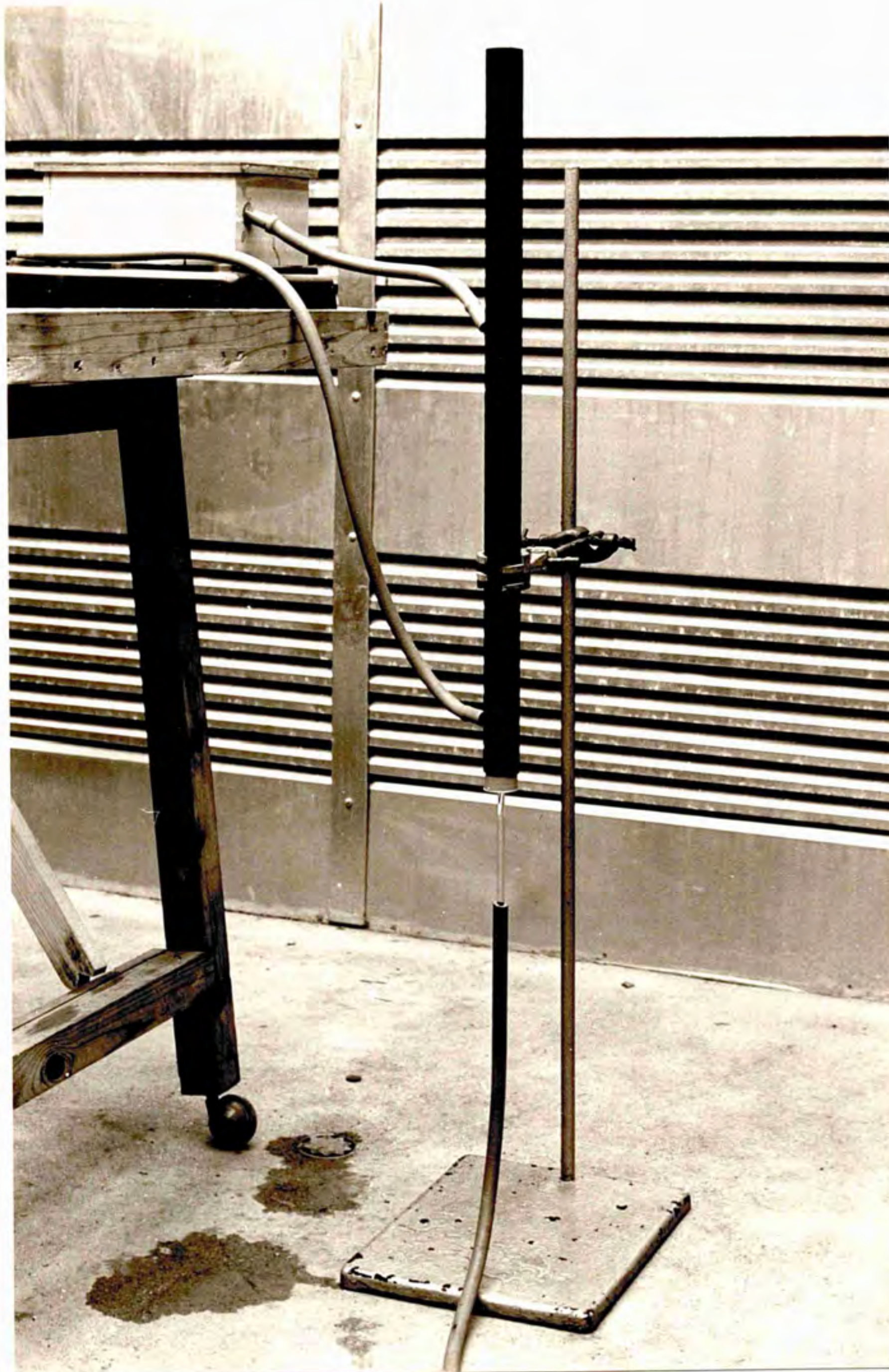


Plate 1. A novel aeration method for the culture of *A. maritima* seedlings.

Germinated seeds were removed from the tray by scooping out the individual seedling and the sand around the roots with a spatula. The sand was removed by dipping the seedlings into a small beaker of demineralized water whilst holding one of the dicotyledons gently between thumb and forefinger. Gentle agitation of the seedling resulted in almost complete removal of sand particles from the root hairs. The seedlings were immediately transferred to a plastic box specifically designed for their initial solution culture. The box 180 x 125 x 65 mm made of opaque polythene had two spigots diametrically opposed as indicated in Plate 2 . These were connected by rubber tubing to two side arms of a glass tube. At one end of this glass tube was fitted a rubber bung through which a gas distribution tube, porosity 3, had been inserted and to this an air line had been attached. The glass was made opaque by using black polythene sticking tape.

The apparatus was then set up as indicated in Plate 1 with the tube clamped upright alongside the box. The air pump was switched on and the apparatus was fitted with demineralized water, containing 0.5 g/litre $Ca(NO_3)_2.4H_2O$, to a level just below the top of the box and the glass tube was then raised or lowered so that the upper spigot was approximately 100 mm below the level of the solution. The air pressure was adjusted, by utilising an air leak, so that a constant gentle flow of air bubbles resulted in the side arm. This flow produced a constant renewal of aerated water in the box by drawing water through the lower piece of tubing as indicated.

The lid of the box was 3 mm thick and 108 holes were drilled in it at 11 x 11 mm intervals and countersunk. The seedlings were lowered through the holes so that their dicotyledons were supported by the edges of the countersunk holes and their roots were suspended in the solution below. After a few days of growth in this container sufficient seedlings were large enough for differential nutrient solution applications to test tolerance to copper.

Plate 2. Initial culture vessel for *A. maritima* seedlings.

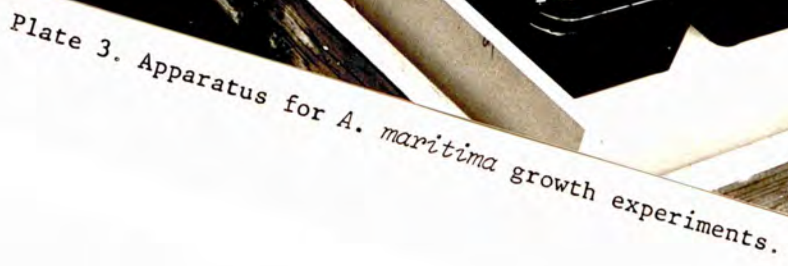


Plate 3. Apparatus for *A. maritima* growth experiments.

Seedlings were selected for uniformity of growth, root lengths between 10 mm and 30 mm, and for the absence of root branching. They were transferred into 1 litre polystyrene freezer cartons, 180 x 130 mm, which had been painted black on the outside. Each container had a tight fitting lid similarly painted. The lids had twelve symmetrically positioned 10 mm diameter holes drilled in them. The plants were held in position inside a wedge of black polystyrene foam. These wedges could be lodged inside the holes in lids.

The containers were filled with solution to within 5 mm of the foam. Using this arrangement the parts of the roots not in solution were in a moist environment yet the foam did not remove solution by capillary action.

Aeration of each nutrient solution was brought about by bubbling air through gas distribution tubes 90 mm long, porosity 3, supported by rubber bungs placed in the corner holes of the box lids. Each box was supplied with air by polythene tubing attached with a junction piece to a main air line. The air line was ringed for evenness of air pressure and an air leak fitted to allow for adjustment of bubbling rates in the solutions.

Photographs of the apparatus described above are presented, see Plates 1 - 3.

Figure 20. Timetable for Experiment A.M.(i)

Day 1 Seeds soaked and sown.

Day 15 Start of seedling transferral to the initial growth box containing $Ca(NO_3)_2.4H_2O$, 0.5 g/litre.

Day 29 Termination of seedling transferral and further transferral of uniform seedlings to containers for differential application of nutrient solutions containing 0.5 g/litre $Ca(NO_3)_2.4H_2O$.

Day 32 Solutions containing $Ca(NO_3)_2.4H_2O$, 0.5 g/litre and copper(II) ions at various concentrations, see Fig.22, were applied to the containers. First root length measurements were made.

Day 36 Second root length measurements were made.

Day 39 Third root length measurements were made and solutions were renewed.

Day 43 Fourth and final root length measurements were made.

Day 47 Half strength full nutrient solution, see Fig.21, plus copper(II) ion levels as above.

Solutions reapplied at weekly intervals until harvesting.

Day 119 Plants harvested.

Figure 21. Full Strength Balanced Nutrient Solution used in Tolerance Experiments

Compound	Concentration of stock soln. M/mM	Volume of soln. per litre of final soln. ml	Essential Element	Final Conc. of Element μM	Final Conc. of Element p.p.m.
Macronutrients	M		N	16000	224
KNO_3	1.00	6.0	K	6000	235
$Ca(NO_3)_2.4H_2O$	1.00	4.0	Ca	4000	160
$NH_4H_2PO_4$	1.00	2.0	P	2000	62
$MgSO_4.7H_2O$	1.00	1.0	S	1000	32
Micronutrients*	mM		Mg	1000	24
KCl	50		Cl	50	1.77
H_3BO_3	25		B	25	0.27
$MnSO_4.H_2O$	2.0	1.0	Mn	2.0	0.11
$ZnSO_4.7H_2O$	2.0		Zn	2.0	0.13
$Na_2MoO_4.2H_2O$	0.5		Mo	0.5	0.05
Fe-EDTA**	20	1.0	Fe	20	1.12

* A combined solution is made of bracketed micronutrients.

** Ferrous dihydrogen ethylenediamine tetraacetic acid

4.4. Results and Discussion

A complete timetable of the experiment, A.M.(i) is provided in Fig. 20 and the results of root length measurements are given in Figs. 22 & 23 . Calculation of the indices of tolerance would seem to indicate that tolerance to copper is very high. The normally accepted level at which tolerance to copper becomes obvious is 0.5 p.p.m. copper. In work with tolerant and non-tolerant *Agrostis tenius* McNeilly and Bradshaw (1968) observed indices of tolerance ranging from 5 - 30 for non-tolerant and 40 - 80 for tolerant populations. *Armeria maritima* with an index of 66 is highly tolerant of copper at this level. At the highest level of copper applied, 3 p.p.m., initial growth was severely affected and many seedlings died. When a balanced nutrient solution was applied, however, new root growth was resumed and the plants remaining continued to grow slowly.

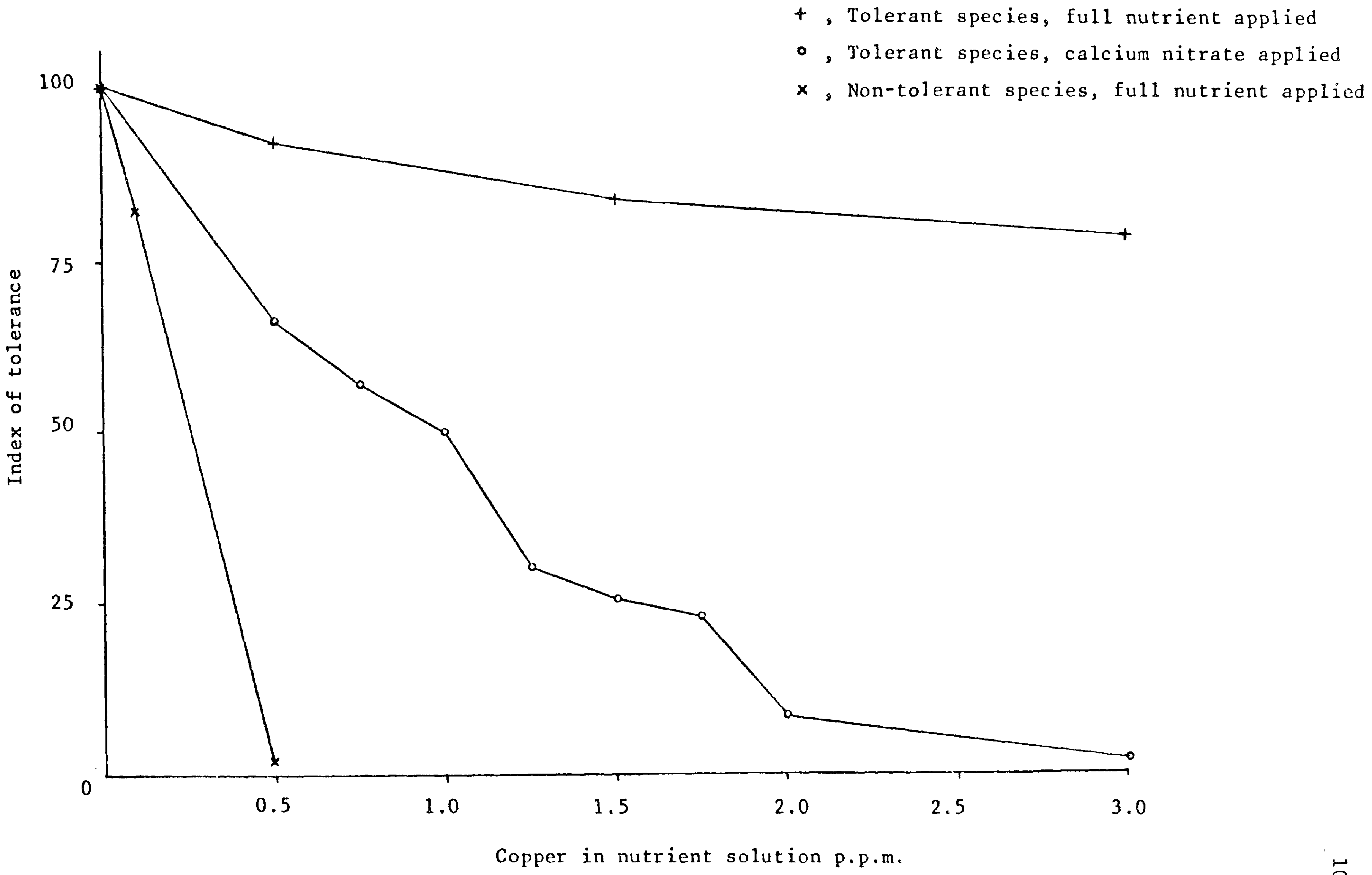
Ernst (1968) pointed out that lead toxicity was enhanced by the use of a calcium nitrate solution rather than a full nutrient solution. Davies and Snaydon (1973) made a direct comparison of rooting techniques, finding that the composition of the nutrient solution influenced the effect of aluminium on the root extensions of populations of *Anthoxanthum odoratum*. A study of root extension of two populations of *Holcus lanatus* by Coughtrey and Martin (1977) indicated that growth in Hoagland's medium and calcium nitrate solutions was very different for one population.

A better indication of tolerance may, therefore, be gained from the application of a balanced nutrient solution at the start of the growth period. So a second experiment, A.M. (iii), was carried out, similar in every detail except that a half strength full nutrient solution was used throughout the growth period. The results of the root length measurements are given in Fig. 24 . The population tested now appears to be scarcely affected at the 0.5 p.p.m. level and indeed highly tolerant at all the levels tested.

Figure 22 Results of Measurement of Tolerance of *Armeria m.* Experiment with Calcium Nitrate, A.M.(i).

Test Solution Box No.	No. of Plants in Box	Concentration of Copper (p.p.m.)	Mean of Increase in Root Lengths over 11 Days (mm)	L. S. D. P = 0.05	Index of Tolerance (%)
1	8	0	33.5	7.3	100
2	10	0.50	22.1	7.4	66.0
3	4	0.75	19.0	6.4	56.7
4	8	1.00	16.7	7.7	50.0
5	6	1.25	10.2	7.8	30.4
6	7	1.50	8.6	7.5	25.6
7	8	1.75	7.8	7.4	23.1
8	6	2.00	2.8	3.5	8.4
9	5	3.00	0.6	0.8	1.8

Figure 23. The effect of varying levels of copper concentration on the index of tolerance of tolerant and non-tolerant *Armeria maritima*.



A third extended growth experiment, A.M.(ii) was carried out with full nutrient solution but this time using seed from a typical unmineralized maritime site at Aberaeron Bay. Levels of applied copper up to 3.0 p.p.m. were used but all concentrations above 0.5 p.p.m. eventually killed the seedlings. The results of the root length measurements are presented in Fig. 24 . Plants were cropped for analytical purposes on Day 74 of the growth programme.

The toxic effects observed, both for the non-tolerant plants at and above the 1 p.p.m. level and in experiment A.M.(i) before full nutrient solution was applied, were those typical of heavy metals. The roots were brown with dark brown tips and were stunted and deformed having a 'barbed' appearance. Secondary root growth was inhibited, the only real growth being in the moisture laden air above the solution. The first true leaves were tinged or fully purple. Leaf growth was limited and chlorosis was observed later.

A graphical representation of the effect of varying levels of copper concentration on the index of tolerance of tolerant and non-tolerant populations of *A. maritima* (Fig.23) shows dramatically the difference in tolerance of the two ecotypes. Clearly demonstrated also is the enhancement of the toxic effects of copper brought about by the lack of essential elements in the growth solution. A correlation was attempted between wet yield and the index of tolerance in calcium nitrate solution for experiment A.M.(i). The correlation coefficient was calculated to be -0.42, indicating that the index of tolerance in calcium nitrate gives no measure of a plant's ability to grow under realistic nutrient conditions. It appears that for *A. maritima*, as well as for many other species, calcium alone is not enough to ensure healthy root growth at the earliest stages.

Results of Measurement of Tolerance of *Armeria m.* (tolerant species) Experiment with Full Nutrient A.M.(iii).

Test Solution Box No.	No. of Plants in Box	Concentration of Copper (p.p.m.)	Mean of increase in Root Lengths over 9 Days (mm)	L. S. D. $p = 0.05$	Index of Tolerance (%)
1	4	0	36.8	14.6	100
2	5	0.50	33.8	9.6	92.0
3	4	1.50	30.8	9.0	83.7
4	5	3.00	28.6	7.7	77.8

Results of Measurement of Tolerance of *Armeria m.*(non-tolerant species) Experiment with Full Nutrient A.M.(ii).

Test Solution Box No.	No. of Plants in Box	Concentration of Copper (p.p.m.)	Mean of increase in Root Lengths over 11 Days (mm)	L. S. D. $p = 0.05$	Index of Tolerance (%)
1	8	0	25.0	7.7	100
2	6	0.10	20.5	8.5	82.0
3	8	0.50	0.6	0.4	2.4

Figure 24

Figure 25.
Plant Analyses from Experiment A.M.(i).

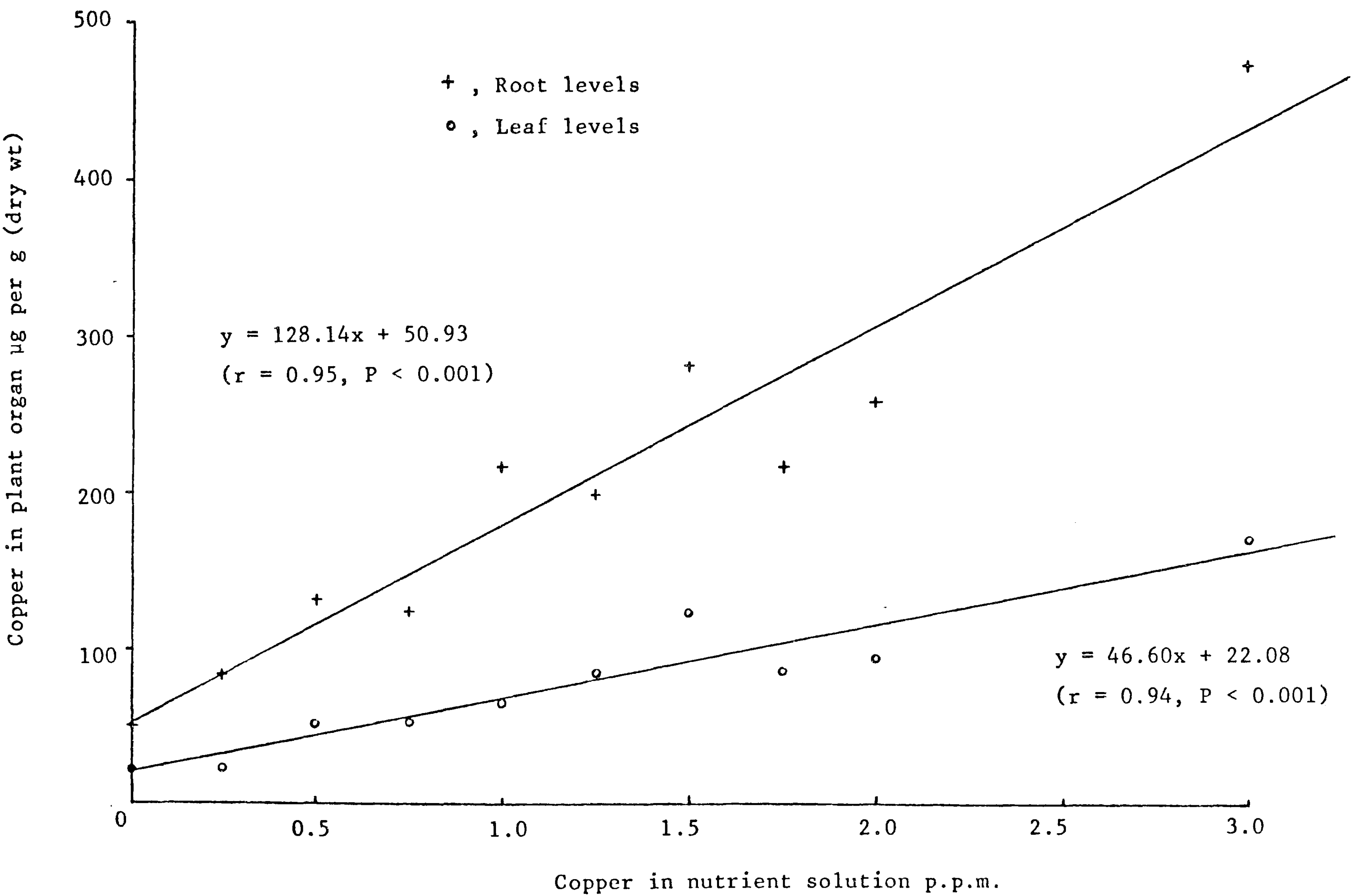
Roots

Conc. of copper ppm	Wet yield per plant g	Cu ppm (dry)	Fe ppm (dry)	Ca ppm (dry)	Mg ppm (dry)
0.0	0.2058	50	2294	4421	1942
0.25	0.2595	85	1865	3954	1849
0.50	0.1433	135	2907	4327	2096
0.75	0.2933	125	1446	3802	2020
1.00	0.2364	218	3730	6371	1772
1.25	0.2787	200	2865	4264	2092
1.50	0.1853	282	2223	6402	1636
1.75	0.3528	217	1715	4760	1913
2.00	0.2960	260	3045	4360	1592
3.00	0.1848	475	5608	8365	1787

Leaves

Conc. of copper ppm	Wet yield per plant g	Cu ppm (dry)	Fe ppm (dry)	Ca ppm (dry)	Mg ppm (dry)
0.0	0.6439	23	250	6654	7538
0.25	0.6144	25	208	6640	7055
0.50	0.3550	54	203	7095	7566
0.75	0.7054	54	201	6465	7901
1.00	0.5449	66	237	7446	7578
1.25	0.8685	85	226	8236	8392
1.50	0.5536	124	262	9029	9618
1.75	0.8855	86	204	8175	8707
2.00	0.5747	93	215	8826	9615
3.00	0.4470	170	209	8090	8977

Figure 26. Copper uptake by *Armeria maritima* from various nutrient solutions.



4.5 Plant Metal Levels

When the plants were harvested at the end of experiment A.M.(i) wet yields were calculated and dried samples were ashed to obtain solutions for metal analyses by Atomic Absorption Spectrophotometry. Copper levels in the roots and leaves were determined. Since copper interferes with iron translocation and heavy metal toxicity may be alleviated by calcium, the levels of iron and calcium were calculated. Magnesium levels are also presented (Fig. 25).

Statistical treatment of the data in Fig. 25 indicated the lack of any inter-element correlations for either roots or leaves of tolerant *A. maritima*. Analyses of regression of copper nutrition levels on to copper root and leaf levels were both significant beyond the $P = 0.001$ level. Linear regression lines are plotted in Fig. 26.

It is apparent from the results in Fig. 25 that copper at the levels present in the roots and leaves is not affecting other metal levels in the roots or their translocation within the plants. Particularly noteworthy is the fact that even with high levels of copper in the roots translocation of iron is not being suppressed. The roots accumulate between 2.3 and 3.4 times as much copper as do the leaves.

The copper levels in the non-tolerant plants (Fig.27) are broadly comparable with those for the tolerant plants grown at the same levels of copper nutrition. This is in accord with the widely held belief that tolerant plants do not exclude toxic levels of metals but rather have an internal tolerance mechanism for dealing with them.

4.6 Salt Excretion

A characteristic feature of the *Plumbaginaceae*, of which *A. maritima* is a member, is the occurrence of special epidermal glands which secrete mucilage and/or calcium salts. Ernst (1969) has reported that some European species excrete heavy metals by means of these glands. However, little interest has been shown in these glands as a means of protection against heavy metal toxicity, since most toxicity studies have involved grasses which do not possess such glands.

During these growth experiments a pale blue powdery excretion was observed on the leaves of the plants. This was not observed on the plants in their natural state probably because the regular and heavy rainfall of the bog area would be sufficient to prevent a build-up of the deposit.

The salt excretion was carefully washed off all the plants in each nutrient solution. The solutions were evaporated and the residue wet ashed with nitric acid and prepared for analysis. The levels of copper secreted by tolerant plants in experiment A.M.(i) are presented in Fig. 27 and a graph of secreted copper per gramme of fresh leaf was plotted against the copper concentration of the solutions (Fig. 28). The results indicate that the amount of secreted copper is directly proportional to the concentration of copper experienced by the roots. A significant quantity of the copper absorbed by these plants is being removed from metabolic sites by gland secretion. This constant removal of copper does not prevent the build-up of high levels of copper in the plant and can only represent part of a more complex internal tolerance mechanism.

Figure 27.
Plant Analyses from Experiment A.M.(ii).

Roots

Conc. of copper ppm	Wet yield per plant g	Cu ppm (dry)
0.0	.1535	84.3
0.1	.2020	110.4
0.5	.0949	185.3
1.0	.0430	481.5

Leaves

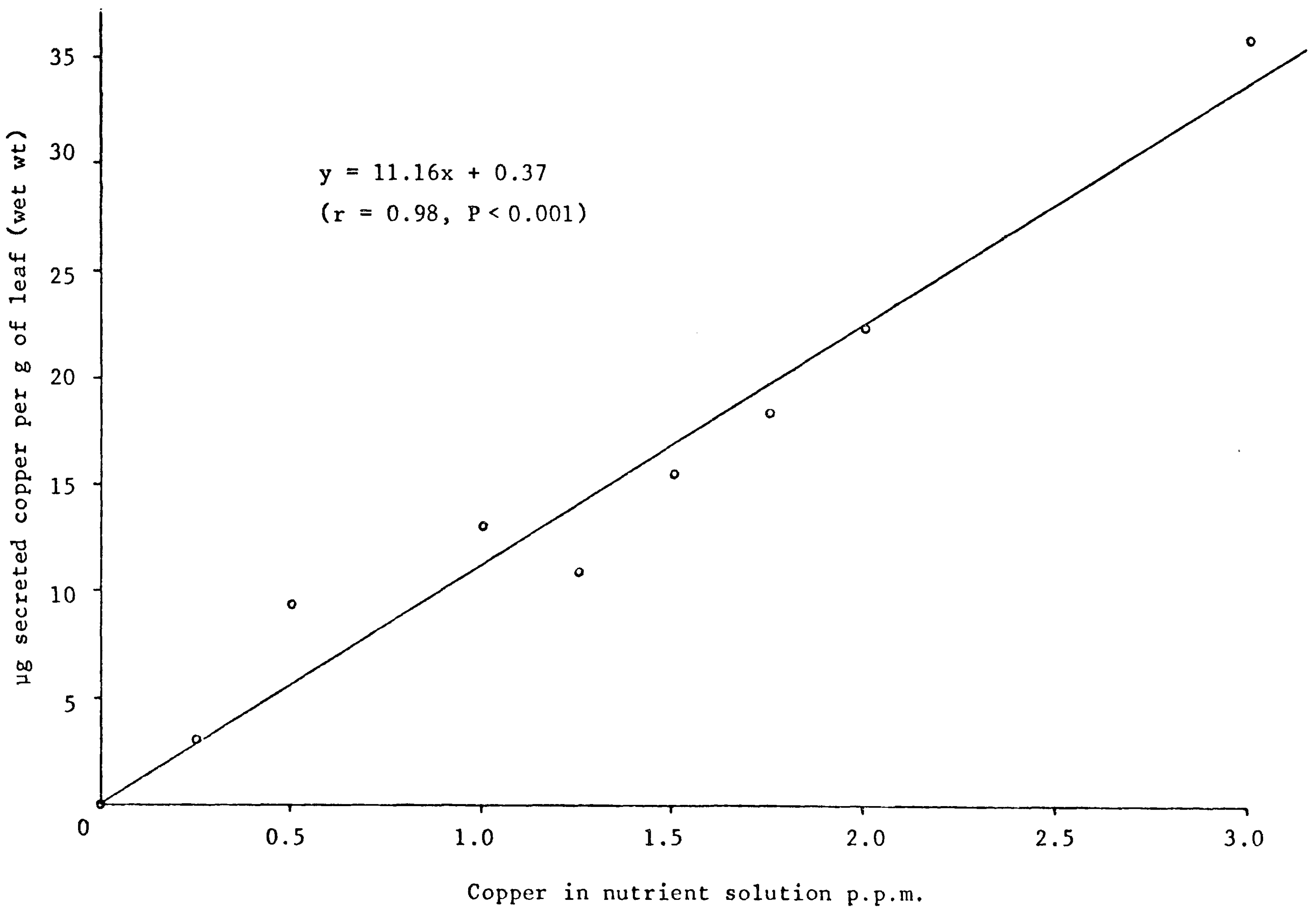
Conc. of copper ppm	Wet yield per plant g	Cu ppm (dry)
0.0	.5732	21.3
0.1	.7106	25.9
0.5	.3921	72.1
1.0	.1296	198.3

The Relationship between Secreted Copper and Nutrition Levels.

Copper nutrition level ppm	Total leaf mass g	Total secreted copper µg	Total secreted copper per g of leaf (wet weight) g
0.00	5.795	0	0
0.25	5.529	16.7	3.02
0.50	3.550	33.3	9.38
* 1.00	3.814	50.0	13.11
1.25	4.342	47.6	10.96
1.50	4.429	69.0	15.58
1.75	6.199	114.2	18.42
2.00	2.873	64.3	22.38
3.00	1.788	64.3	35.96

* 0.75 level sample was lost

Figure 28. The variation in copper secretion with levels of applied copper



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CHAPTER V

COPPER DISTRIBUTION THROUGHOUT TOLERANT *ARMERIA MARITIMA*

Introduction

Only the tolerant grasses have been studied extensively and exhaustively to find how their tolerance mechanisms operate. *Armeria maritima* has long been known as a considerable accumulator of copper and provided a readily available source of research material. In order to fully understand how the plant tolerates copper it was necessary to possess an appreciation of both the gross morphology and the cellular structure of the plant. Direct observations of copper were undertaken using the technique of X-ray micro-analysis and histochemistry. A rough sectioning of the root into cortical and stelar parts followed by copper analyses also provided evidence for the sites of localization of copper within *Armeria maritima*. Initial analyses indicated that the major accumulation site within the plant was the root and studies were largely limited to its investigation.

Armeria maritima (Mill.) Willd. is a perennial herbaceous plant belonging to the family *Plumbaginaceae*. *A. maritima* sub-species maritima is widely distributed on sea cliffs and rocks, estuaries and salt-marshes and up to 1280 m on inland mountains.

5.1 Morphology

Armeria maritima (Miller) Willdenow, var. maritima (typica)
Thrift, Sea Pink. (Clapham et al., 1962).

A. maritima is a dicotyledonous perennial herbaceous plant of low tufted habit having a stout, erect, woody rootstock. The short stem is more or less branched, with short internodes, each branch ending in a rosette of long, narrow, complete leaves, normally subtended by remains of old leaf bases.

The leaves are from 2 - 15 cm long, up to 1.5 mm wide, flat, single (or triple)-veined, fleshy, punctate and glabrous or ciliate.

The scape is 5 - 30 cm long, erect, leafless, tormentose to glabrous and similar in width to the leaf.

The involucral sheath is 8 - 14 mm in diameter. The outer involucral bracts, more or less green on the back, are usually mucronate.

The flowers are grouped in a single, terminal, hemispherical head, 1.5 - 2.5 cm in diameter. The flower stalk is usually as long as the calyx tube, which is pubescent on five vertical ribs and may or may not be hairy between them. The calyx parts are marginless, obtuse to triangular, 0.2 - 1.4 mm long with a 0.1 - 0.5 mm cusp (occasionally absent), but having no spur. The corolla measures 8 mm, varying in shade from rose-pink to almost white, frequently exceeding the calyx tube. The flowers, consisting of five sepals, five stamens, five styles and a single ovule, vary in number from four to ten per head.

The fragrant scent, due to coumarin, attracts pollinating insects. The male and female organs mature more or less simultaneously. The fruit produced is an albuminous one-seeded utricle.

5.2 Distribution and Reproduction

The genus *Armeria* is almost restricted to Europe and North Africa but *A. maritima* (Mill.) Willd. has a discontinuous distribution with stations in N. Asia and the New World in addition to Europe.

Excepting those in the far north, the European forms of *A. maritima* reproduce by means of a dimorphic self-incompatibility system (Baker, 1966). In a natural population there will be a 1:1 ratio of plants with A and B-type flowers. Type-A flowers bear smooth stigmas with coarsely reticulate pollen whilst type-B flowers bear papillate stigmas and punctate pollen. Type-A pollen germinates only papillate stigmas and B pollen only smooth stigmas. American, Arctic and certain N. European populations are monomorphic and thus self-fertile but separation between most types is not always clear.

5.3 Heavy Metal Tolerant Populations

Self-fertility has been shown to be a factor in heavy metal tolerant populations of *Agrostis tenuis, Anthoxanthum odoratum* (Antonovics, 1968) and *Armeria maritima* (Lefèbvre, 1970), but was tested in experimental plots not on natural populations. In the case of *A. maritima* growing on a zinc-lead mine strict out-breeding was in fact observed (Lefèbvre, 1976).

The plants studied in this thesis collected from Dolfrwynog bog are composed of individuals with hairy or smooth scapes, with generally hairy leaves up to 1 mm wide and single-veined. Plants with lengthened outer bracts appear sporadically (8%) and completely hairy calyxes are found in 12% of the total. Overall their dimensions are reduced, but some have scapes up to 34 cm and bracts measuring up to 11 mm (Lefèbvre, 1972).

Approximately two thirds of this population exhibited features typical of *A. maritima* ssp. m. This is in contrast to *A. maritima* from Central and Eastern European mine sites which are included in a 'metallophyte' taxon, *A. maritima* ssp halleri (Lefèbvre, 1974). The nearness to the coast where *A. maritima* ssp.m. is abundant may help to explain the close morphological similarities between the copper tolerant and the typical maritime population. Pollen analyses by Ernst (1969) have shown that heavy metal tolerant plants have existed at the site since the 12th century at least.

5.4 Histochemistry

This technique has been of limited use in the past to those studying metals in plants, simply because general metal concentrations in plant organs are well below detection limits for most reagents. In the case of large levels of accumulated metals, however, this technique can be most revealing. Where metals accumulate at specific sites the concentration may be above the sensitivity limit of the indicator. Various modern stains for copper have been produced, namely Zincon[5-(o-carboxyphenyl)-1-(2-hydroxy-5-sulphophenyl) 3-phenylformazon], Cuprazone[biscyclohexanone oxalydihydrazone] and

P.A.N. [1-(2-pyridylazo)-2-naphthol].

Plants were killed and fixed and in some cases preserved. The part of the plant under study was then sectioned and stained either to show anatomical details up more clearly or simply to locate the metal in question. The sections were then photographed. The stain chosen for metal indentification must be specific and ideally produce a colour distinct from any natural pigmentation in the section. The stain-metal complex should be insoluble in the solvent used otherwise a rapid translocation of the metal will occur. This problem may be overcome by the use of photography.

Method

Plant samples were separated into root stocks and leaves and washed. Killing and fixing was accomplished in one step by putting samples into absolute ethanol or equal proportions by volume of ethanol and glycerol. Without glycerol added, material tends to become brittle after a time. Sections were made free-hand using stainless steel disposable razor blades. A microtome was not employed for this purposes for a number of reasons. Since exact cytological detail was not necessary, ultra-thin sections were not required. The numerous extended procedures necessary for paraffin embedding are likely to cause the redistribution of metals within the tissues.

Since copper was not detectable in leaf material only the root stock of *A. maritima* was studied photomicrographically. Two types of staining technique were employed; firstly standard staining methods to show up differences between cells as an aid to anatomical study and secondly specific methods for copper identification.

A phloroglucinol-hydrochloric acid stain (Gurr, 1953) was used to stain lignified tissue, which took on a red coloration. A coloured photomicrograph was taken, plate 4 and a simplified labelled diagram is presented below. (Fig.29).

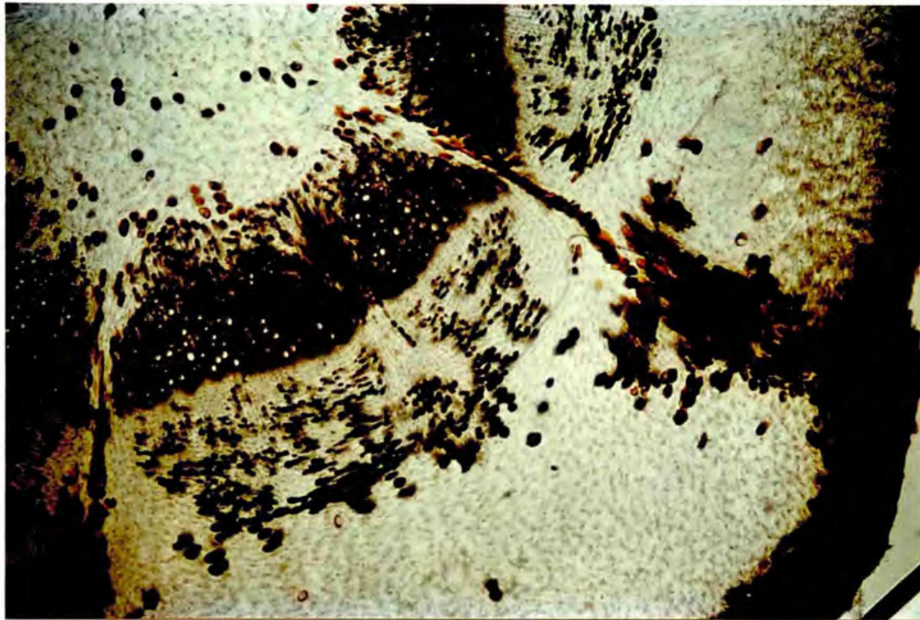


Plate 4. *A. maritima* root cross-section (x 75) stained for lignified tissue.

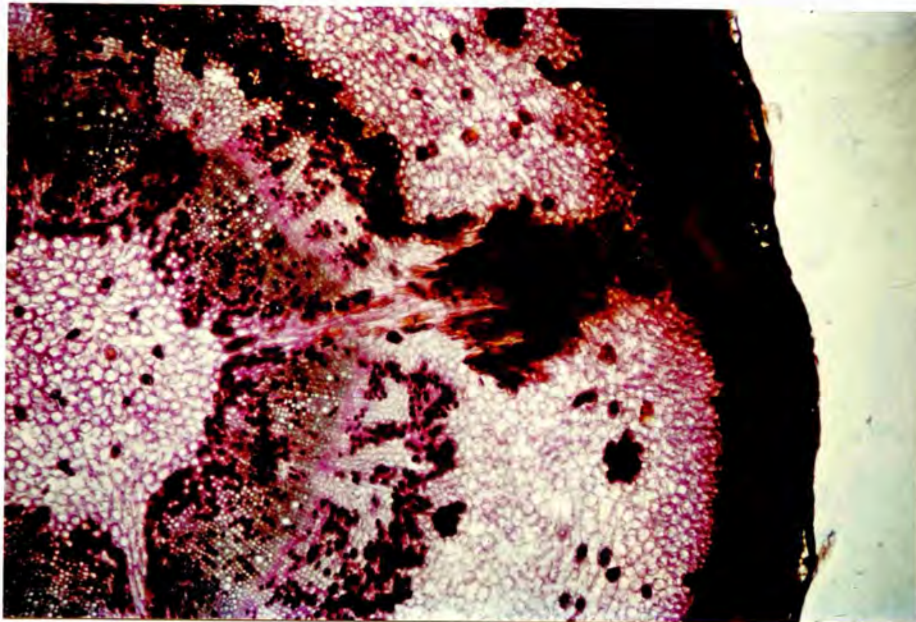
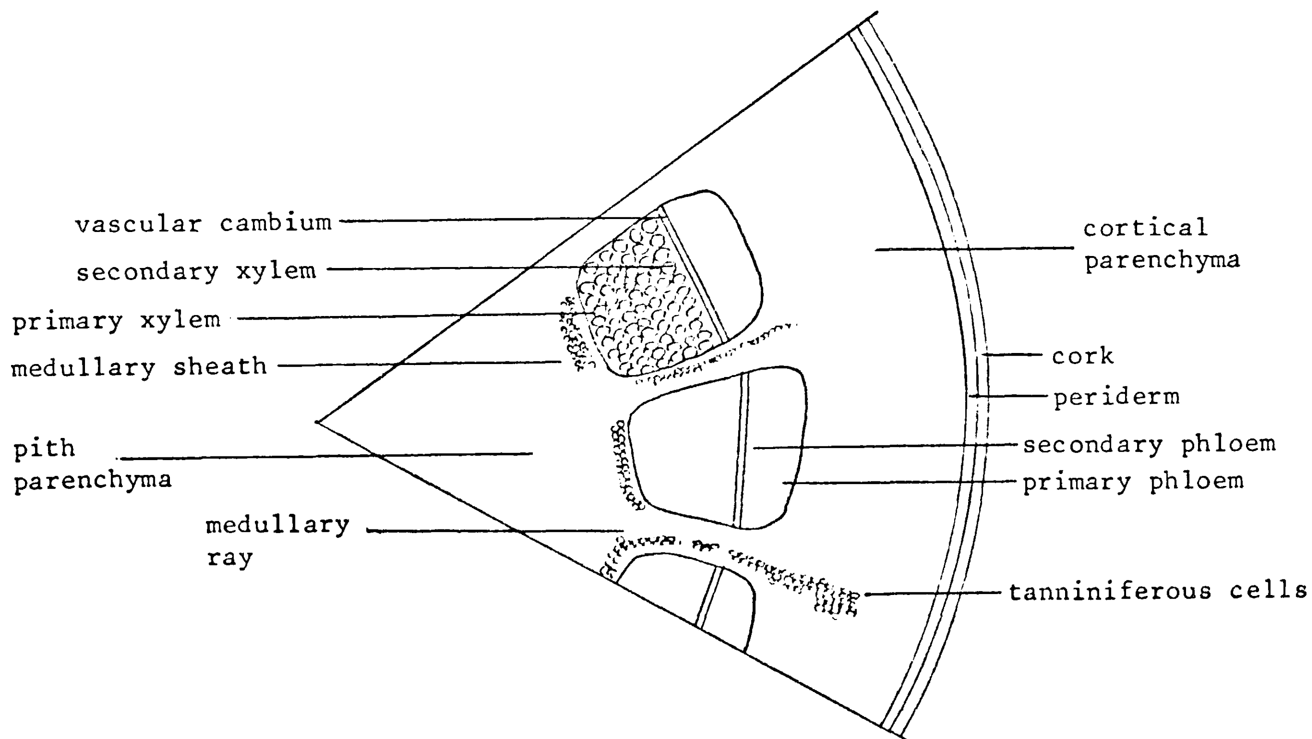


Plate 5. *A. maritima* root cross-section (x 75) stained for copper.



Results

The salient features of the above cross-section are as follows: the pith consists of intact parenchyma cells surrounded by a number of bundles of vascular tissue, inconspicuous phloem vessels are separated from xylem by a narrow band of vascular cambium, beyond the vascular bundles parenchymatous cortex is bordered by periderm which gives rise to an outer layer of cork and medullary rays pass between the vascular bundles.

Numerous tannin containing cells are found associated with or in the phloem and endodermis, the medullary sheath, the periderm, the cells of the rays and scattered in small groups within the cortex.

Attempts to locate copper by staining with Zincon and Cuprazone were unsuccessful. Both reagents failed to produce distinct colours, either because copper concentrations were too low or because the copper was present as a more stable complex in the plant. However, sections stained with a drop of an ethanolic solution of P.A.N. and observed immediately (Smith, 1953) showed a purplish-red colour indicative of copper readily distinguishable from the orange red colour which forms with zinc (Plate 5).

Tissues stained lightly were cells in the medullary sheath and cells containing tannin deposits. The most heavily stained were hypodermal cells and considerable levels of copper were found in and around phloem and endodermal cells.

5.5 Electron Microscopy

A general background to this technique has been presented in Chapter II . The use of the electron microscope in microanalysis has been of great value in the understanding of the rôles played by macronutrient elements in plant metabolism. Although the much lower levels of the micronutrients are normally well below detection limits, the copper levels in the roots of tolerant *A. maritima* are sufficiently high for the technique to be employed.

Method

A section of root of *A. maritima* was dried and sectioned longitudinally so as to avoid any copper displacement. The sample was cut to a suitable size (about 1 cm^2), glued to a base plate and coated as previously described (Chapter II). The sample was then placed in the electron microscope. An electron accelerating voltage of 30 kV was used and a specimen current of 10^{-7} A. The copper Kα X-ray line was monitored.

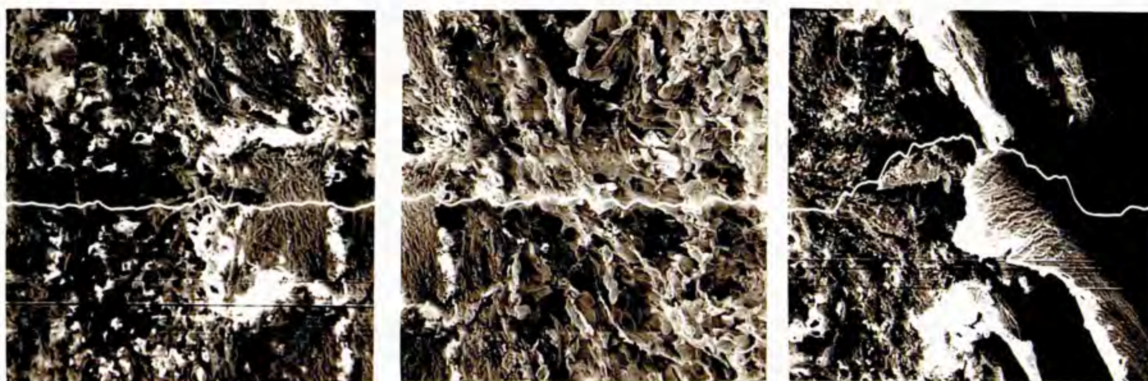
Results

Plate 6a is a primary electron photograph (10 kV accelerating voltage) and shows a cross-section of a piece of mature root stock with severed leaf bases evident. A high energy electron beam is scanned across from the centre of the root to its edge, as indicated on plate 6a . In plates 6b, c and d the white lines show the copper concentration in the tissue. A constant copper level, close to the detection limit of the instrument, is observed across the sample except in the hypodermis where a significantly higher level is observed.

Plate 6 (composite)

Electron microprobe photomicrographs of high copper root material of *A. maritima*.

Microprobe track across root (x 20), overlay indicates path of beam. Plate 6a.



Copper distribution across root (x 50) showing peak at epidermis. Plate 6b, c & d.

5.6 Sectioning of Root into Inner and Outer Parts

Method

A young *Armeria maritima* root stock was carefully cleaned as previously described. The root was then chopped into a number of short sections. Each piece was separated into two parts, an inner and outer part. The outer parts of the root, comprising cortical, hypodermal and epidermal cells, were carefully pared off from the sections. This left the pith, stele and inner cortex as the inner part. The separated parts were dried to constant weight at 40°C, weighed and wet ashed. Both samples were analysed for their copper levels by atomic absorption spectrophotometry.

Results

The method can only furnish a crude idea of copper distribution within the root. But even allowing for large errors 86.4% of the total copper present had been accumulated in the outer cells. Still more dramatic were the relative copper levels, there being 7.4 times more copper in the outer parts than in the inner parts. The results are presented in Fig. 30.

Figure 30. Distribution of copper between the inner (cortex/stele) and outer (epidermis/hypodermis) plant parts.

Sample	Plant part	Plant material (dry wt) g	Copper content ppm (dry wt)	Percentage of total copper %
A	inner	0.1350	444	13.6
B	outer	0.1162	3,270	86.4
Total	whole	0.2512	1,752	100.0

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CHAPTER VI

DETERMINATION OF THE SITE AND THE GENERAL FORM OF COPPER DEPOSITION WITHIN TOLERANT *A. MARITIMA*

Introduction

Several methods have been devised to determine the sites and/or forms in which plants store toxic metals. A knowledge of where the metal is accumulated is a prerequisite for the elucidation of the method and form of complexation. The techniques employed here: differential ultracentrifugation, sequential extraction and dialysis are based upon the use of certain physical phenomena, namely those of sedimentation, solubility and ion exchange capacity respectively. These properties of sub-cellular components vary considerably so that plant parts can be fractionated in different ways. Each fraction may then be assayed to find its ability to accumulate copper.

6.1 Extraction Schemes for the Removal of Copper from Roots and Leaves of *A. maritima*

The first chemical fractionation scheme was carried out to give some initial idea of how the metal was bound, and is not considered to be a rigorous investigation of the nature of the compounds with which copper is associated within the plant. Leaf material was used in the study because, containing approximately seven times less copper than was found in root material, detection problems could be immediately highlighted. The scheme is based on the use of solvents of increasing polarity and is presented in Fig. 31 , together with chemical substances extracted.

Method A

Approximately 2 g of young floret leaves from samples of plants taken from Dolfrwynog bog were carefully picked. Any obviously contaminated or dead leaves were discarded. The leaves were washed in 400 cm^3 of a 2.5%

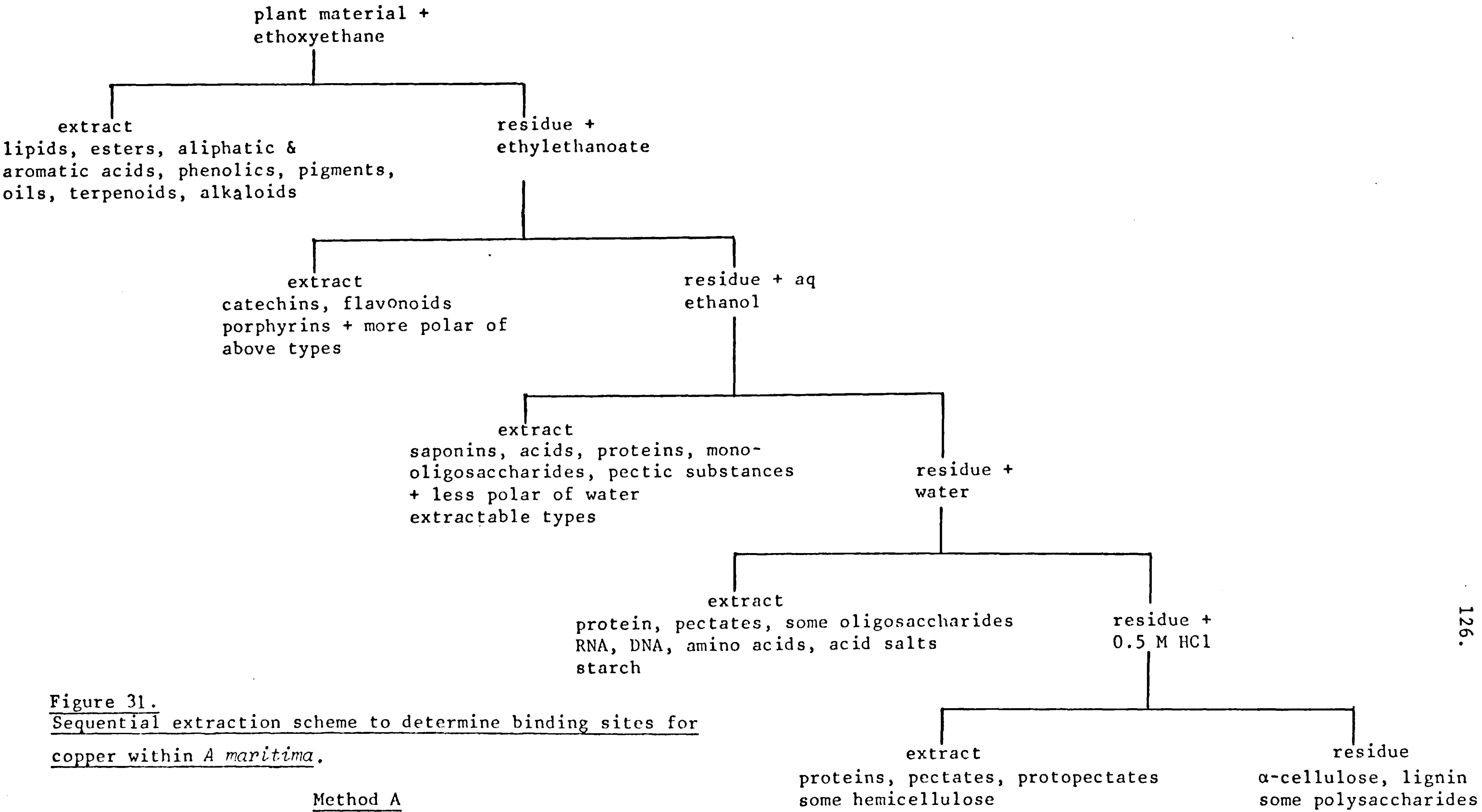

Figure 31.
Sequential extraction scheme to determine binding sites for copper within *A maritima*.

Method A

solution of Teepol in deionized water for ten minutes. The liquid was decanted and the leaves further washed with 400 cm^3 of deionized water. The leaves were finally rinsed with 200 cm^3 of deionized water on a Buchner filter.

The leaves were dried to constant weight in an oven at 70^oC, and powdered using a ball mill. 1.977 g of material was put into a soxhlet apparatus and mounted on a 500 cm^3 round bottomed flask containing 150 cm^3 of sodium dried ethoxyethane. A similar apparatus was set up alongside with no plant material in the thimble to serve as a blank. The extraction was carried out for four hours. Ether was subsequently removed on a rotary evaporator and the flasks were placed on sand trays on hot plates. The contents of the flasks were wet ashed.

Further soxhlet extractions were made firstly with 150 cm^3 of ethy-ethanoate for six hours and then with 150 cm^3 of 80% ethanol for eight hours. The solvents were removed as above and wet ashings carried out.

The thimbles were dried and the plant material was transferred to a stoppered 500 cm^3 conical flask. 150 cm^3 of deionized water was added and the flask shaken for sixteen hours. The mixture was centrifuged at 1500 r.p.m. for one hour and the clear supernatant was pipetted off. The plant material was twice washed and recentrifuged and the supernatant pooled, dried and ashed.

The residue was finally extracted by shaking with 190 cm^3 of 0.51 M analar hydrochloric acid in a 500 cm^3 flask for sixteen hours. Centrifugation, washing, drying and ashing were carried out as above. The final residue was also wet ashed.

The results are set out in Fig. 32 and discussed with those of the following extractions.

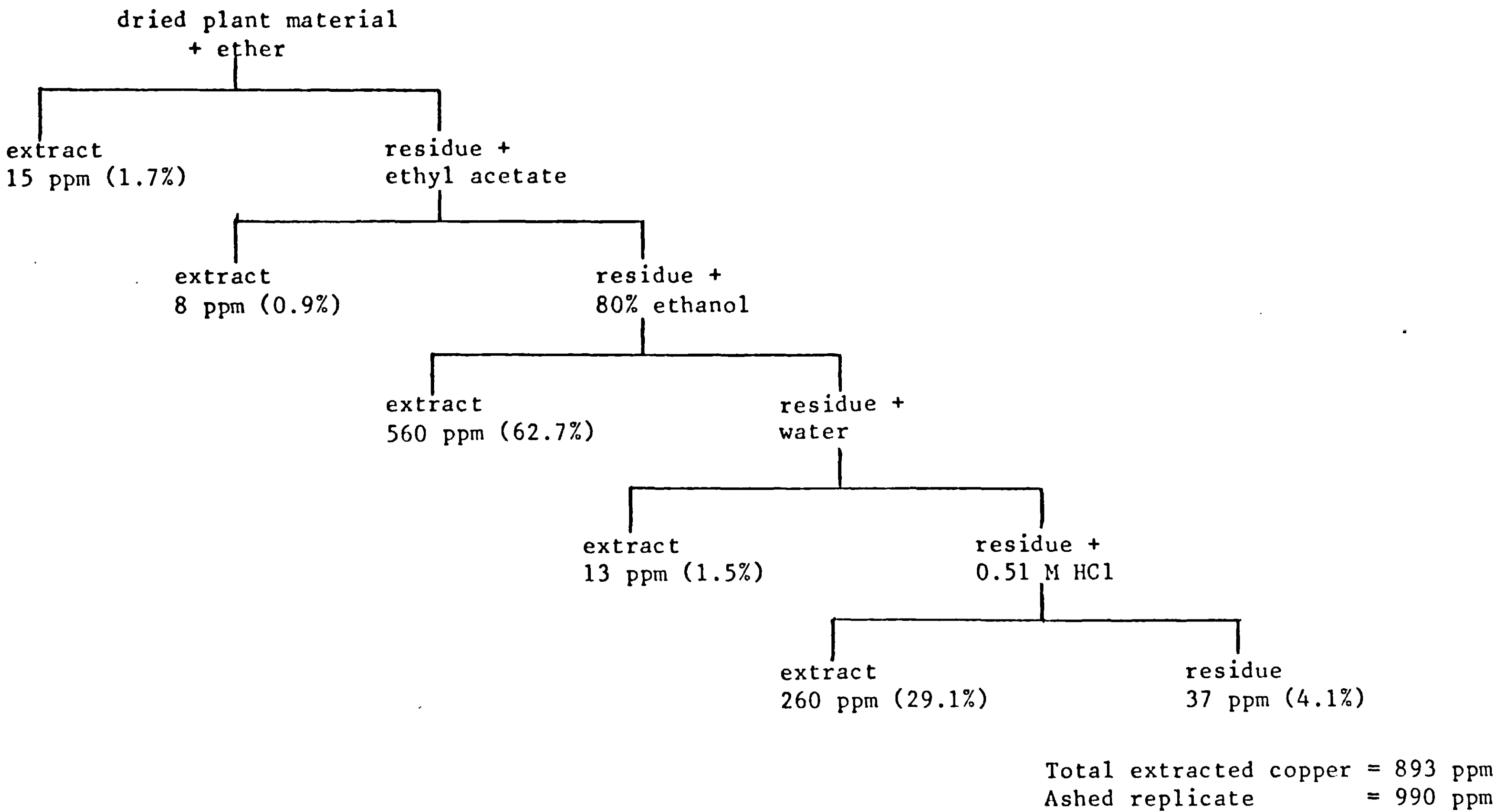

Figure 32.

Extraction scheme for powdered leaves of *Armeria maritima* ssp maritima

Copper levels indicated in parts per million, percentages of total copper given in brackets.

Consideration of the results from the first extraction scheme led to the adoption of a scheme which pays particular attention to acid hydrolysable and water soluble material. The scheme is based on that of Peterson (1969) with some modifications. The water extract of the dried 80% ethanol extract and the water extract proper were both further investigated. Sevag et al. (1938) described a simple method for removing protein from aqueous solutions as a stable gel. By shaking the solution with trichloromethane and a foam preventing substance like pentan-1-ol, a protein gel is formed in the organic layer. This procedure was employed on both of the water extracts.

Further to this soluble pectic substances were precipitated by the addition of an equal volume of acetone to the solution (Hinton, 1939).

The scheme was used on both root and leaf samples and is presented in Fig. 33 together with a simple breakdown of the major classes of compounds extracted.

Method B

Samples Nos. 2001 - 8 were used for extraction purposes. From the analyses presented in Fig. 17 the level of copper present in the roots should be approximately 1170 ppm of copper. Significant levels of copper would be 0.5% (5.8 μg/g) of the total present. With accurate detection limits of 1 ppm using 25 cm^3 of ashed solution for atomic absorption analysis the required amount of root material was 4 g. Only 3 g of leaf material was available so a replicate analysis was not undertaken.

The root and leaf material had both been sifted through a 210 μm gauge stainless steel gauze. Two 4 g root and one 3 g leaf samples were used. The following extraction procedure applied similarly to all three samples. The first extraction solvent was an 80% absolute ethanol solution.

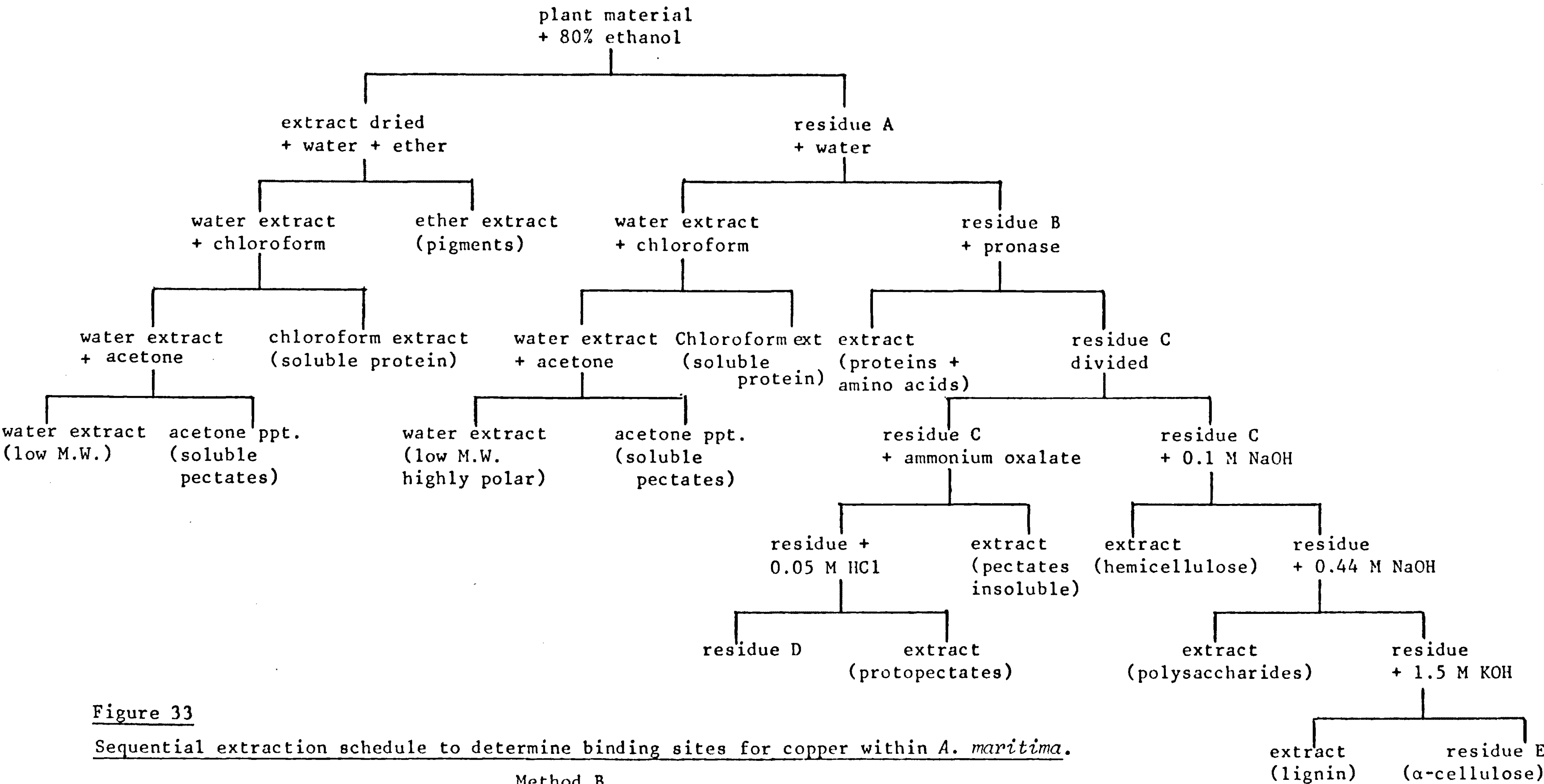

Figure 33

Sequential extraction schedule to determine binding sites for copper within *A. maritima*.

Method B

The sample was refluxed for fifteen minutes with 150 cm^3 of the solvent in a 500 cm^3 flask with a condenser. The suspension was filtered using a "millipore" type Buchner filter with a fine glass fibre filter pad to minimise sample loss. The extraction was repeated twice and the extracts pooled. The residue (A) was dried to constant weight in an oven at 40°C.

Not at any stage of these analyses were aqueous extracts left for any longer than a few hours without treatment, either refrigeration or freeze drying. This was not only because chemical reactions in solution are possible but mainly because bacterial growth was rapid under such conditions.

The 80% ethanol extract was taken to dryness firstly by rotary evaporation at 40°C to remove the ethanol and further by freeze drying to remove the water. The dried extract was dissolved in 300 cm^3 of water and introduced into a separating flask to which was also added 125 cm^3 of sodium dried ether. Separation of the extract between the two phases was effected by vigorous shaking and running off the lower aqueous layer. The ether extract was taken to dryness on a rotary evaporator and the residue wet ashed.

To the 300 cm^3 of water (1 part) was added 0.1 parts of analar pentan-1-ol and 0.25 parts of analar trichloromethane. The whole was shaken for 15 minutes in a 1 litre separating flask. The lower white trichloromethane-pentanol-protein gel was run off and taken to dryness on a rotary evaporator and subsequently ashed. The upper (mainly water) layer was rotary evaporated to remove any traces of the organic solvents and an equal volume (100 cm^3) of analar acetone was added to form a precipitate. The solution was centrifuged and the supernatant carefully decanted.

The precipitate was wet ashed as was the solution after evaporation to dryness. This completed the treatments of the 80% ethanol soluble components.

Residue A was weighed and placed in a 500 cm^3 flask with a condenser. To this was added 100 cm^3 of water. Refluxing was carried out for 10 minutes. Filtration was again done using a "millipore" filter and twice repeated. Residue B was dried to constant weight as before. The extracts were pooled and directly freeze dried. The dried extract was dissolved in 50 cm^3 of water and the trichloromethane-pentanol and acetone treatments were carried out as before.

Residue B, together with 1 g of the proteolytic preparation pronase, 10 mg of chloroamphenicol and 200 cm^3 of phosphate buffer (0.01 M, pH 7.4) was placed in a 500 cm^3 conical flask and shaken for 40 hours. The sample was filtered and reincubated with pronase. The possibility of the phosphate buffer acting as an extracting agent was considered. So, to remove any doubts, a blank was run on a sample of plant material, previously extracted using ethanol and water, with the pronase excluded. Only 4 ppm (dry weight) of copper were found in the extract. Another possible source of error might have been copper present in the proteolytic enzyme. An ashed sample, however, indicated the absence of copper. The extract was freeze dried and wet ashed, whilst the residue (C) was washed and dried to constant weight.

Residue C was accurately weighed and separated exactly into two parts, each of which were subjected to a different series of extraction procedures.

The first portion was shaken with 100 cm^3 of a 2% ammonium oxalate solution in a 250 cm^3 conical flask for 2 hours. After centrifugation and decantation the residue was treated similarly with the same volume of 0.05 M hydrochloric acid. The final residue (D) was wet ashed.

The second portion was extracted as for the first portion using successively 0.1 M NaOH, 0.44 M NaOH and boiling 1.5 M KOH. The final residue (E) was also wet ashed.

Atomic absorption analysis of the copper levels in each extract and the final residues were made. These results are presented in Figs. 34-36 Also presented are the amounts of material extracted at various stages of the schedule (Fig.37).

Results and discussion

The initial extract scheme indicated that 82% of the copper within leaf material was present in association with acid and 80% aqueous ethanol extractable materials. Since the 80% aqueous ethanol extract was carried out by the soxhlet method, the composition of the extracting mixture had more than 80% of ethanol present. Judging by the very low level of water extractable copper it seemed likely that much of the hot aqueous ethanol extractable material was also water soluble. Preliminary extracts with ethoxyethane and ethylethanoate were considered superfluous for future studies. The two important fractions were further studied by using extraction and precipitation methods on the water and 80% aqueous ethanol extractable fractions and replacing the harsh hydrolysis by a series of more gentle forms of solubilization.

A general assessment of the accuracy of the second extraction scheme can be made by a comparison of total metal extracted with the levels of metals found in the same plant sample by wet ashing. For root material, taking an average value for both schemes A and B, a level of 1112 ppm was extracted which compared well with the average of the two replicates 2001-8 R1A and R1B (Fig. 17) of 1175 ppm. A similar correlation for leaf material gives a level of 178 ppm extracted compared with 171 ppm in the plant tissue. It appears therefore that losses, contamination and atomic absorption interferences have been kept to a minimum.

The amounts of material removed at each stage of extraction were calculated and are presented below, Fig. 37 .

Sequential extraction schedules to determine the binding sites for copper within roots of *Armeria maritima*.

Method B

Scheme A	after 80% ethanol extraction				after water extraction							Total
	Ether extract	Chloroform extract	Acetone ppt.	Water soluble	Chloroform extract	Acetone ppt.	Water soluble	Pronase extract	$(NH_4)_2C_2O_4$ extract	0.05 M HCl extract	Residue D	
Compounds extracted	pigments	soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W highly polar	Proteins & amino acids	Pectates insol.	Proto-pectates		
Cu ppm (dry wt) a	2.2	31.1	56.4	290.0	2.7	27.4	20.3	130.0	450.0	99.1	15.3	1124.5
extracted replicates b	1.1	40.8	27.4	325.6	3.4	11.3	22.8	137.5	450.0	115.0	14.4	1149.3
Average Cu level ppm	1.7	36.0	41.9	307.8	3.0	19.4	21.6	133.8	450.0	107.0	14.9	1137.1
Percentage of total copper	0.1	3.2	3.7	27.1	0.3	1.7	1.9	11.8	39.6	9.4	1.3	100.1%

Figure 34

Sequential extraction schedules to determine the binding sites for copper within roots of *Armeria maritima*.

Method B

Scheme B	after 80% ethanol extraction				after water extraction								Total
	Ether extract	Chloroform extract	Acetone ppt.	Water soluble	Chloroform extract	Acetone ppt	Water Soluble	Pronase extract	0.1 M NaOH extract	0.44 M NaOH extract	1.5 M KOH extract	Residue E	
Compounds extracted	pigments	soluble protein	soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W highly polar	Proteins & amino acids	hemi-cell-ulose	poly-saccharide	lignin	α-cell-ulose	
Cu ppm (dry wt) extracted replicates a	2.2	31.1	56.4	290.0	2.7	27.4	20.3	130.0	456.3	37.3	22.5	7.6	1083.8
Cu ppm (dry wt) extracted replicates b	1.1	40.8	27.4	325.6	3.4	11.3	22.8	137.5	447.5	39.3	22.5	8.3	1087.5
Average Cu level ppm	1.7	36.0	41.9	307.8	3.0	19.4	21.6	133.8	451.9	38.3	22.5	7.9	1085.8
Percentage of total copper	0.2	3.3	3.9	28.3	0.3	1.8	2.0	12.3	41.6	3.5	2.1	0.7	100.0%

Figure 35

Figure 36

Sequential extraction schedules to determine the binding sites for copper within leaves of *Armeria maritima*

Method B

Scheme A	after 80% ethanol extraction				after water extraction							Total
	Ether extract	Chloroform extract	Acetone ppt	Water soluble	Chloroform extract	Acetone ppt	Water soluble	Pronase	$(NH_4)_2C_2O_4$	0.05 M HCl	Residue D	
Compounds extracted	pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W highly polar	Proteins & amino acids	Pectates insoluble	Proto-pectates		
Cu extracted ppm (dry wt)	3.0	15.9	9.7	30.9	2.1	11.2	4.8	15.0	63.3	26.0	7.0	188.9
Percentage of total copper	1.6	8.4	5.1	16.4	1.1	5.9	2.5	7.9	33.5	13.8	3.7	99.9%

Scheme B	after 80% ethanol extraction				after water extraction								Total
	Ether extract	Chloroform extract	Acetone ppt	Water soluble	Chloroform extract	Acetone ppt	Water soluble	Pronase extract	0.1 M NaOH	0.44 M NaOH	1.5 M KOH	Residue E	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W	Soluble protein	Soluble pectates	Low M.W highly polar	Proteins & amino acids	Hemi-cell-ulose	Poly-saccharides	lignin	α-cell-ulose	
Cu extracted ppm (dry wt)	3.0	15.9	9.7	30.9	2.1	11.2	4.8	15.0	36.6	20.0	14.0	3.3	166.5
Percentage of total copper	1.8	9.5	5.8	18.6	1.3	6.7	2.9	9.0	22.0	12.0	8.4	2.0	100.0%

Figure 37

Material	Percentage material removed by		Percentage of material remaining prior to pronase extract
	80% aq. ethanol	water extract	
Root a	27.9	18.2	53.9
Root b	24.3	21.1	54.6
Leaf	25.1	18.3	56.6

Material removed after pronase extraction could not be measured because the protease was more or less retained on filtration. A visual impression of the amounts of soluble protein and pectates removed by 80% aqueous ethanol and water extraction indicated that most was removed by 80% aqueous ethanol for both leaves and roots.

An assessment of extraction Scheme A for root material reveals that the water soluble proteins and pectates account for only 3.5% and 5.4% of the copper removed in contrast to the 29% removed in association with low molecular weight water extractable materials. Of the water insoluble material, which can be considered largely cell wall material, 11.8% is solubilized by pronase, this material will consist of protoplasmic proteins, polypeptides and possibly cell wall protein. The largest portion of copper, 49% is associated with pectic and protopectic materials. The residue, probably consisting of α-cellulose, lignin, neutral hemicellulose (polysaccharides) and less readily hydrolysed acidic hemicelluloses, appears remarkably copper free.

As much as 54.4% of copper extracted is therefore associated with materials based on uronic acids. Scheme B presents a broadly similar picture, the 0.1 M NaOH extract releasing pectic as well as hemicellulose material. Since there is no dividing line between neutral and acidic hemicelluloses, extractions with increasing concentrations of alkali yield a series of fractions with increasing contents of uronic acid (Robinson, 1969).

Information from Scheme A concerning the binding sites for copper in leaves of *A. maritima* shows that 58.3% of the metal solubilized was linked with pectic and protopectic substances, 17.4% is removed with soluble and insoluble proteins or polypeptides. Copper associated with low molecular weight material in the water extract, 18.9%, is considerably lower than that found in the roots of the same plants. Materials extracted by 0.44 M sodium hydroxide and 1.5 M potassium hydroxide in Scheme B are likely to include also acidic hemicellulose and pectic materials.

The possibility of co-precipitation of copper with pectin or protein is a criticism which may be levelled at the technique. However, copper ions were not found in water extracts used for chromatographic study (Chapter VII), thus eliminating the possibility.

6.2 The Sub-cellular Distribution of Copper within the Roots of Tolerant *A. maritima* using Differential Ultracentrifugation

Method

Approximately 40 g fresh weight of *Armeria maritima* roots were washed in cold deionized water, followed by 0.01% Teepol, 0.02 M calcium chloride and finally in cold deionized water. The roots were then homogenized in 200 cm^3 of 0.1 M sodium phosphate/0.4 M sucrose buffer (Gomori, 1955) at pH 7.2. The homogenate was poured into a muslin bag; the residue being retained as the crude debris. The supernatant was centrifuged to obtain the cell wall debris and further ultracentrifuged to spin down the mitochondrial and microsomal cell fractions. The final liquid was the high speed supernatant. Results are presented in Figs. 38 and 39.

Results and discussion

The crude debris happened to comprise the bulk of the root material. This was perhaps not surprising since the roots appeared quite woody and much of the debris was in the form of woody splinters.

The differential ultracentrifugation procedure for homogenized roots of *Armeria maritima*.

Subcellular Fraction Isolated	Fraction Title*	Separation Procedure		Composite Units **	
		xg Average Force	Time Minutes	g.min	s_{min} (Svedburg Units)
Crude Debris	CD	Muslin filtration	-	-	-
Cell Wall Debris	0.5P5	500	5	2500	317 000
Mitochondrial pellet	10P20	10,000	20	200 000	3960
Microsomal pellet	100P120	100,000	120	12 000 000	66
High speed supernatant	100S120	>100,000	120	>12 000 000	<66

* Fraction titles after Cartwright (1966).

** Composite units calculated as recommended by de Duve and Berthet (1953).

Figure 38

The results of subcellular fractionation of root material from *Armeria maritima*.

Subcellular fraction Isolated	Fraction Title	Copper concs. in subcellular fractions ppm (dry wt)	Percentage distribution of dry weight materials	Copper distribution in subcellular fractions % total copper
Crude Debris	CD	1570	78.4	82.7
Cell Wall Debris	0.5P5	630	8.9	3.8
Mitochondrial pellet	10P20	1240	3.7	3.1
Microsomal pellet	100P120	930	2.4	1.5
High speed supernatant	100S120	2000	6.6	8.9
Whole root	-	1480	100.0	100.0

Figure 39

The whole root had a higher copper concentration than did Sample Nos. 2001 - 8. In general, samples collected at different times from different parts of the bog showed wide variations in copper levels.

Copper concentrations in the subcellular fractions, whilst not being of much interest from a bulk accumulation point of view, do show that higher levels of copper are not excluded from the more sensitive parts of the cell. The greatest portion of the copper in the root was located in the crude debris where it was least able to interfere with the normal metabolic processes. The crude debris consisted of hypodermal and epidermal material, pith, dead cells and parts of the larger cell walls, all these being ideal sites for toxic metal deposition.

Direct comparisons between these results and any previous work would necessarily be tenuous as deposition has been shown to be dependent not only on nutrition levels (Turner, 1969) but on other factors such as plant species, ecotype and plant age. Most of the data published suggests that accumulation occurs at the cell wall. Cartwright (1966) reported 64% of total copper in subterranean clover nodules to be in the cell wall fraction. Turner showed that in copper tolerant *Agrostis tenuis* at high copper nutrition levels, 61% of copper was deposited in the crude debris and cell wall fractions.

6.3 Dialysis with Powdered Roots of Copper Tolerant *Armeria maritima* to Determine Binding Sites for Copper

Method

Replicate 1 g samples of powdered root material, particle size $\leqslant$ 210 µm, were placed in bags made of Visking tubing. The bags were formed by tying both ends with black cotton. To one bag 40 cm^3 of deionized water was added and to the other 40 cm^3 of 0.02 M tartaric acid was added.

The first bag was suspended in a beaker containing 100 cm^3 of deionized water for 36 hours. The other bag was suspended firstly in 0.02 M tartaric acid for 24 hours, followed by a 24 hour period in deionized water. In both cases the solutions were stirred and replaced every four hours. After dialysis the solutions were freeze dried and wet ashed as previously described. Care was taken when ashing the solid tartaric acid as frothing occurred. Finally, the samples were analysed for copper using the atomic absorption technique (see results below).

Results and discussion

Differences observed in the relative amounts of copper extracted by the dialysis of root material against water and tartaric acid were quite dramatic. Dialysis with water alone removed only 9.7% of the total copper. This would include water soluble organic-copper complexes as well as any unbound or weakly bound ionic copper that may have been present. When, however, dialysis was performed with tartaric acid as well as water a total of 44.8% of the copper was removed. The discrepancy between these two results must be due to more tightly bound cation exchange complexes.

Solubility of copper ions using water and tartaric acid as extracting agents.

Sample	Extraction agent	Plant material dry weight g	Copper extracted ppm (dry wt)	Copper extracted as percentage of total %
A	tartaric acid	1.0015	875	44.8
B.	water	1.0020	190	9.7

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CHAPTER VII

A STUDY OF WATER SOLUBLE COPPER COMPLEXES OF TOLERANT *A. MARITIMA*

7.1 Complexes in Plants

Since it has been adequately demonstrated that plants do not exclude or control the uptake of large quantities of toxic ions, they must, therefore, be truly tolerant of high metal concentrations. Furthermore, heavy metal tolerance must involve a specific internal metabolic process to prevent metals reaching and interfering with metal sensitive sites.

Before any understanding can be achieved of the means which plants employ for keeping heavy metals from metabolically sensitive sites, it is first necessary to consider the various types of known heavy metal complexes in plants. Heavy metals have been shown to be involved in numerous enzymes, coenzymes and other functional constituents of normal cell activity. Complexed and uncomplexed forms of heavy metals, as intermediate transportable forms, have been demonstrated in plant exudates. Finally and of most relevance to the present study are the forms in which metals, in excess of physiological levels and therefore unnecessary and indeed injurious to metabolism, are rendered unavailable.

Many transition metals have been shown to be involved in organo-metallic enzymes in plants. Enzymes occur in very small quantities and certainly are not relevant to a consideration of means of complexing toxic quantities of heavy metals. However, the fact that these metals can bind quite strongly with proteins was a starting point for many workers in the consideration of which organic compounds would be most likely to act as complexing agents.

Most of the early work in this field was limited to merely indicating that complexes existed in plants and that many plant constituents, notably proteins, had high chelatogenic capabilities. Studies indicating heavy metal

complexes were those by Dawson and Nair (1950) who used a copper-amalgam electrode to identify copper complexes in grass tissue and Levitt and Todd (1952) who linked copper and zinc with the crude protein fraction in potato tubers. Mills (1954, 1956) in a series of experiments on copper complexes in grassland herbage showed that a number of complexes were present and that addition of copper(II) ions to a herbage homogenate resulted in the total removal of ions at pH 4.

A number of investigations suggesting possible chelating agents included that of Mills (1954) who listed proteins, amino acids, porphyrins, purines, pterines, flavins, catechol and its derivatives, tannins and lignin as being plant and animal products which form stable complexes with copper. Dekock (1956) suggested that heavy metals combined with protein chelates in the roots of mustard plants at toxic levels of nutrition, but offered no conclusive evidence. In model experiments Timberlake (1959) demonstrated complex formation between copper and some organic acids, phenols and phenolic acids occurring in fruit. A slightly different approach was that of Ennis (1962) who studied the chemical nature of copper complexes in peat. She found that phenolic hydroxyl and carboxylic acid groups were those mainly responsible for chelation.

7.2 Exudate Studies

Studies of metal translocation have been a major source of information on metal complexes in plants. Since iron forms insoluble precipitates with phosphate and hydroxyl ions at physiological pH's, a natural complexing agent in plant sap seemed essential. Schmid and Gerloff (1961) demonstrated a naturally occurring chelate of iron in xylem exudate of *Nicotiana tabacum* stumps, but were unable to identify it.

Initial studies by Tiffin and Brown (1962) suggested that iron was present as the malate complex in soyabean exudate, but this was not confirmed. More recent work by Tiffin (1966) demonstrated the presence of iron-citrate

complexes in soyabean, tomato, cucumber and sunflower. Further to this he showed that manganese, cobalt and zinc are transported as inorganic cations in stem exudates of tomatoes (Tiffin, 1967). Nickel was, however, found as an anionic complex in various crop species (Tiffin, 1971). Maize was another plant in which iron-citrate was the predominant transportable form of iron (Tiffin, 1973).

Hoefner (1966) investigated organic metal complexes in exudates of *Helianthus annuus* using various techniques. Electrophoresis of labelled metal ions indicated complexes of zinc and cobalt with oligopeptides and three anionic copper complexes. He further showed cobalt to be in an amino acid complex and zinc to be peptide linked (Hoefner, 1968). He could not illustrate the participation of organic acids such as glucuronic, maleic malonic or citric acid in trace element complex formation.

In work on 80% ethanol extracts of root, stem and leaf tissue of *Leptospermum scoparium*, Lyons, Peterson and Brooks (1969) indicated the presence of a trisoxalatochromate(III) ion.

7.3 Accumulator Species Studies

Considering the large number of heavy metal accumulators reported in the literature, very few studies of the nature of the accumulated metals within the plants have been presented. Reilly (1969) working with *Becium homblei* found a significant correlation between the copper content and the total nitrogen in leaves. This rather suggests that protein might be acting as a complexing agent for copper in plant tissues. Chromatography of water extracts indicated copper-amino acid complexes which may or may not have been a result of copper-peptide dissociation (Reilly, 1972). Studies of nickel accumulator *Hybanthus* species by Kelly et al. (1975) indicated that nickel was present both as the ion and also as a low molecular weight complex. Farago and Pitt (1977) have identified a zinc-galacturonic acid complex in *Polycarpaea glabra*.

7.4 Methods Employed and their Limitations

The methods used for investigating soluble heavy metal complexes are thorough cleaning of fresh samples followed by extraction of the fresh or dried material. This is normally followed by initial purification stages including adsorption chromatography, ion exchange chromatography, gel filtration and solvent extractions. Final identification requires the use of paper chromatography, thin layer chromatography, electrophoresis and ideally comparison with synthesised complexes.

The inherent problems involved in this work are indeed formidable. The possibility of formation of artefacts is great since the destruction of cellular integrity brings about an entirely artificial situation. The constituents and pH's of various parts of the plant are subtly different. During analyses the plant extract can undergo drastic changes in the forms of frequent dilution and concentration, warming and cooling, and irradiation. Heavy metals are known to complex strongly with many plant constituents (Mills, 1956) so that weak kinetically labile complexes, in a delicately balanced environment, would inevitably become dissociated in the extract and hence form new more stable complexes. The likelihood of weak complexes existing at all is, however, not very great, because in moving through the plant the metals in question would undoubtedly encounter many strong chelating agents.

Purification techniques are liable to be rather harsh. Silica gel, alumina and ion-exchange colums are all notorious for dissociating even fairly strong complexes. Cellulose although milder can still cause dissociation of weak complexes. Gel filtration, however, appears not to create too much cause for concern in these respects. Obviously problems can be minimised by carrying out all work at the lowest temperatures possible and by concentrating solutions where necessary by freeze drying. Final

identification of complexes with paper chromatography and thin layer chromatography can cause complex fission in three ways: the adsorption process itself could cause dissociation, the pH of the system used might be one at which the complex is unstable, or the solvent may react with the complex. It would therefore seem highly likely that only stable complexes would be discovered using these methods.

7.5 The Chromatographic Analysis of Water Soluble Complexes of *A. Maritima*

Detection reagents

1-(2-pyridylazo)-2-napthol (P.A.N.),5-(o-carboxyphenyl)-1-(2-hydroxy-5-sulphophenyl)-3-phenylformazan (Zincon), biscyclohexanone-oxalydihydrazone (Cuprazone), diethyldithiocarbamate and 8-hydroxyquinoline were all considered as reagents for the detection of copper. The last two were discarded because the nature of the colours produced with copper made them difficult to observe. The yellow colours given by these reagents combining with copper could easily be confused with the yellow/brown streaks produced in the chromatography of plant water extracts. Zincon, although very sensitive, produces similar colours for both copper and zinc and was discounted since zinc was present in the extract in significant quantities. Cuprazone was specific for copper but not very sensitive. However, P.A.N. was very sensitive and further distinguishes between copper and zinc giving a mauve colour for copper and a pink colour for zinc against an orange background. Unless otherwise stated P.A.N. was used for detecting copper in the water extracts of *A. maritima* root stocks.

Preparation of extract for chromatography

Armeria maritima ssp. maritima root stocks were cleaned, dried and powdered as previously described. 2 g of this powdered material was shaken for 6 hours at room temperature with 200 cm^3 of demineralised water, in a stoppered 500 cm^3 quickfit conical flask. The resultant solution was filtered

using a "millipore" filter and the root material further extracted twice as above and the liquors pooled. The water extract was freeze-dried and stored in a fridge in an airtight container, until required. Immediately prior to chromatography the extract was taken up in a minimum of demineralised water and used as described.

Chromatography

Preliminary one-dimensional chromatography to decide which methods and solvents would be most useful, was carried out on 300 x 300 mm Whatman No.1 chromatography paper in Shandon Glass Tanks. Spots of concentrated water extract and copper ions were applied to the paper and developed in the ascending mode. The following three solvent systems, commonly utilised in natural product investigations, were employed:

(i) phenol-water, 4:1, w/v;

(ii) butanol-pyridine-water, 1:1:1, v/v/v;

(iii) methylethylketone-conc. hydrochloric acid-water, 25:3:2, v/v/v.

The chromatographic results are presented in Fig. 40.

From the results it would seem that decomposition of the complex was taking place both in strongly acidic and basic media but not in a nearly neutral media. Phenol-water, 4:1, w/v was therefore selected as a solvent system for this complex.

A second solvent system was required to confirm the results produced using the above method. The search for this was begun using cellulose MN300 polythene thin layer sheets in thin layer chromatography glass tanks in the hope of speeding up the investigation. Spots of the extract and copper ions were applied to the sheets and the chromatograms developed in the ascending mode. The solvent systems used were:

(i) acetone-water, 1:1, v/v;

(ii) butanol-ethanoic acid-water, 12:3:10, v/v/v upper layer.

Ascending chromatography of *A. maritima* root extract using 300 x 300 mm Whatman No.1 chromatography paper.

Spots Applied	Detection Reagents Used	R_F values in various solvent system		
		phenol-water, 4:1, w/v	butanol-pyridine-water, 1:1:1, v/v/v	methylethylketone-HCl-water, 25:3:2 v/v/v
Copper ions	P.A.N.	0	63	73
Extract	P.A.N.	93	63	73
Extract	Zincon	92	63	72

Figure 40

Ascending chromatography of *A. maritima* root extract using MN300 cellulose thin layer chromatography sheets .

Spots Applied	Detection Reagents Used	R_F values in n-propanol-water, 1:1, v/v
Copper ions	P.A.N.	75 comet tail to origin →
Extract	P.A.N.	37
Extract	Ninhydrin	55 ←streak→ 35 ←streak→ 21

Figure 41

Both of these systems were rejected since dissociation of the copper complex occurred.

Another system, n-propanol-water, 1:1, v/v, gave better results as presented in Fig. 41 and was selected as a second solvent system. To ascertain the nature of the complexing agent a duplicate was sprayed with ninhydrin and heated with a hairdrier to develop the colours produced. The main spots were found at R_F 55, 35 and 21, but were not distinct since they were linked by ninhydrin positive streaks. The central spot was shaped similarly to, and had the same R_F value as copper in the former chromatogram.

There was a likelihood of a ninhydrin positive ligand being the complexing group. A further phenol-water paper chromatogram was run to test this hypothesis. The results are presented in Fig.42 with tentative assignations of the spots.

At this stage it seemed probable that either an amino acid or peptide might be involved. To find out which grouping was involved the removal of uncomplexed ninhydrin positive compounds was necessary. This was done by streaking concentrated extracts on to five sheets of Whatman No.3 MM chromatography paper and developing in the ascending mode with phenol-water, 4:1, w/v as the solvent. To isolate the complex, strips were cut off along the edges of the papers and sprayed with P.A.N. Three bands were identified: band 3, $R_F = 93 \pm 5$, band 2, $R_F = 63 \pm 1$, and band 1, $R_F = 46 \pm 1$. These bands were cut out and eluted with demineralised water in the descending mode . Finally the eluants were freeze dried.

The eluants from bands 1, 2, and 3 were rechromatographed on Whatman No.1 chromatography paper as above and the results presented in Fig.43. It appears that bands 2 and 3 are due to zinc complexes and band 1 is composed of a copper and a zinc complex. This was confirmed by using dithizone with masking agents. The relative proportion of zinc to copper was 4:10 as found

Chromatography of *A. maritima* root extract in phenol-water, 4:1, w/v on Whatman No.1 paper 300 x 300 mm.

Reagent Used	R_F Value of Spot	Colour of Spot	Assignation
P.A.N.	92, 62 (faint)	Purple, pink	Copper, zinc
Ninhydrin	91	Grey-violet	Phenylalanine
"	88	Yellow	Proline
"	78	Violet	Valine
"	70	Violet	Arginine
"	57	Violet	Glutamine
"	39	Violet	Asparagine
"	34	Violet	Lysine
"	26	Violet	Glutamic acid
"	16	Blue-violet	Aspartic acid

Figure 42

Chromatography bulk separation of *A. maritima* root water extract in phenol-water, 4:1, w/v on Whatman No.3 MM paper, 300 x 300 mm.

Band No.	R_F Indicated by P.A.N.	Colour with P.A.N.	R_F Indicated by Dithizone	Colour with Dithizone
1	92 ± 6	Purple	95, 85	Grey, pink
2	57	Pink	60	Pink
3	49	Pink	50	Pink

Figure 43

by atomic absorption spectroscopy of the water extract.

Amino acid analysis

If an amino acid was indeed complexing with copper, an amino acid analysis of the extract would provide information on the most likely acids to be involved. A complete amino acid analysis of the root extract was undertaken. A commercial amino acid analyser was used, operating on the principle of liquid chromatography of the extract and subsequent spectroscopic ninhydrin determinations of the separated components. Prior to analysis water soluble proteins were removed using Sevag's method (Sevag, Lackman and Smolens, 1938) to simplify the results. Extraction studies had indicated that little copper was linked with water soluble proteins. The results of the analysis are presented in Fig. 47.

Chromatography

The concentration of amino acids present in the concentrated water extract used for the chromatographic investigations was approximately 20 mM and that of copper approximately 1 mM. Given these facts and also that one copper complex had been observed, only the amino acids accounting for approximately 10% or more of the total could possibly be involved in complexation. Assuming 2:1 amino acid to copper stoichiometry.

In the light of these findings it is hardly surprising that copper was detected far from the origin on chromatography of root extract with phenol-water as the solvent, because copper ions and amino acids form very stable compounds (Albert, 1950) (Mills, 1956).

To find out which amino acids, if any, were involved, marker solutions of the ten most plentiful amino acids present in the extract and copper complexes of these, were chromatographed on 300 x 300 mm Whatman No.1 chromatography paper in the ascending mode. The complexes were made by mixing two volumes of 0.01 M amino acid to one volume of 0.01 M copper(II)

Chromatograph of copper-amino acid complexes and amino acids in phenol-water, 4:1, w/v on Whatman No.1 300 x 300 mm chromatography paper.

Amino acid	R_F of free acid with Ninhydrin	Colour with Ninhydrin	Copper complex of amino acid	
			R_F with P.A.N.	R_F with Ninhydrin
Arginine	62	Violet	56 →79 streaks	70
Asparagine	42	Orange-brown	54	46
Aspartic acid	18	Blue-violet	21	19
Glutamic acid	32	Violet	33	30
Glutamine	59	Violet	78	80,58
Lysine	48,32	Violet	28 →58 streaks	27 →53 streaks
Phenylalanine	87	Grey-violet	98	98,85
Proline	91	Yellow	97	97
Threonine	46	Violet	79	79,47
Valine	80	Violet	96	97

Figure 44

A comparison of the chromatographic behaviour in phenol-water, 4:1, w/v of the water extract with copper-proline and copper proline:proline mixtures on Whatman No.1 chromatography paper.

Spot applied	R_F value with P.A.N.	R_F value with Isatin
Extract	93 ± 1	90 ± 3
Proline	-	91 ± 2
Copper-proline (i)	95 ± 3	93 ± 4
(ii)	96 ± 3	93 ± 5
Copper-proline:proline, 1:10	93 ± 1	90 ± 3
Copper-proline:proline, 1:20	93 ± 1	90 ± 3

Figure 45

chloride solution. The results are presented in Fig. 44.

The only copper-amino acid complexes that ran far enough up the paper to bear comparison with the water extracted complex were copper-proline, copper-valine and copper-phenylalanine. Of these only proline is present in significant quantity in the water extract to be a possible chelating agent for the copper.

The presence of free amino acids and possibly other plants products were probably responsible for the diffuse nature of the complex spot. To provide positive identification of the complex, further purification of the extract was required. A weak cation exchange resin, Zeolit 225, was used in an attempt to remove plant acids from the extract but unfortunately the complex dissociated during this treatment. Sephadex G25 gel filtration using water as the eluant was followed, after reconcentration, by another gel filtration with phenol-water, 4:1 w/v. Subsequent results indicated that the complex had not dissociated. The solvent was removed by drying in a stream of air and the residue taken up in a minimum of water.

Chromatography of the purified extract indicated that the complex was associated with proline, but the complex had an R_F value below that expected for the copper-proline complex. It seemed quite likely that the behaviour of the complex might depend on the quantity of uncomplexed proline present. To test this hypothesis mixtures of copper-proline and proline were run alongside the purified extract, copper-proline and proline. The results in Fig. 45 indicated that this hypothesis was correct.

Final proof was provided by chromatographing copper-proline and proline mixtures alongside the water extract in n-propanol-water, 1:1, v/v, and again in phenol-water, 4:1, w/v. The results in Fig. 46 clearly demonstrate that the purified extract is a mixture of proline and a copper-proline complex.

A comparison of the chromatographic behaviour in phenol-water, 4:1, w/v, and n-propanol-water, 1:1, v/v of the water extract with copper-proline and proline mixtures on Whatman No.1 300 x 300 chromatography paper.

Spot applied	Solvent System		
	Phenol-water, 4:1	n-propanol-water, 1:1	
	R_F with P.A.N.	R_F with P.A.N	R_F with Isatin
Proline:Cu-proline, 2.5:1	96	71	71
Proline:Cu-proline, 5:1	95	70	70
Proline:Cu-proline, 10:1	94	62	62
Extract	94	64	64
Proline	-	-	61
Cu^{++} ions	0	0 $\xrightarrow{\text{streak}}$ 18	

Figure 46

7.6 Amino Acids in Plants

The discovery of an amino acid-copper complex in the previous section prompted a more detailed study of the free amino acid content of *Armeria* roots. The surprisingly high level of proline in the roots of copper tolerant *A. maritima* raised two important questions. Are high proline levels always found in tolerant plants and are they a direct response to copper stress? Since free amino acid pools vary considerably according to the age and stage of growth of the plant and the time of year, direct comparisons between plants could only be made if they were grown under carefully controlled growth conditions.

Amino acids may be divided into two groups; members of one group are found in all living matter either in the free state or combined in the form of proteins or peptides while the second group appear only in certain

Amino acid analysis of root extract of copper tolerant *A. maritima*.

Amino acids	Quantity of amino acid present in 5 mg of extract (μM)	Percentage of total ninhydrin +ve material (%)
Alanine	0.0147	1.08
Arginine	0.0885	6.50
Asparagine	0.1471	10.80
Aspartic acid	0.0354	2.60
Cysteic acid	<0.001	-
Cystine	<0.001	-
Glutamic acid	0.1049	7.70
Glutamine	0.1049	7.70
Glycine	<0.001	-
Histidine	0.0128	0.94
Isoleucine	0.0064	0.47
Leucine	0.0057	0.42
Lysine	0.0268	1.96
Methionine	<0.001	-
Methionine sulphone	<0.001	-
Phenylalanine	0.0184	1.35
Proline	0.5509	40.45
Serine	0.0158	1.16
Threonine	0.0346	2.54
Tyrosine	0.0079	0.58
Valine	0.0214	1.57
Ammonia)	0.1011	7.42
Unknown 1)	0.0096	0.70
Unknown 2)	0.0232	1.70
Unknown 3)*	<0.001	-
Unknown 4)	0.0013	0.09
Unknown 5)	0.0307	2.25

1 g of root material yielded 0.24 g of water extractable material

* other ninhydrin positive substances.

Figure 47

organisms and not as building blocks for proteins.

There are 24 amino acids which comprise the monomeric units of proteins. They are listed in Fig. 47 with the exception of cysteine, methionine sulphoxide and tryptophan. They are all described as alpha amino acids, that is in each molecule the amino and carboxylic groups are linked to the same carbon atom, and all but glycine are optically active usually occurring in the L-form.

The other group consists of non alpha amino acids and members have a scattered distribution in higher plants. 4-Aminobutyric acid is a common constituent of most plants and pipecolic, 3,4-dihydroxyglutamic and 2-amino-adipic acids all have wide distributions.

There are numerous others which are often limited to a single species or a family. Qualitative and quantitative amino acid determinations may be carried out using chromatography with ion exchange resins.

7.7 Amino Acid Analyses

Method

The system used was a LKB 4101 single column analyser. The column was 35 cm long with 6 mm diameter and was packed with Ultrapac II, a sulphonated polystyrene cation exchange resin. Before use the column was regenerated with 0.4 M sodium hydroxide for 10 minutes and equilibrated with a pH 3.25 buffer for 45 minutes.

Samples were introduced at the top of the column and eluted by three separate buffers. The buffers consisted of different amounts of sodium citrate and hydrochloric acid producing solutions of pH 3.25 (buffer A), 4.25 (buffer B) and 6.45 (buffer C). Elution of the sample with these buffers brought about the consecutive removal of acidic, neutral, aromatic and basic amino acids. Buffers A, B and C were passed for 10, 41, and 65 minutes respectively. The buffers also contained traces of thiodiglycol to prevent oxidation and phenol and an antibacterial agent. The buffer then flowed through a photocell at a

rate of 50 cm^3 per hour, through which ninhydrin also flowed at a rate of 25 cm^3 per hour. The absorbance of the mixture in the cell was measured at 440 and 570 nm. The resulting series of absorbance peaks was checked against those made by a standard mixture of common amino acids all present in 25 nanomole amounts.

Sample preparation

Finely powdered *A. maritima* root material (approximately 30 mg) was shaken in 50 cm^3 of distilled water for 3 hours. The extract was filtered and treated with trichloromethane and pentan-1-ol to precipitate proteins (see page 129). The procedure was repeated and the resulting solution freeze-dried. The residual material was redissolved in 4 cm^3 of water prior to analysis.

1.1 cm^3 Of the sample solution was added to 0.2 cm^3 of aqueous norleucine as an internal standard and this solution was then divided into two equal parts. The first was made up to 3 cm^3 with a pH 2.2 citrate buffer and 0.5 cm^3 of this solution was introduced on to the column for analysis. The second portion was hydrolysed with 2 M hydrochloric acid at 110°C for 5 hours, evaporated and dissolved in 3 cm^3 of the citrate buffer. 0.5 cm^3 of this solution was also used in amino acid determination. Both hydrolysed and non-hydrolysed samples contained 16.7 nanomoles of norleucine.

Results and Discussions

Amino acid analyses were carried out on root extracts of tolerant and non-tolerant plants grown at various levels of copper nutrition and the results are presented in Fig. 48. In two cases insufficient material was available so two extracts were combined. In each extract the most common protein amino acids were found in large quantities, namely: alanine, arginine, glutamine, glutamic acid, asparagine and aspartic acid. Particularly noteworthy is the relatively high proportion of proline present in all the

Figure 48. Amino acid composition of various *A. maritima* root extract.

Free amino acids in aqueous root extract	Percentage of total amino acids present				
	Tolerant *A.maritima* Level of copper nutrition			Non-tolerant *A.maritima* Level of copper nutrition	
	0.0 ppm	1.75 & 2.00 ppm	3.0 & 5.0 ppm	0.0 ppm	0.1 ppm
Alanine	7.8	5.0	10.4	13.1	15.3
4-aminobutyric acid	5.0	7.9	7.5	7.4	4.7
2-amino-2-methylpropionic acid	1.3	1.1	1.2	2.1	0.8
Arginine	6.6	6.0	8.2	13.7	8.7
Asparagine	5.1	10.2	3.5	4.0	7.3
Aspartic acid	4.6	6.0	11.3	3.1	5.1
Cystine	0.3	1.0	-	0.4	0.7
Glutamic acid	6.1	6.2	3.0	18.1	9.9
Glutamine	18.2	16.4	18.5	13.3	21.1
Glycine	3.0	1.0	3.0	2.4	1.2
Histidine	3.4	1.8	3.2	2.2	1.6
Isoleucine	1.8	3.1	2.3	2.4	1.8
Leucine	3.3	2.8	1.9	1.7	1.4
Lysine	3.4	2.2	2.6	3.3	1.7
Phenylalanine	3.4	1.5	1.0	0.1	0.9
Proline	11.4	10.4	10.6	1.5	3.1
Serine	4.1	5.2	4.5	1.4	2.9
Threonine	1.5	2.6	2.6	1.1	2.0
Tyrosine	1.0	1.1	1.0	0.4	0.6
Valine	6.4	4.4	2.3	4.2	3.3
Unknown	2.3	4.1	1.4	4.1	5.9

tolerant extracts. Proline is one of the less common plant protein components and as such is present here in surprisingly large amounts.

Although statistical evidence is lacking, it would seem that the high levels of proline present in tolerant plants are not produced as a response to copper stress. The non-tolerant plants on the contrary have much lower levels of proline. This result, together with the identification of a copper-proline complex and the enormously high level of proline found in a mature sample of tolerant *A. maritima* root from Dolfrwynog bog, reinforced the belief that proline is produced by tolerant plants as a chelating agent functioning as part of a complex tolerance mechanism.

Proline would seem to be a good candidate for the rôle of a strong complexing agent for copper. An indication of its relative complexing ability is found by comparing the log stability constant of the bisprolinato copper(II) dihydrate complex with those of other copper complexes (Fig. 49).

Figure 49. Relative stabilities of copper complexes of various plant products.

Class	Compound	Ionic Strength mol litre^{-1}	Log Stability Constant	Notes
Amino acids	Glycine	0.2	15.0	
	L-alanine	0.1	14.9	
	L-serine	0.1	14.5	
	L-cysteine	-	-	Reduction of Cu^{2+} by ligand
	L-cystine	0.15	13.7	Isomerisation not stated
	L-threonine	0.1	14.7	
	L-valine	0.1	14.9	
	L-methionine	0.1	14.7	
	L-leucine	0.2	14.9	$20^{o}C$
	L-isoleucine	0.5	15.4	
	L-proline	0.1	16.4	
	L-phenylalanine	0.05	14.8	
	L-tyrosine (H_2L)	0.1	14.7	$M(HL)_2/M.(HL)^2$
	L-tryptophan	0.37	15.5	$20^{o}C$
	L-asparagine	0.1	14.4	

Figure 49 continued

Class	Compound	Ionic Strength mol $litre^{-1}$	Log Stability Constant	Notes
Amino acids	L-glutamine	0.1	14.2	
	L-aspartic acid	0.1	15.4	$30^{o}C$, DL mixture
	L-glutamic acid	0.1	14.2	
	L-arginine (H_2L^+)	0.1	14.6	$M(HL)_2/M.(HL)^2$
	L-lysine	0.1	14.0	$20^{o}C$
	L-histidine	0.1	18.1	
	β-alanine	0.1	12.5	
	L-hydroxyproline	0.1	15.4	Isomerism not stated
Organic acids	Quinic	0.05	2.4	ML/M.L
	Oxalic	0.1	9.2	
	D-tartaric	1.0	8.2	$M_2L_2/M^2.L^2$
	Malonic	0.1	7.8	
	β-hydroxybutanoic	2.0	2.9	Isomerism not stated
Aromatic compounds	Protocatechuic acid	0.1	22.6	$30^{o}C$, also ML/M.L, 12.8
Flavonoids	Quercetin	0.1	12.8	Medium 40% v/v ethanol

Figure 49 continued

Class	Compound	Ionic Strength mol litre^{-1}	Log Stability Constant	Notes
Nucleic acids and derivatives	Purine	0.05	11.5	Does not occur free
	Adenine	0.05	13.3	
	Riboflavin	0.1	6.5	$35^{o}C, K_1$
	Riboflavin monophosphate	0.1	8.3	$35^{o}C, K_1$
	Cytosine	0.05	2.7	
Amines				Not available
Miscellaneous N & S compounds	Nicotinic acid	0.5	3.5	$20^{o}C, K_1$
Peptides	Glycyl-L-proline	0.16	11.4	As model
	Triglycine	1.0	9.7	As model
	Glycyl-DL-histidylglycine	3.0	19.9	As model
Alkaloids				Not available
Porphyrins				Not available

Unless otherwise stated log stability constants refer to the equilibrium $M^{2+} + 2L^{-} \rightleftharpoons ML_2$ at $25^{o}C$. Data sources were (i) Critical stability constants, Vol.1 Amino acids (1974) compiled by Martell, A.E. and Smith, R.M., Plenum Press, New York. (ii) Stability constants of metal-ion complexes, Part B, Organic ligands (1979) compiled by Perrin, D.D., Pergamon Press, Oxford.

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CHAPTER VIII

THE STATUS OF THE COPPER AVAILABLE TO *A. MARITIMA* FROM DOLFRYWNOG BOG

8.1 Metal Forms in Soil Solution

Dykeman and De Sousa (1966) reported that a bog which contained as much as 7% copper supported a normal vegetation with no ill effects. This they considered to be due to the immobilization of the copper into forms unavailable to the plants. The immobilization was brought about by chelation with organic substances. Other studies have shown that heavy metals are complexed by organic material and as such become less available to plants (Lucas, 1948) (Miller and Ohlrogge, 1958).

In peat bogs organic material behaves as an ion-exchange medium. The ion-exchange capacity depends on the ion under consideration and in the case of copper and other heavy metal ions partly covalent bonded complexes are involved. This capacity is thought to be due to carboxyl groups on humic and fulvic acids and at higher pH's to phenolic groups (Given, 1975).

In the soil solution copper may occur in soluble, solid or colloidal forms. Particulate copper may be present as the sulphide, oxide or basic copper carbonate. It may also be present in an adsorbed form on organic or clay particles or as insoluble complexes. Clays, hydroxides and polypeptide complexes may also occur as colloids. The soluble forms include the aqueous copper ion, a soluble copper carbonate complex and other soluble complexes.

Stiff (1971) investigated forms of soluble copper in model systems as well as from filtered environmental samples. Forms predominant in the presence of peat were the copper carbonate complex, amino acid/polypeptide or similar labile complexes and inert humic complexes.

Hodgson and co-workers carried out a series of studies into the forms of copper and zinc in the soil solutions. Hodgson *et al.*(1965) found that

for copper as much as 99% may exist in a complexed form. Work on twenty calcareous soils showed that 98-99% of the copper was complexed but that rarely was more than 75% of the zinc present in complexed forms. Complexing of copper by water or carbonate was not included in the above figure and was thought to make up the bulk of the remainder (Hodgson *et al.*,1966).

Complexing of copper was directly related to the soluble organic fraction and not the total level of organic matter in the soil. The soluble organic fraction itself not being related to the total organic matter (Hodgson *et al.*,1966). Gangwar and Vithal (1971) also could find no link between extractable copper and total organic matter.

In an attempt to characterize some of these ligands Geering and Hodgson (1969) found at least three groups of complexing agents for zinc and copper in a silt loam. The dialyzable ligands were thought to be aliphatic and amino acids. The average metal-ligand ratios were 1:1 in all cases. The estimated log stability constants of the non-dialyzable complexes were 4.3 and 5.5 for zinc and copper respectively.

Coleman et al. (1956) also determined the log stability constant for a copper-peat ligand complex finding it to be 6.5.

Uptake of copper and nickel from peat soil by crop species was found by Roth et al. (1971) to be independent of the form of the metal present (water extractable, potassium chloride extractable and residual). It was concluded that if water soluble metal is the source of supply to plants the other reserves must maintain a constant supply.

Bloomfield and co-workers studied the anaerobic and aerobic mobilization of trace elements from their oxides using decomposing plant material. The aerobic study (Bloomfield et al.,1971) showed that the initial rapid mobilization of copper was due to the formation of cuprammino complexes and that humic materials were found in solution. The metals were thought to have

dissolved initially into true solution but subsequently to have combined with insoluble organic residues and colloidal humic materials.

In peat and other organic rich soils high levels of micronutrients may be immobilized whilst at the same time organic ligands contribute to metal mobility. The resulting complexes being more or less available to plants.

8.2 Experimental Determination of the Nature and Stability of the Copper Species present in Bog Solution

Introduction

Two experiments were carried out on bog material from Dolfrwynog in an attempt to determine the nature of the complex formed between copper and organic ligands. The first experiment investigated the influence of the bog extract on the removal of copper from other soils. The second gave an indication of the thermodynamic stability of the complex or complexes formed by Cu^{2+} and organic substances in the extract. The methods were similar to those of Miller and Ohlrogge (1958).

Preparation of extract

Two separate bog samples were collected, care being taken to collect both material and attendant surface liquid. One sample was a light brown bog material (A) and the other a blackish brown bog material (B). In both cases the bog material consisted mainly of decaying plant material (largely *Armeria maritima* as this is the dominant species in the bog) and some loam.

The bog samples were subsequently buchner filtered. The filtrates were found to contain 0.7 ppm of copper in filtrate (A) and 0.1 ppm of copper in filtrate (B). Since bog sample (A) appeared to have a higher chelatogenic capacity it was used in subsequent investigations.

The bog filtrate plus washings had a total volume of 1 100 cm^3 and a pH value of 7.5 as opposed to 5.5 when measured in situ. This change is

almost certainly due to the loss of carbon dioxide caused by vacuum filtration. The filtrate was concentrated on a rotary evaporator. The dried bog material, 70.3 g, was soxhlet extracted at between 70°C and 80°C for 5 hours. The concentrated filtrate and the extract were combined producing a total volume of 820 cm^3 of bog extract. This was used in the following experiments as the 100% bog extract.

Preparation of soils

A Regent's Park loam (A) and a Sonning Common clay loam (B) were used for this investigation. Neither soils having a high organic content.

Both soils were dried at 40°C and powdered in a Glen Creston ball mill. They were then sifted through a sieve allowing a maximum particle size of 306 μm. Copper was added to both soils at the level of 400 μ moles of copper ions per gramme of soil. In both cases 20 g of soil was placed in a 500 cm^3 conical flask with the requisite quantity of copper ions as solid analar $CuSO_4.5H_2O$. To this was added 250 cm^3 of pure water. The flasks were stoppered and shaken at room temperature for three days at 6 hours per day.

The soils were filtered and washed several times with 2 M KCl solution until no more copper was removed. After numerous washings a low constant level of copper was being leached each time, this was taken into account in the subsequent calculations. Excess KCl was removed by washing with pure water. The amounts of copper fixed in the soils were determined by subtraction of the levels removed from the original quantity applied (the actual levels in the soil being negligible in comparison). Soil sample (A) contained 210 mg of copper ions and soil sample (B) contained 195 mg of copper ions.

Removal of copper from soils

In order to find the capacity of the extract for removing copper from soils, 2 g samples of soil were placed in plastic vials, to these were added

10 cm^3 of 1.0 M KCl solution to maintain constant ionic strength and 10 cm^3 of extract solution suitably diluted. These suspensions were shaken in a mechanical shaker for three hours a day for three days. The quantities of copper removed in each case were determined by direct Atomic Absorption analysis, a blank was also run to allow for copper leaching from the soils.

Determination of the nature of complex formation using resin exchange equilibria

The nature of a complex formed between copper and the organic chelating agent(s) was determined by using the principle of resin exchange equilibria. The method depends on the quantity of metal bound to a known weight of resin at equilibrium being proportional to the concentration of free ions in solution. The stability constant of the complex or complexes was determined using;

$$\log_{10}(\lambda_o/\lambda - 1) = \log_{10}K + x\log_{10}(Ch)$$

λ_o = distribution constant in absence of chelating agent;

λ = distribution coefficient in presence of chelating agent;

log K = stability constant;

x - number of moles of chelating agent combining with 1 mole of the metal;

(Ch) = concentration of chelating agent in moles.

The quantities λ_0 and λ are the values by which the concentration of free plus complex ions in solution must be multiplied by to obtain the concentration of the ion bound to a definite amount of resin at equilibrium.

The distribution of metal between the resin and the solution was determined by placing 1 g of Zerolit 225, SRC15 $R\text{-}SO_3\text{-}H^+$ type cation exchange resin, which had been acid and base washed and finally saturated with potassium ions, in a plastic vial and adding 10 cm^3 of test solution plus 10 cm^3 of 1.0 M KCl to maintain constant ionic strength. All solutions had a pH value of 6.0

Removal of copper from Cu^{2+}/bog extract mixture by cation exchange resin at pH 6.0 (Total Cu, 3.0 ppm).

Dilution of extract %	Copper left in solution ppm	Copper removed from solution ppm	λ	$\lambda_o/\lambda - 1$	$Log_{10}(\lambda_o/\lambda - 1)$	Log_{10} (Extract dilution)	$Log_{10}[Ch]$	$Log_{10}K$
0	0.33	2.67	8.0909 = λ_o					
2	0.43	2.57	5.9767	0.3537	-0.4514	0.3010	-5.6554	6.05
5	0.61	2.39	3.9180	1.0651	0.0274	0.6990	-5.2557	6.07
10	0.85	2.15	2.5294	2.1987	0.3422	1.0000	-4.9547	6.04
25	1.40	1.60	1.1429	6.0793	0.7839	1.3980	-4.5567	6.02
50	1.98	1.02	0.5152	14.7044	1.1675	1.6990	-4.2557	6.06

Figure 50

and a copper content of 3 ppm. The vials were shaken for four hours. The resin was allowed to settle and the supernatant decanted. The amount of copper removed by the resin was calculated, thus the distribution of the copper between the resin and the solution was found for each concentration of the chelating agent. (Fig. 50).

Results

The relationship between the concentration of extract and the amount of copper removed from the soil is seen in Fig. 51.

At the lower extract concentrations similar quantities of copper were removed from both soils and an approximately linear relationship was observed.

Removal of copper from soil complexes must involve an equilibrium process. In which copper is exchanged between soluble and insoluble complexing agents. Such a straight line relationship would only exist if one of the reactants, in this case the chelating agent, was all consumed in the reaction.

The maximum complexing ability was calculated from Figure 51. The 10% extract complexed .61 ppm of copper, therefore, the maximum complexing ability would be 6.1 ppm for the full strength extract.

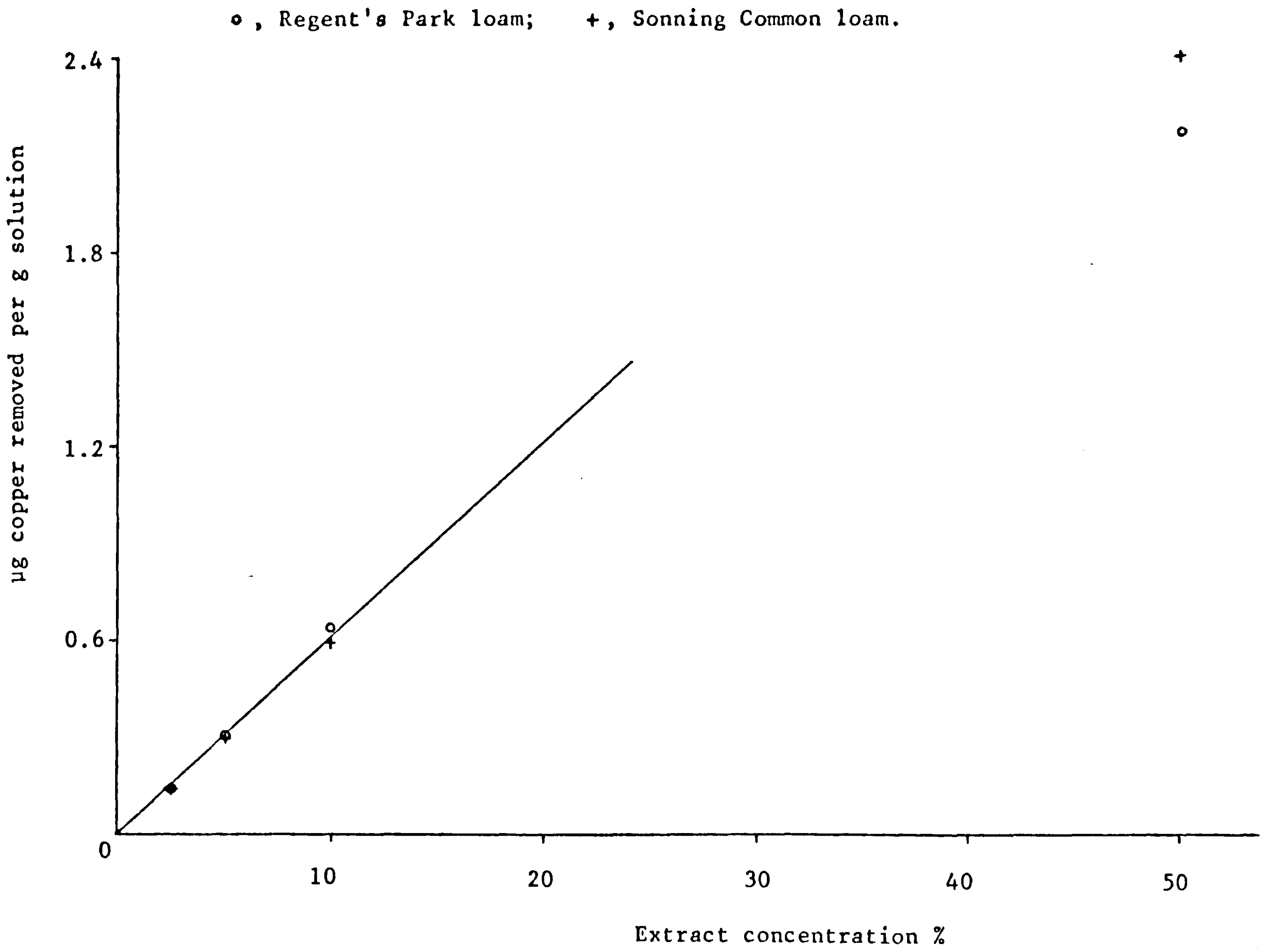
The relationship $\log_{10} (\lambda_0/\lambda-1)$ vs. $\log_{10}(Ch)$ cannot be plotted directly since (Ch) is unknown. If, however, relative values of (Ch) are assumed, by using extract dilution values, x may be obtained.

The concentration of the complexing agent can be calculated from a knowledge of the maximum complexing ability of the extract and the value of x, which is given by the slope of the line in Figure 52 . Thus a value for log K can be determined.

The value of 1.15 for x as taken from the gradient of the slope in Figure 52 indicates that more than one complexing agent is present.

Figure 51. Copper extracted from two soils by bog extract solutions.

o , Regent's Park loam; + , Sonning Common loam.



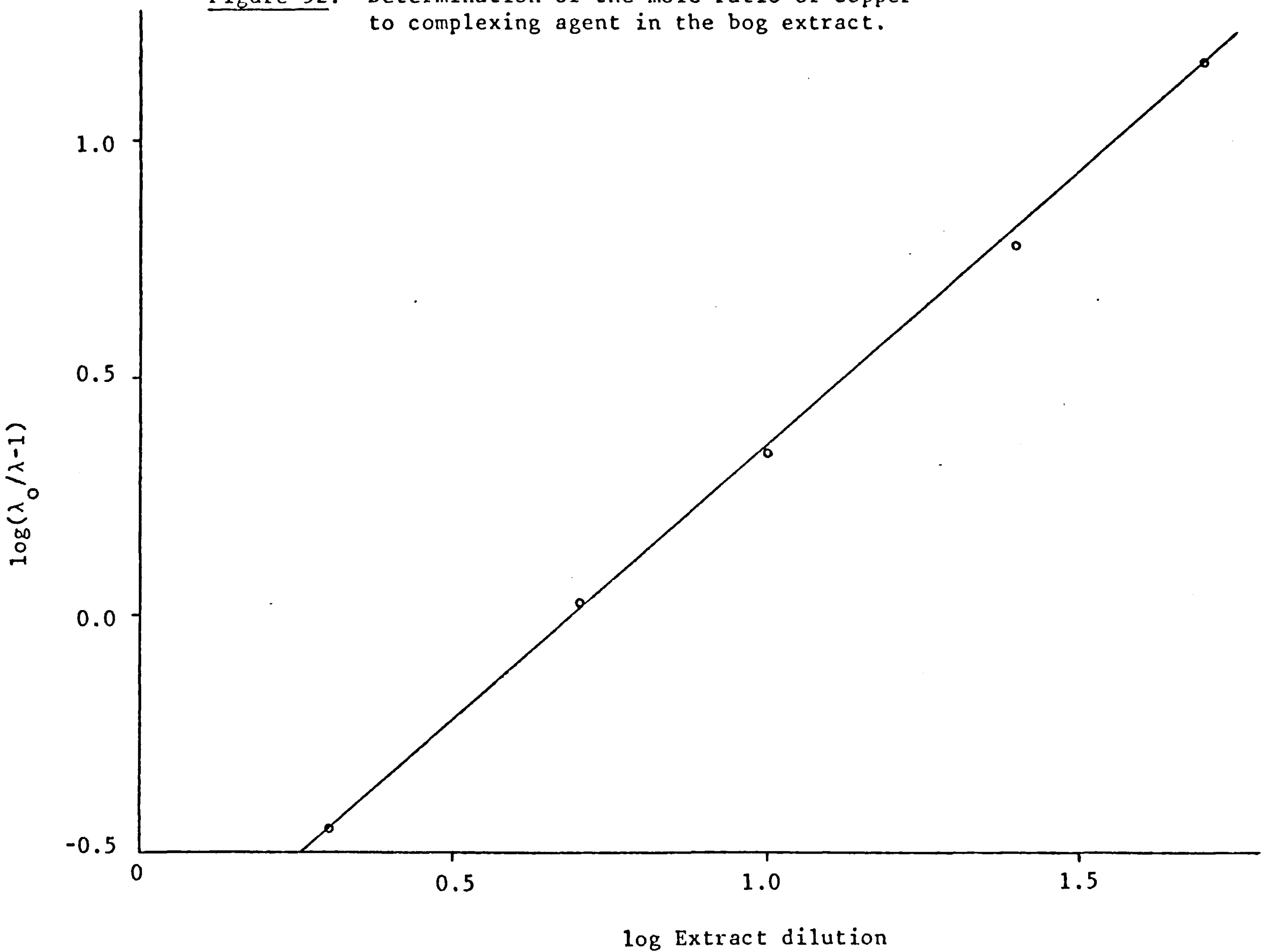
Using the value of 6.1 ppm as the complexing ability of the 100% extract, the concentration of complexing agent(s) in the 100% extract was calculated to be 1.11×10^{-4} mol dm^{-3}. A series of values of log K were calculated using the relationship;

$$\log_{10}K = \log_{10}(\lambda_o/\lambda) - 1.15 \log_{10}(Ch).$$

The average value of the stability constant was found to be 6.0.

It would appear that the copper is available to the plants as various organic complexes with an average stability constant of 6.0.

Figure 52. Determination of the mole ratio of copper to complexing agent in the bog extract.



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CHAPTER IX

THE UPTAKE OF PLATINUM GROUP METALS BY TOMATO, BEAN AND CORN

9.1 Background

The work so far presented in this thesis has been concerned with the uptake and subsequent detoxification processes which occur within the plant. Whilst final deposition sites and a soluble complex have been identified, the labile nature of copper compounds means that the metal may form one or more associations with plant constituents during the life of the plant. It would be of interest to investigate the effect of elements which form non-labile compounds and to see also if the elements when applied to plants in different complexed forms produce similar effects.

The relatively small scale world production of the platinum group metals: platinum, iridium, osmium, palladium, rhodium and ruthenium of 84,989 kg per year (annual average 1961-1972), (West 1972) has been used mainly in the manufacture of electrical and analytical equipment and jewellery, which requires metals with high melting points and low chemical reactivity. In addition to this platinum group metals are exceptionally good catalysts. But unless wastage is small other cheaper types are often used. Two recent developments have served to increase both the interest in and environmental levels of these metals.

As early as 1903 the antibacterial activity of iridium chloride solution was reported (Zinno and Cutolo, 1903). But it was not until 1965 that Rosenberg and co-workers studied the toxic effects of platinum group metals on *E. coli* and more importantly found that platinum species affected normal cell growth producing elongated cells (Rosenberg et al., 1965). Rosenberg et al. (1969), further to this, discovered that certain platinum species showed anti-tumour activity in mice. Following this, extensive trials have resulted in the marketing of chemotheraputic anti-tumour

drugs by American and British drug companies. The drugs based on cis-[$Pt(NH_3)_2Cl_2$] have been shown to be particularly effective against testicular and ovarian cancers. Recent reviews by Rosenberg (1978) and LeRoy (1975) have covered the other chemotheraputic studies on platinum group metals.

The anti-pollution laws of many highly industrialised nations have resulted in the development of various types of platinum group metal containing catalytic CO and NO_x after-burners for automobiles. The amounts of platinum lost from after-burners have been tentatively estimated by Newkirk and Goluba (1975) at 1 g per car. Whilst levels in soils will be low and the forms uncertain, but most likely elemental, the possibility of entry into man's food chain clearly cannot be ruled out. The deleterious effects of platinum group metals on man have long been observed as occupational hazards of the relevant industries (Brubaker *et al.*, 1975). Since the advent of cancer chemotherapy unavoidable clinical side effects have also been reported (Bristol Myers Co. Ltd., 1979) (Williams and Whitehouse, 1979).

The existence of platinum group metal tolerant species has not been reported and indeed is not surprising considering the almost complete unavailability to plants of the metals from ore tailings (Parsons, 1979) (Payne, 1978). Hamner (1942) studied the effects of platinum applied as chloroplatinic(IV) acid on bean and tomato. In the present study his observations have been confirmed and in addition the levels of platinum and calcium in roots and tops are presented. Tso *et al.* (1973) investigated the effects of platinum and palladium on the nicotine content of tobacco plants. But neither of these studies reported plant metal levels. Conversely in other studies by Fuchs and Rose (1974), Kothney (1976) and Sarwar *et al.* (1970) trace levels of platinum group metals have been given for uncultivated species without the occurrence of toxicity. In addition Bumbalek (1977) (1978)

claims the usefulness of banana and citrus fruit in accumulating platinum group metals and indeed other more esoteric elements from soils.

In an independent study carried out at about the same time as that presented here Pallas and Bentom-Jones (1978) investigated the uptake of platinum by crop species. They found evidence of chlorosis and a decrease in dry weight at the 5.7 p.p.m. level. Although increases in dry weight were reported for some species at the 0.057 p.p.m. level the increases were not dramatic and the number of plants in each treatment (2 x 4 replicates) were too few to be statistically significant. With the same reservations a possible growth stimulatory effect has been noted in some species with rhodium applied (this thesis, p.195)(Parson, 1979). All species accumulated significant levels of platinum in their roots but only translocated small or undetectable levels to their tops. Particular attention was paid to water relations which were found to be dependent on the level of platinum applied.

The possibility of cultivating South African grass species which grow around the spoil heaps of the platinum mines at Rustenberg, South Africa, was investigated (Payne, 1978). The plants were grown in spoil heap material with and without added nutrient solution. Growth was very poor without nutrient solution and in both cases significant levels were not detected in roots or shoots. In the apparent absence of indigenous tolerance common crop species were chosen for study. Bean, tomato and corn were treated with platinum, palladium and rhodium and also with various cationic and anionic forms of platinum.

9.2 Uptake of Platinum, Palladium and Rhodium by Tomato

(a) Platinum

Experimental

The experiment was conducted in a controlled environment growth room. 'Eurocross BB' F_1 hybrid tomato seeds were used and germination was

carried out in acid washed Loch Aline sand. Conditions in the growth room were maintained throughout the experiment. These were: 1,000 ft-candles of illumination, a day temperature of 20 ± 1 ^{o}C and a night temperature of 15 ± 1 ^{o}C, a 14 hour day, relative humidity of 65%. The tomatoes were grown hydroponically to provide uncontaminated root samples for analysis and to provide accurately maintained conditions over the whole duration of the experiments.

The seeds were placed in rows on a 355 x 217 mm seed tray of acid washed sand and covered lightly with a further 1 mm layer. The tray was then placed in a trough of demineralized water for a matter of seconds until the sand was moistened. A layer of cotton material lining the tray prevented the sand from being washed away. After any excess moisture had been allowed to drain away the seed tray was transferred to an environmentally controlled growth room.

The tray was covered with polythene sheeting to prevent loss of moisture during the germination period. Germinated seedlings were removed from the tray when the first true leaves became established, by scooping out the individual seedling and the sand around its root with a spatula. The sand was removed by dipping the seedling into a small beaker of demineralized water whilst holding one of the dicotyledons gently between thumb and forefinger. Gentle agitation of the seedling resulted in almost complete removal of sand particles from the root hairs.

The seedlings were selected for uniformity of growth and were transferred to 2 dm^3 plastic boxes which had been made opaque by covering with black adhesive tape. Each container had a tightly fitting lid, painted black. The lids had four symetrically positioned 15 mm holes drilled in them, a fifth smaller hole was made for aeration purposes. The plants were held in position inside a wedge of black polystyrene foam.

Plate 7. Tomato plants before application of platinum to nutrient solutions.

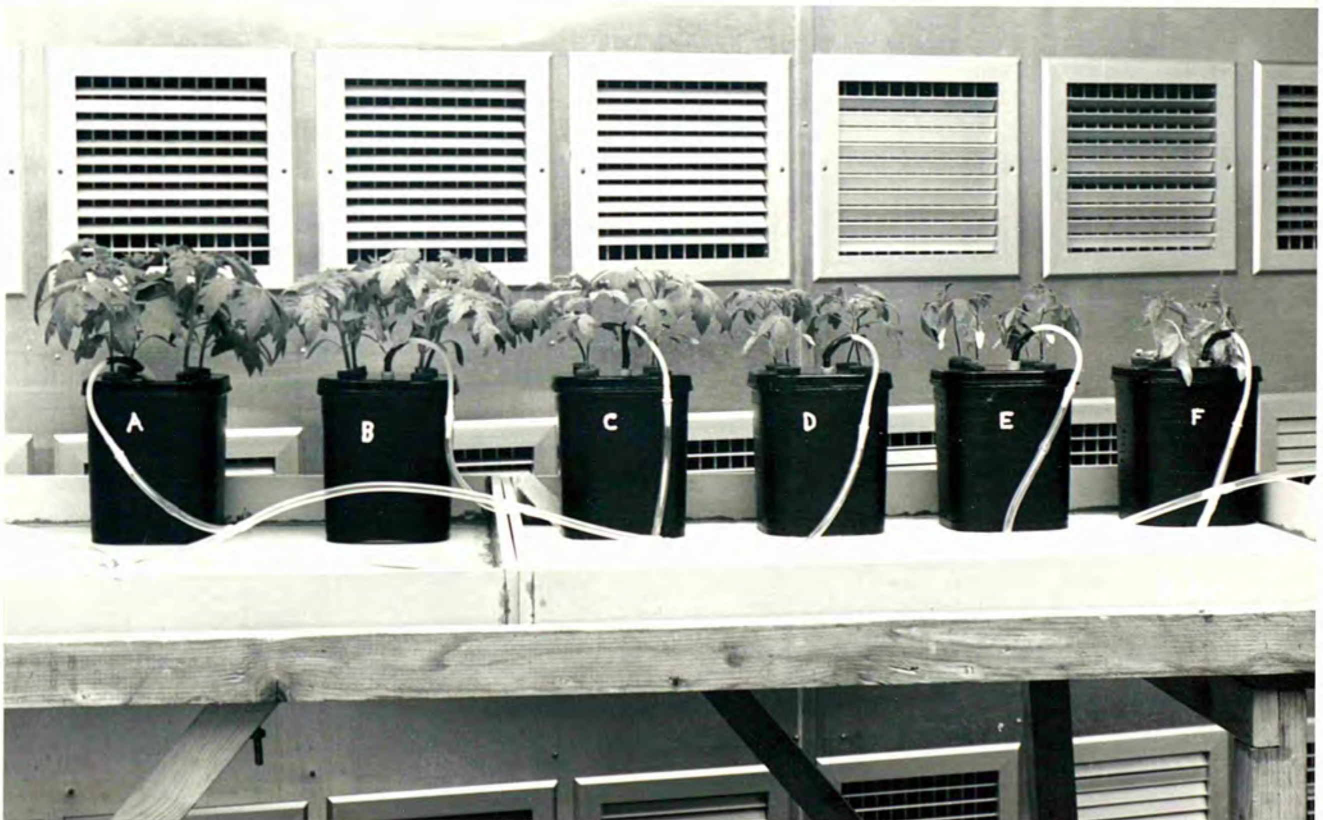

Plate 8. Tomato plants after 10 days of growth in platinum solutions.

These wedges could be lodged inside the holes in the lids. The containers were filled with solution to within 5 mm of these wedges.

Aeration of each solution was brought about by bubbling air through gas distribution tubes 90 mm long, porosity 3, supported by rubber bungs placed in the smaller holes in each of the lids. Each box was supplied with air by polythene tubing attached via a junction piece to a main air line. The air line was ringed for evenness of air pressure and an air leak fitted to allow for adjustment of bubbling rates in the solutions.

Identical half strength nutrient solutions were applied to the six boxes each containing four plants. When the plants were well established the boxes were treated with full strength nutrient solutions and the following concentrations of platinum, in the form of $(NH_4)_2[PtCl_6]$: 0.05, 0.5, 2.5, 5.0, 10.0, 30.0 p.p.m. The composition of the full strength nutrient is given in Fig. 21. Solutions were renewed twice during the experiment.

Results

After three days normal healthy growth was observed at the 0.05 nutrient level. At the 0.5 and 2.5 levels there was evidence of chlorosis and a slowing of growth. Little growth was observed at the 5.0 p.p.m. level, roots were stunted and appeared yellow/brown. The highest levels showed no growth, complete stunting of roots and severe browning. Necrotic spots appeared on the lower leaves. Only one plant was killed at the 30 p.p.m. level.

After 14 days the plants were harvested. Wet weights of the plant tops and roots were taken. They were then dried at 40°C and their dry weights determined. The dried plant material was wet ashed using concentrated nitric acid. After ashing was completed the solutions were taken to dryness and made up as described in Appendix 1. The solutions were analysed for their platinum and calcium contents and the results presented in Fig. 53.

Figure 53.

Yields and analysis of tomato plants treated with $(NH_4)_2[PtCl_6]$.

Tops	A1	B1	C1	D1	E1	F1 *
Applied Pt/ppm	0.05	0.50	2.50	5.00	10.00	30.00
Wet weight/g	63.15	58.91	21.44	7.27	3.53	2.10
Dry weight/g	4.060	3.630	1.473	0.752	0.433	0.265
Weight of water/g	59.09	55.28	19.97	6.52	3.10	1.83
Water present/%	93.6	93.8	93.1	89.7	87.7	87.4
Total Pt/μg	6.0	23	32	10	19	40
Pt (dry wt)/ppm	1.5	6.3	21.7	13.3	43.8	151
Total Ca/mg	105	92	34.5	12.4	7.8	4.3
Ca (dry wt)/%	2.59	2.53	2.34	1.65	1.80	1.62

Roots Box	A1	B1	C1	D1	E1	F1 *
Applied Pt/ppm	0.05	0.50	2.50	5.00	10.00	30.00
Wet weight/g	11.60	11.23	7.00	3.47	1.11	0.24
Dry weight/g	0.720	0.764	0.540	0.318	0.170	0.050
Weight of water/g	10.88	10.47	6.46	3.15	0.94	0.19
Water present/%	93.8	93.2	92.3	90.8	84.7	79.2
Total Pt/μg	12	480	950	1425	1625	850
Pt (dry wt)/ppm	16.7	630	1760	4480	9560	17000
Total Ca/mg	6.5	5.9	4.6	2.1	1.3	0.43
Ca (dry wt)/%	0.90	0.77	0.85	0.66	0.76	0.86

* Data presented here is for 3 plants only.

Photographs were taken before and after platinum application and are presented in plates 7 and 8.

Discussion

With an experiment of this kind the limited growth period gives an indication of how much the final yields and therefore the total amount of platinum uptake will be. Drop off in the yields of both roots and tops of the tomatoes occurs between the 0.5 and 2.5 p.p.m. levels. At the higher levels little or no growth was made after application of platinum. The optimum uptake of platinum occurs between 0.5 and 2.5 p.p.m.

It would seem surprising from these results that such high levels of platinum are taken up by the roots at the 5.0, 10.0 and 30.0 p.p.m. levels when little or no root growth took place. This could be explained by the precipitation of platinum-hydroxy species in the outer free spaces of the roots thus giving rise to a brownish appearance of the roots.

Although the solutions were renewed three times during the course of the experiment the percentages of total platinum removed by the plants were: A1 - 14%, B1 - 29%, C1 - 14%, D1 - 5%, E1 - 3%, F1 - 1%. They are thus reasonably efficient at the removal of platinum from dilute solution.

(b) Palladium and rhodium

Experimental

Apparatus and conditions were as described above. When the tomato plants, two per container, were well established, the boxes were treated with full strength nutrient solutions and the following concentrations of palladium, in the form of $Na_2[PdCl_4]$: 0.05, 0.5, 2.5, 5.0 p.p.m. and rhodium in the form of $Na_3[RhCl_6]$: 0.05, 0.5, 2.5, 5.0 p.p.m.

Results

Two tomato plants were cropped at the start of the experiment as controls. Yields were: tops, wet weight, 3.361 g, dry weight 0.232 g, percentage water 93.1%; roots, wet weight 0.999 g, dry weight 0.072 g, percentage water 92.8%. After 10 days the plants were harvested. All plants

Yields and analysis of tomato plants treated with $Na_2[PdCl_4]$.

Tops	Box	G1	H1	J1	K1
Applied Pd/ppm		0.05	0.50	2.50	5.00
Wet wt/g		36.88	38.50	8.037	7.340
Dry wt/g		2.325	2.317	0.624	0.572
Water present/%		93.7	94.0	92.2	92.2
Total Pd/μg		1.5	2.3	2.4	3.2
Pd (dry wt)/ppm		0.64	0.99	3.8	5.5
Roots	**Box**	**G1**	**H1**	**J1**	**K1**
Applied Pd/ppm		0.05	0.50	2.50	5.00
Wet wt/g		8.515	9.897	1.765	2.142
Dry wt/g		0.607	0.670	0.210	0.184
Water present/%		92.9	93.2	88.2	91.5
Total Pd/μg		40.0	149	525	3170
Pd (dry wt)/ppm		65.9	222	2504	17230

Figure 54

Yields and analysis of tomato plants treated with $Na_3[RhCl_6]$.

Tops	Box	L1 *	M1	N1	P1
Applied Rh/ppm		0.05	0.50	2.50	5.00
Wet wt/g		12.80	34.08	36.85	37.19
Dry wt/g		0.857	2.025	2.309	2.149
Water present/%		93.0	94.0	93.7	94.2
Total Rh/μg		0.75	10.5	46.0	65.0
Rh (dry wt)/ppm		0.87	5.2	19.9	30.3
Roots	**Box**	**L1**	**M1**	**N1**	**P1**
Applied Rh/ppm		0.05	0.50	2.50	5.00
Wet wt/g		4.944	6.554	8.651	7.676
Dry wt/g		0.259	0.418	0.532	0.500
Water present/%		94.8	93.6	93.9	93.5
Total Rh/μg		10.0	142	450	385
Rh (dry wt)/ppm		38.7	339	847	771

* Only one top was used for analysis

Figure 55

gained weight during the experiment, boxes J1 and K1 gaining little. In box L1 normal healthy growth was observed but one plant was damaged due to mishandling. For purposes of analysis both roots but only one top was used. In boxes M1, N1, P1, G1 and H1 normal growth was also observed. In boxes J1 and K1 there was very little growth with new roots developing in the moist air above the solutions. The older roots were dark brown and some light precipitation was observed in solution. The tops were wilted and necrotic. Analyses were carried out as already described and the results presented in Figs. 54 and 55.

Discussion

For palladium drop off in yields occurs between the 0.5 and 2.5 p.p.m. levels as was observed for platinum, but was far more drastic. It appears that there is a clear threshold for palladium toxicity. Levels of total and p.p.m. dry weight palladium in the plants are broadly comparable to those observed for platinum except that much smaller, practically negligible levels were translocated to the tops of the plants.

Rhodium at the levels investigated appeared to have no deleterious effects upon the plants. Sigificant levels of rhodium were translocated whilst high levels were not associated with the roots. The rhodium levels in the plants are similar to those which would be expected for a micronutrient. In subsequent experiments higher levels of rhodium were applied in order to see if toxicity could be established.

9.3. Uptake of Platinum by Bean (*Phaeseolus vulgaris*)

Experimental

The apparatus and conditions were similar to those used for the tomato experiment. The cultivar chosen for the experiment was 'Masterpiece'. The beans were germinated on vertical sheets of moist chromatography paper in glass beakers, this facilitated accurate selection of uniform seedlings. The levels of platinum applied were: 0.5, 0.5, 2.5, 5.0, 10.0, 30.0 p.p.m.

Yields and analysis of bean plants treated with $(NH_4)_2[PtCl_6]$.

Tops	Box	A3	B3	C3	D3	E3	F3
Applied Pt/ppm		0.05	0.50	2.50	5.00	10.00	30.00
Wet wt/g		24.16	15.08	12.03	13.22	7.72	3.61
Dry wt/g		2.214	1.279	1.386	1.578	0.986	0.511
Water present/%		90.8	91.5	88.5	88.1	87.2	85.9
Total Pt/μg		1	5	9	40	28	65
Pt (dry wt)/ppm		0.4	3.9	6.5	25.3	28.4	127
Total Ca/mg		55.0	23.5	21.8	20.5	11.8	4.6
Ca (dry wt)/%		2.48	1.84	1.57	1.30	1.19	0.90

Roots	Box	A3	B3	C3	D3	E3	F3
Applied Pt/ppm		0.05	0.50	2.50	5.00	10.00	30.00
Wet wt/g		8.53	5.40	2.71	2.38	1.63	1.21
Dry wt/g		0.527	0.301	0.336	0.303	0.221	0.121
Water present/%		93.8	94.4	87.6	87.3	86.4	89.9
Total Pt/μg		40	340	880	1650	1400	3100
Pt (dry wt)/ppm		76	1130	2620	5440	6330	25600
Total Ca/mg		2.4	1.7	1.6	1.5	0.9	1.0
Ca (dry wt)/%		0.46	0.56	0.48	0.49	0.41	0.83

Figure 56

Results

After 11 days the plants, 2 per box, were harvested. In boxes A3 and B3 healthy growth was observed. In box C3 slight chlorosis was seen with secondary roots stunted and darkened. In addition to the symptoms observed in box C3, root clubbing with little growth became evident in box D3. Even less growth was made in E3 with clubbing and browning of roots. In box F3 there was severe chlorosis of the first true leaves and no new root growth after the application of the platinum solution. The roots became dark brown and the tops extensively necrotic. The yields and elemental analyses are presented in Fig. 56 . A brown precipitate was observed in solution at the two highest platinum levels.

Discussion

Insignificant levels of platinum were delivered to the tops of the plants, whilst large levels were deposited at the roots. The results show similarities to those observed in the tomato experiment. One obvious difference between the species was in the levels of translocated calcium. Significant drops in the calcium level in the aerial parts was noted even at the 0.5 p.p.m. level for bean. This plant would appear to be particularly susceptible to platinum and was not considered as a suitable species for subsequent studies with other platinum group metals.

9.4 Uptake of Platinum, Palladium and Rhodium by Corn.

Experimental

The apparatus and conditions were as previously described. 'John Innes' F_1 hybrid corn seeds were used and their germination carried out in sand trays. The amount of platinum group metals found to be toxic for tomato and bean provided a basis for choosing the levels to be applied to the corn plants. Two corn plants per box, once established, were treated with full strength nutrient and the following concentrations of platinum as $(NH_4)_2[PtCl_6]$:

0.05, 0.5, 2.5, 5.0; palladium as $Na_2[PdCl_4]$: 2.5, 5.0 and rhodium as $Na_3[RhCl_6]$: 10.0, 30.0.

Results

After 14 days the plants, two per box, were harvested. In boxes A2 and B2 normal growth was observed. In C2 and D2 growth was slower but there was no evidence of chlorosis. Only in box D2 was any root browning observed and then only slight. A decrease in yield was noted in boxes G2 and H2 where top growth became weak. Browning and stunting of the roots was also noted. Healthy growth was observed in boxes L2 and M2 treated with rhodium.

Two corn plants were cropped at the start of the experiment as controls. Yields were: tops, wet weight 1.938 g, dry weight 0.129 g, percentage water 93.3%, roots, wet weight 1.878 g, dry weight 0.169 g, percentage water 91.0%. The percentage of water in the control root was lower than would be expected for a mature plant because the root, separated from the top at the base of the coleoptile, included remains of the starchy endosperm from the seed, thus increasing the dry weight of the root. Analyses were carried out and presented in Figs. 57 and 58.

Discussion

The uptake of platinum by corn appears to be similar to that found for tomato. Translocation is very small compared with the levels retained by the roots. Browning at the 5 p.p.m. level would seem to be directly related to the high platinum content of the roots. The water contents of the roots both of tomato and corn drop significantly at the higher levels of platinum nutrition.

Palladium was toxic to corn at the levels tested. Very large levels were deposited at the roots with negligible amounts translocated.

Yields and analysis of corn plants treated with $(NH_4)_2[PtCl_6]$.

Tops	Box	A2	B2	C2	D2
Applied Pt/ppm		0.05	0.50	2.50	5.00
Wet wt/g		7.76	10.486	4.446	4.557
Dry wt/g		0.473	0.672	0.344	0.346
Wt of water/g		7.292	9.813	4.102	4.211
Water present/%		93.9	93.6	92.3	92.4
Total Pt/µg		2.5	3.0	4.5	8.5
Pt(dry wt)/ppm		5.3	4.5	13.1	24.6
Roots	**Box**	**A2**	**B2**	**C2**	**D2**
Applied Pt/ppm		0.05	0.50	2.50	5.00
Wet wt/g		3.137	3.716	2.003	3.056
Dry wt/g		0.178	0.219	0.201	0.308
Wt of water/g		2.959	3.425	1.803	2.749
Water present/%		94.3	92.2	90.0	89.9
Total Pt/µg		25	100	300	1100
Pt (dry wt)/ppm		140	343	1496	3575

Figure 57

Yields and analysis of corn plants treated with $Na_2[PdCl_4]$.

	Tops	G2	H2	Roots	G2	H2
Applied Pd/ppm		2.5	5.0		2.5	5.0
Wet wt/g		4.187	5.439		2.029	3.056
Dry wt/g		0.306	0.404		0.190	0.297
Water present/%		92.7	92.6		90.7	90.3
Total Pd/μg		1.5	4.5		410	2150
Pd (dry wt)/ppm		4.9	11.1		2160	7237

Yields and analysis of corn plants treated with $Na_3[RhCl_6]$.

	Tops	L2	M2	Roots	L2	M2
Applied Rh/ppm		10.0	30.0		10.0	30.0
Wet wt/g		13.44	8.134		4.406	3.632
Dry wt/g		0.783	0.540		0.261	0.247
Water present/%		94.2	93.4		94.1	93.2
Total Rh/μg		22.3	45		45	108
Rh (dry wt)/ppm		28.4	83.3		173	438

Figure 58

Rhodium produced no ill effects that could be observed. In contrast to the other metals relatively large amounts of rhodium were translocated.

9.5 Uptake of Cationic and Anionic Platinum Species by Corn

Introduction

In an attempt to ascertain whether the important factor in platinum toxicity is the platinum atom itself, the complex or simply a molecular size effect corn plants were grown, as before, with the addition of various platinum complexes to the normal nutrient solutions.

Experimental

Apparatus and conditions were as previously described. The platinum species chosen for this study were: $[PtCl_4]^{2-}$, $[PtCl_6]^{2-}$, $[Pt(NH_3)_4]^{2+}$, $[Pt(NH_3)_6]^{4+}$. The levels chosen were those at which normal growth was observed for corn using $[PtCl_6]^{2-}$ as the platinum source.

Results and discussion

Two plants were cropped at the start of the experiment as controls. Yields were: tops, wet weight 3.685g, dry weight, 0.225 g, percentage water, 93.9%; roots, wet weight 1.697 g, dry weight, 0.149, percentage water, 91.2%. After 11 days growth the plants were harvested. Healthy growth took place in all boxes except for E4 in which there was poor root growth with slight browning and clubbing and less than normal top growth. In addition to this a slight brown precipitate was present in the nutrient solution, E4. Results and analyses are presented in Fig. 59 .

Normal water relations were maintained except with $[PtCl_4]^{2-}$ at the 2.5 p.p.m. level. Root metal levels are broadly comparable with no obvious differences between the various species. Low levels of platinum were translocated to aerial parts except for $[Pt(NH_3)_4]^{2+}$ most especially at the 2.5 p.p.m. level. Too much emphasis cannot be placed upon one value and this result should be further investigated.

Yields and Analyses of corn plants treated with platinum in different forms

Box	Type of Complex	Applied Pt ppm	Wet Weight g	Dry Weight g	Weight of Water g	Water Present %	Total Pt μg	Pt (dry wt) ppm
TOPS								
A 4	$K_2[Pt(II)Cl_4]$	0.5	13.691	0.893	12.798	93.5	~ 1	~ 1
B 4	$(NH_4)_2[Pt(IV)Cl_6]$	0.5	14.723	0.810	13.914	94.5	10.5	13.0
C 4	$[Pt(II)(NH_3)_4]Cl_2$	0.5	19.925	1.127	18.798	94.3	21.3	18.9
D 4	$[Pt(IV)(NH_3)_6]Cl_4$	0.5	21.644	1.254	20.390	94.2	2.0	1.6
E 4	$K_2[Pt(II)Cl_4]$	2.5	10.954	0.816	10.138	92.5	2.5	3.1
F 4	$(NH_4)_1[Pt(IV)Cl_6]$	2.5	16.952	1.073	15.879	93.7	~ 1	~ 1
G 4	$[Pt(II)(NH_3)_4]Cl_2$	2.5	21.355	1.286	20.069	94.0	175	136
H 4	$[Pt(IV)(NH_3)_6]Cl_4$	2.5	18.193	1.087	17.107	94.0	4.1	3.8
ROOTS								
A 4	$K_2[Pt(II)Cl_4]$	0.5	4.054	0.286	3.768	92.9	175	612
B 4	$(NH_4)_2[Pt(IV)Cl_6]$	0.5	4.551	0.327	4.224	92.8	175	535
C 4	$[Pt(II)(NH_3)_4]Cl_2$	0.5	5.644	0.355	5.289	93.7	100	281
D 4	$[Pt(IV)(NH_3)_6]Cl_4$	0.5	6.689	0.438	6.251	93.5	150	343
E 4	$K_2[Pt(II)Cl_4]$	2.5	4.041	0.447	3.594	88.9	200	447
F 4	$(NH_4)_2[Pt(IV)Cl_6]$	2.5	7.026	0.473	6.553	93.3	175	370
G 4	$[Pt(II)(NH_3)_4]Cl_2$	2.5	7.161	0.407	6.755	94.3	275	676
H 4	$[Pt(IV)(NH_3)_6]Cl_4$	2.5	5.971	0.391	5.580	93.4	350	895

Figure 59

9.6 General Discussion

Both tomato and corn proved to be suitable species for the investigation of the effects of platinum group metals on plants. Toxicity symptoms were similar for all metal species tested and plants treated. New root production was halted with older roots turning brown. Tops were wilted, chlorotic and necrotic.

An order of relative toxicity for some of the platinum group metals was tentatively established as folows: Pd(II) > Pt(IV) > Rh(III). Rhodium produced no toxic symptoms at all the levels tested. Indeed there is a possibility that a growth stimulatory effect is operating at low levels (Figs. 55 and 58). Palladium was toxic to both corn and tomato at the 2.5 p.p.m. level and negligible amounts of metal were translocated. Platinum in the form of $[Pt(IV)Cl_6]^{2-}$ was toxic at around the 2.5 p.p.m. level.

Although Pt(II) and Pt(IV) complexes are relatively kinetically inert, over the course of growth experiments various reactions may occur. Rosenberg et al. (1967) found that $[Pt(IV)Cl_6]^{2-}$ reacts with NH_4^+ ions in aqueous solution resulting in the formation of several mixed chlorammine complexes. Blasius and Preetz (1965) also suggested that $[Pt(IV)Cl_5(H_2O)]^-$ was formed and further that OH^- substitutions for Cl^- ligands occurred. Sigler and Blow (1965) found that NH_3 ligands displaced Cl^- ligands from $[Pt(II)\ Cl^-{}_4]^{2+}$ and that phosphate also displaced Cl^- ions. Interaction with organic material is known to occur in at least two different ways; positively charged species may form electrostatic complexes with carboxylate groups and all platinum species are known to bind to proteins, generally at $R{-}S^-$ groups (Blundell and Jenkins, 1977). At the levels tested great differences in the effects of different forms of platinum were not found, except for $[Pt(II)Cl_4]^{2-}$ which interfered seriously with growth at the 2.5 p.p.m level.

Similar studies carried out on the vascular aquatic *Eichhornia crassipes* have shown growth stimulation with low levels of Pt(II) complexes and the

familar toxic effects at higher levels. Rhodium(III) stimulated growth at 20 p.p.m. but produced slight chlorosis and reduced stimulation at 30 p.p.m. Toxicity orders for platinum group metals at 10 p.p.m. are presented below and confirm those found for non-aquatic species. (Parsons, 1979).

Order of Toxicity at 10.p.p.m. Pd(II) $\approx$ Pt(II) > Ru(III) > Os(IV) $\approx$ Pt(IV) $\approx$ Ir(III) >> Rh(III).

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CHAPTER X

SUMMARY

Heavy metal tolerant plants have been mentioned regularly over the last few decades in the literature. But with few exceptions, notably *Agrostis tenuis* and *Becium homblei*, extensive and detailed chemical investigations have been missing. The studies presented in this thesis developed from a number of analyses performed on plants collected from copper rich soils. *Armeria maritima* proved to be an obvious candidate for more detailed chemical analysis. Although all parts of the plant have been studied here, particular attention has been paid to the root; not only because it accumulates the major portion of the copper taken up, but also because it most likely provides the site of an initial tolerance mechanism.

It was deemed important to establish the tolerance and non-tolerance of *A. maritima* ecotypes, both for further chemical comparative studies and also in order to know with which nutritional level of copper the plants could cope. The initial use of Wilkins's (1957) method, using a solution containing the metal under investigation and calcium nitrate, was found to be totally unsatisfactory. Wilkins (1978) has recently reviewed the measurement of tolerance to edaphic factors by root growth and states that in the light of certain failures of the method (Davies and Snaydon, 1973) a full nutrient solution should be used whenever possible. A significant difference in tolerance to copper was established between *A. maritima* from a copper rich environment and a morphologically similar population from a low copper maritime soil.

An anatomical and histochemical study of the root of *A. maritima* provided evidence for sites of copper accumulation. An alcoholic solution of 1-(2-pyridylazo)-2-napthol produced a strong purple-red coloration with copper. The metal was found to be associated with the hypodermis, phloem,

endodermis, medullary sheath and some of the tannin bearing cells. At least partial confirmation of this was provided by crude sectioning and electron microprobe analysis, both of which implicated the hypodermis as the major accumulating region.

The single most important tool for the elucidation of the chemical binding sites within the plant, despite all its attendant problems, is the extraction scheme. A chemical fractionation scheme is presented, based on several earlier schemes. It was devised to obtain maximum information about the chemical environment of the metal, whilst limiting the possibility of more than one distinct class of compounds being extracted or precipitated by the same treatment. Extraction Scheme A for root material revealed that 29.0% of the copper was removed with the fraction which included low molecular weight water soluble materials. 11.8% of the metal was solubilised by the proteolytic enzyme pronase. The copper was thus considered to have been associated with protoplasmic proteins, polypeptides or possibly cell wall proteins. But by far the largest amount, 49.0%, was associated with pectic, protopectic and similar polysaccharidic material. The leaf also contains copper mainly involved with pectic substances. But it has substantially less copper removed in the low molecular weight extractable fraction.

These three copper fractions in the root extracts represent at least three distinct forms in which the metal probably exists within the root. Therefore the simplistic, but attractive, view of a tolerance mechanism whereby copper is removed from sensitive metabolic sites and deposited in some convenient 'sink' must be rejected. Heavy metal tolerance has been shown to be a hereditable characteristic which is often dominant with several genes involved (Wilkins, 1960) (Urquhart, 1971). The mechanisms whereby a plant achieves tolerance are highly complex (Wainwright and Woolhouse, 1975).

Differential ultracentrifugation indicated that the bulk of the copper was associated with cell wall material. Since the pectic substances are found almost entirely in the cell wall this provides supportive evidence that copper is linked largely to cell wall pectates. Several other studies have implicated cell wall pectates as the major deposition site for toxic metals (Turner and Gregory, 1967) (Peterson, 1969) (Turner and Marshall, 1971) (Farago *et al.*, 1975). Dialysis with tartaric acid resulted in the removal of 44.8% of the copper present in the root material. This metal is lightly complexed probably to cell wall pectates and has a greater affinity for the introduced tartarate ligands.

Since the transition from living plant to bog material is not clearly marked, the decomposing plant material may provide the living plant with a means of restricting entry to copper. The metal exists in several forms which may be considered in the following physical terms:

Total soil Cu; plant available (NH_4Ac extractable) Cu; water soluble; $Cu^{2+}(aq)$

5430 p.p.m. > 79 p.p.m. > 0.4 p.p.m. > 0.004 p.p.m.

Actual levels were obtained from Chapter III with the cuprous ion concentration a rough estimate based on work by Stiff (1971) and Hodgson *et al.* (1965, 1966). Ernst (1972) carried out an experiment in which he applied copper to *Silene cucubalus* in three forms; as a soil extract, as a copper - E.D.T.A. complex and as aqueous copper sulphate. Copper uptake was 1, 52 and 200 μg/plant respectively. From this he inferred that the water soluble complexing agents for copper in the soil extract were probably quite large. It appears that for *A. maritima* growing in Dolfrwynog bog most of the copper is unavailable to the plant and that fraction which is available is present as various organic complexes. A complex in the bog solution between copper and organic ligands was found to have a ratio of molecules of complexing

agents to molecules of metal of 1.15 and a log stability constant of 6.0. These values are consistent with those found by other workers and strong indications are that the organic ligands responsible for bonding are humic or fulvic acids (Stiff, 1971) (Ernst, 1972) (Schnitzer and Skinner, 1966).

Humic and fulvic acids are highly polymeric, ill-defined substances which have so far defied complete structural analysis. Chen and Schnitzer (1976) have purified these substances and found both to contain carboxylic acid, phenolic, alcoholic, ketonic and quinoid functional groups. An e.s.r. study by McBride (1978) into the nature of the bonding in a humic acid-copper complex obtained signals consistent with single bonds between the metal and the oxygen ligands of the organic functional groups. Goodman and Cheshire (1973) have presented evidence that porphyrin groups in humic acid make a small contribution to the total number of metal binding sites. Gamble and Schnitzer (1973) found that copper was chelated to phenolic groups in a fulvic acid preparation and Langford et al. (1979) have found copper bound to humic or fulvic acids from an organic rich natural water.

The next and perhaps least understood step in the movement of the toxic metal into the plant, is the way in which the metal is transferred from a soil solution ligand to an organic site in the outer spaces of the root. Ramamoorthy and Leppard (1977) suggested that rhizoplane fibrils with a pectic surface may be responsible for extracting the metal and thence transporting it to the root cell walls. Once the metal has gained entry to the plant there are numerous potential binding sites. Mills (1954) and Vallee and Ulmer (1972) have considered ranges of plant products which might be expected to complex strongly with copper. Possible classes of complexing agents are as follows: acidic carbohydrates and pectic substances; aliphatic and aromatic acids; phenolics; flavonoids; amino acids, peptides and proteins; purines, pterins and flavins; porphyrins; amines; and

miscellaneous nitrogen and sulphur compounds involving imino and sulphydryl groups. Requirements of chelating agents, other than their prosthetic groups, are that they must occur in relatively large quantities and must be well distributed throughout the plant at all stages of growth. Stability constants of copper organic ligand complexes are not available for representatives of all of these groups. Some of the smaller molecules have, however, been extensively studied and log stability constants for likely chelating agents are presented in Fig. 49 . On the evidence of stabilty constants amino acids appear to be good candidates for copper chelating ligands. They are also ubiquitous and plentiful components of all living organisms.

Water extractable copper from roots of tolerant *A. maritima* was found to be associated with the amino acid proline, which is a particularly good complexing agent for copper. In addition to this proline makes up a high proportion of the free amino acid pool in tolerant *A. maritima*, even when the plant is not under copper stress. Non-tolerant plants have much lower levels of proline. It has been shown by Stewart and Lee (1974) that most halophytes contain proline as the major component of their amino acid pools. *A. maritima* from saline and non-saline environments were found to differ considerably, the maritime population having a dramatically higher proline content. Both populations were treated with varying saline solutions and at high salinity the coastal population accumulated much higher levels of proline than the inland population. It was mooted that proline was being utilised as a solute for intracellular osmotic adjustments. They suggested also that salt tolerance was correlated with the capacity to accumulate proline. A question which needs to be resolved is whether or not heavy metal tolerance is also correlated with the capacity to accumulate

proline. Clearly an apparatus enabling the plant to increase its free amino acid pool exists within halophytic species and amino acid studies of other metal tolerant halophytes such as *Silene maritima* might be rewarding.

The most important chemical forms of copper in *A. maritima* appear to be as insoluble complexes with pectic substances. The basic unit of all pectic material is the galacturonic acid molecule. Metal pectates are composed of both inter- and intra-molecular forms with both COOH groups and OH groups donating electron pairs to the cation. Schweiger (1965) has shown that copper pectates are composed of mainly the inter-molecular forms.

The material released by pronase may represent protein from various different sources including cell wall glycoprotein, membrane protein and organelle protein. So no real clue is given as to the origin of the bound copper. A cell wall specificity for copper could be accounted for by binding by cell wall glycoprotein, but as yet no sound evidence has been presented for this neat hypothesis. Copper found in association with soluble pectates and soluble proteins may include forms such as the unidentified polyaminopolycarboxylic acids responsible for translocation of copper in tomato xylem exudate (Tiffin, 1972).

Copper is accumulated in the outer free spaces of the cell walls and is transported across the plasmamembranes by specific carriers. An effective restriction of entry could be brought about by the presence of specific efflux carriers (Poole, 1969) or by a reduction in the number of carrier sites or the uptake rate for copper. Either mechanism would result in a build up of metal at the cell wall without the need for specific accumulation sites. Transport systems are ion specific so protein based molecules almost certainly behave as the carriers, but have yet to be isolated. However, modifications to the phosphatase enzymes of cell surfaces associated with the membrane

have been shown in the case of copper tolerant *Agrostis tenuis* by Wainwright and Woolhouse (1975). With improvements in the purification and isolation of membranes may come a clearer picture of what happens at this vital primary uptake site.

Copper once within the cell is firmly complexed probably as the bisprolinato copper(II) dihydrate species and possibly as other highly stable species. Symplasmic movement of the metal into the stele occurs, where significant amounts of copper are deposited in the phloem and medullary sheath regions. Histochemical studies showed that copper was not associated with xylem elements. Some of the metal is translocated to the leaves where it is largely immobilised at the cell walls. Finally guttation of copper takes place from salt glands.

Tannin bearing secretory cells in some cases also appear to accumulate copper. Tannins have adjacent phenolic hydroxy groups which are known to complex copper strongly. It is possible that these secretory cells have a novel function.

A. maritima would seem to be an ideal plant for further study. Valuable evidence might be gathered from xylem exudate studies and the physical and chemical properties of copper pectinates. Physiological work on the rates of copper uptake and cell wall phosphatase behaviour of tolerant and non-tolerant ecotypes would also be of interest.

The major part of this thesis is concerned with the uptake of labile complex form(s) of copper and their removal from metabolic sites. As a contrast to this metals forming non-labile complexes were also studied. The effects of platinum group metals on crop species were investigated and plant metal levels presented. The relative order of toxicity was found to be: Pd(II) > Pt(IV) > Rh(III). A possible growth stimulatory effect due to rhodium was observed. This phenomenon has also been reported for *Eichhornia crassipes* (Parsons, 1979). No clear distinctions were found between different complexed forms of platinum in their effects on corn plants at the levels tested.

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APPENDIX 1

Experimental Conditions for Atomic Absorption Analysis

Elements detected using various atomic absorption spectrophotometers

Element	Shandon Southern A3000	Pye Unicam SP 1900	Pye Unicam SP1900 & Beckman Massmann Carbon Furnace Analyser
Calcium	*	*	
Copper	*	*	
Iron	*		
Magnesium		*	
Palladium		*	*
Platinum		*	*
Rhodium		*	*
Zinc	*		

Flame atomic absorption using a Pye Unicam SP1900

Element	Lamp Current mA	Slit Width mm	Air/Acetylene Flow Rates dm^3/min	Wavelength nm	Solution Compositions	Standard Additions
Calcium	5	0.20	5.0/1.0	422.7	1.0% HNO_3*	0.1% $LaCl_3$*
Copper	3	0.10	5.0/1.0	324.8	1.0% HNO_3	-
Magnesium	3	0.20	5.0/1.0	285.2	1.0% HNO_3	0.1% $LaCl_3$
Palladium	10	0.10	5.0/1.0	247.6	2.0% HCl	0.2% $LaCl_3$
Platinum	10	0.10	5.0/1.0	265.9	2.0% HCl	0.2% $LaCl_3$
Rhodium	10	0.20	5.0/1.0	343.5	2.0% HCl	0.2% $LaCl_3$

* Solution and standard additions used for platinum were also employed.

Flame atomic absorption using a Shandon Southern A3000

Element	Lamp Current mA	Slit Width mm	Air/Acetylene Flow Rates dm^3/min	Wavelength nm	Solution Composition	Standard Additions
Calcium	5	0.50	7/2.5	422.7	1.0% HNO_3	0.1% $SrCl_2$
Copper	6	0.05	7/1.2	324.8	1.0% HNO_3	-
Iron	10	0.025	7/1.5	248.8	1.0% HNO_3	-
Zinc	8	0.25	7/1.5	213.9	1.0% HNO_3	-

Flameless atomic absorption using a Pye Unicam SP1900 with a Beckman-Massmann carbon furnace atomizer

Element	Lamp Current mA	Slit Width mm	Wave-length nm	Step 1 $^{o}C/s$	Step 2 $^{o}C/s$	Step 3 $^{o}C/s$	Step 4 $^{o}C/s$	D_2 Atten-uator Setting	Sample Volume µl	Solution Composit-ion
Palladium	6	0.10	340.4	100/30	600/20	3300/7	3200/1	5	50	2% HCl
Platinum	10	0.10	265.9	100/30	600/20	3300/10	3200/2	5	50	2% HCl
Rhodium	10	0.20	343.5	100/30	600/20	3250/6	3200/1	6	50	2% HCl

PUBLICATIONS - CORRIGENDA

A Study of *Armeria maritima* (Mill) Willdenow growing in a Copper-impregnated Bog. Farago. M.E., Mullen, W.A., Cole, M.A. and Smith, R.F. (1980), Environmental Pollution (Series A), 21, 225.

Data presented in Figs. 1 - 5 was not obtained as a result of research by W.A. Mullen.

The Uptake of Platinum Group Metals by Tomato, Bean and Corn. Farago, M.E., Mullen, W.A. and Payne J.B. (1979), Inorganica Chimica Acta, 34, 151. The paper was not sent to the authors for proof reading and consequently several typographical and print setting errors have resulted. An adjustment was made to the data for box F1 in Table 1 due to the fact that one plant died during the experiment. Wet and dry weights were adjusted, using the average weights per plant, to the values expected for four plants.

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A STUDY OF *ARMERIA MARITIMA* (MILL) WILLDENOW GROWING IN A COPPER-IMPREGNATED BOG

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ABSTRACT

A survey has been made of the Dolfrwynog area in the Coed-y-Brenin Forest, Gwynedd, Wales, and in particular of the Dolfrwynog Bog. The distribution of indicator species, with emphasis on Armeria maritima, *is reported and their relationship to the geochemistry and other environmental factors is discussed.* A. maritima *from the bog is very tolerant to copper, which it stores in the carbohydrate of the cell walls, and in particular in the root.*

INTRODUCTION

The sea pink, or thrift, *Armeria maritima* (Mill) Willd, has been recognised for many years as a metal indicator. Henwood (1857) reported that, in the Dolfrwynog area of Wales, the presence of copper was indicated by its growth.

Copper mineralisation in the Dolfrwynog area occurs in, or emanates from, the Coed-y-Brenin porphyry copper deposit, located on the eastern flank of the Harlech Dome where middle and upper Cambrian sediments, which dip steeply eastwards, have been intruded by diorite sills, stocks and minor dykes. The copper mineralisation occurs as veinlets of chalcopyrite in both the lightly fractured older diorites and the sediment (Rice & Sharp, 1976). The deposit occurs in upland plateau country of moderate relief, which has been deeply dissected by the Afon Wen and the Afon Mawddach, the latter flowing to the Barmouth estuary west of Dolgellau.

In places bedrock is masked by glacial drift and peat. Dolfrwynog Bog, which slopes gently from east to west, occupies such a site within the pre-glacial valley of the Afon Wen. Podzolic soils of variable depth cover much of the area, but there brown earths characterise the steep valley sides.

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Deciduous woodlands, dominated by the sessile oak *Quercus petraea* (Mattuschka) Liebl., cover the valley sides and formerly extended further. Today coniferous plantations and pastures occupy much of the higher land.

Anomalous plant communities characterised by indicator species, especially *Armeria maritima* and *Minuartia verna* L. (Hiern), occupy the copper-impregnated Dolfrwynog Bog (the 'copper turf' of the old miners), and apparently undisturbed sites within the coniferous plantations and pastures. In the 19th century, levels (adits) were driven into the hillsides in attempts to locate the lode which was believed to be the source of copper in Dolfrynog, and the indicator species also grow in the dumps of material left from these excavations. Most of the communities occur either where ground waters with a high copper content are within the rooting depths of plants (Cole, 1979) or on contaminated soil or dump material. The occurrence of copper in considerable concentrations in the peat in the Coed-y-Brenin area was first observed by Henwood (1857). He described the extensive deposit of 'copper turf' at Dolfrwynog where, he stated, 70 acres (28 ha) were worked in the early 19th century —a much larger area than that occupied by the Bog today (about 4 ha). Henwood described a surface bed of peat about 60 cm thick, below which there was a bed of stones of unexplained origin and then a further layer of peat, containing little copper and left unworked. Such a configuration of beds is not apparent today. The peat was burned in kilns and the ashes were sent to Swansea to be processed further. At the time of his visit Henwood records that the remaining peat would not yield more than $2\frac{1}{2}\%$ of copper and its working was not an economic proposition. This remaining peat is presumably the material investigated in the course of this study.

MATERIALS AND METHODS

Collection and treatment of soil samples

Samples were taken of surface (top 10 cm) soil where it was exposed or, where a root mat was present, of material from just below the mat. About 100 g was collected, air-dried and finally dried at 25 °C. Samples (−80 mesh) were digested in 8M nitric acid (3 ml) for 95 min at 95 °C (water bath). Distilled water (7 ml) was added to each sample, which was shaken and allowed to settle before analysis. Since the soils from the bog contain large amounts of peat, a second analysis was carried out on ashed samples as follows: a weighed amount of soil was ashed at 420 °C and the weight loss on ashing determined; the analysis was then carried out as before.

Collection and analysis of plant material

Plant samples were washed on site. In the laboratory the material was dried, freeze-dried and ground using a Glen-Creston mill with agate grinders. Approximately 0·2 g, sieved through 320 μ mesh, was weighed accurately and heated with 'Ultra' grade nitric acid (10 ml) in an ashing tube. The tube was heated for 12 h

at 140 °C in a thermostatically controlled heating block. The resultant mixture was made up to 25 ml for analysis. Analyses were carried out on a Shandon-Southern A30 Atomic Absorption Spectrophotometer using standard methods.

RESULTS

General description of the Dolfrwynog area in the Coed-y-Brenin Forest, Gwynedd, Wales

The distribution of the indicator species between the Afon Mawddach and the Afon Wen in the Coed-y-Brenin Forest has been discussed by Cole (in press). The largest community characterised by both *A. maritima* and *M. verna* is in the Dolfrwynog Bog. Clearly defined corridors of vegetation dominated by *A. maritima* occur over three parallel cupriferous veins in bedrock which outcrops on the sloping ground above the bog. These communities contrast sharply with adjacent coniferous plantations, in which, however, many of the trees are dying.

Between the plantations and both to the east and west of the veins, further corridors of *Armeria maritima* occur along drainage lines, fed from springs issuing beneath the peat cover and containing water from the area of the porphyry copper. Both *M. verna* and *A. maritima* occupy spoil from the levels driven into the hillside. Indicator plants also extend along the access track which has been constructed from spoil material, dividing the bog into an eastern third and a western two-thirds. Drainage channels have been dug on the west side of the track by the Forestry Commission to carry water from the north across the bog to the main stream. This stream, on the northern edge of the bog, has been artificially deepened to lower the water table. There have also been minor disturbances over restricted areas from drilling operations and forestry activities.

Transects

Most of the work in the area was concentrated within the bog. Here species frequency was recorded and plant and peat or soil samples were collected along transects, the approximate positions of which are shown in Fig. 1. Transect 1 (Tr. 1) in the east crossed the bog from south to north; Tr. 2 began at the adit at the western end of the bog and crossed the bog parallel with Tr. 1, while Tr. 3 ran east to west down the centre of the bog, crossing the other transects more or less at right angles. The fourth transect, Tr. 5, crossed two of the mineralised areas on the hillside to the south of the bog and again ran from east to west. The data from Tr. 5 are less variable and less complex than those from other transects. Transects 5 and 2 will be described in this paper. Transect 5 (Fig. 2) crossed the central and western corridors of plants over the mineralised veins and an intervening region of background vegetation. It followed the contour crossing slopes between 7° and 19°; those where the indicator species occurred were steepest. Between 29 and 70 m, where the

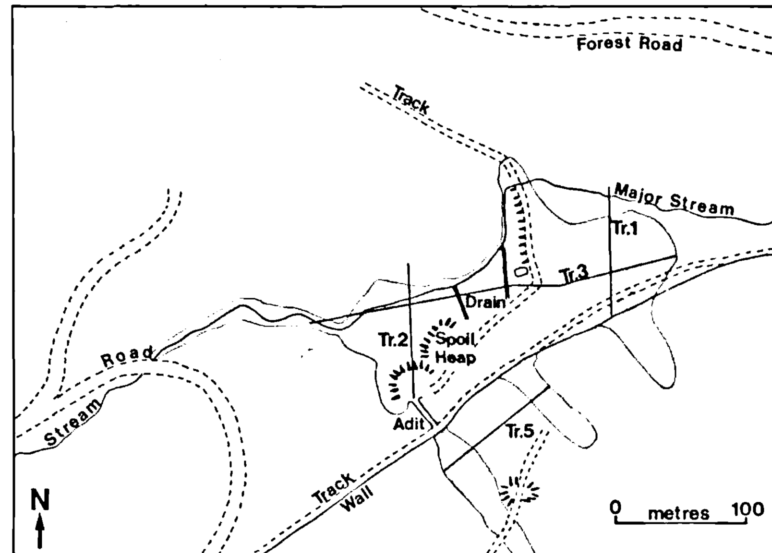

Fig. 1. Approximate locations of the transects and the extent of the major communities of indicator species in Dolfrwynog Bog.

transect ran through a plantation of *Pinus sylvestris* L., ground vegetation was recorded on alternate quadrats. Bare ground has been omitted from this area of the histogram because of the presence of fallen trees, stumps and broken branches.

Figure 2 shows that the areas of indicator plant communities are associated with two well-defined geochemical anomalies. Copper levels are lower in the eastern anomaly (between 0 and 2 m), which coincides with the central corridor of indicator plants. Here there is evidence of some recent excavations north of the transect line, and of disturbance by a track which appears to have crossed the line of the transect at some time in the past.

M. verna is present in both plant communities but is more frequent in the eastern anomaly. There is a very high percentage cover of *A. maritima* between 75 and 100 m. *Calluna vulgaris* (L.) is generally present over the western anomaly but other species such as *Vaccinium myrtillus* L. and *Ulex gallii* Planchon are completely absent where the concentrations of copper are high.

Dykeman & de Sousa (1966) observed that copper was present in a bog in New Brunswick at concentrations up to 7 % but that it appeared to have no visible effects on the vegetation. This they related to the chelation of copper by organic matter in the soil. It thus seemed possible that the toxicity of the soil on Tr. 5 was not a function of copper concentration alone but of a relationship between copper concentration and the organic matter content of the soil. This point will be discussed further in subsequent publications. The copper content of some plants on the western anomaly of Tr. 5 is shown in Fig. 3.

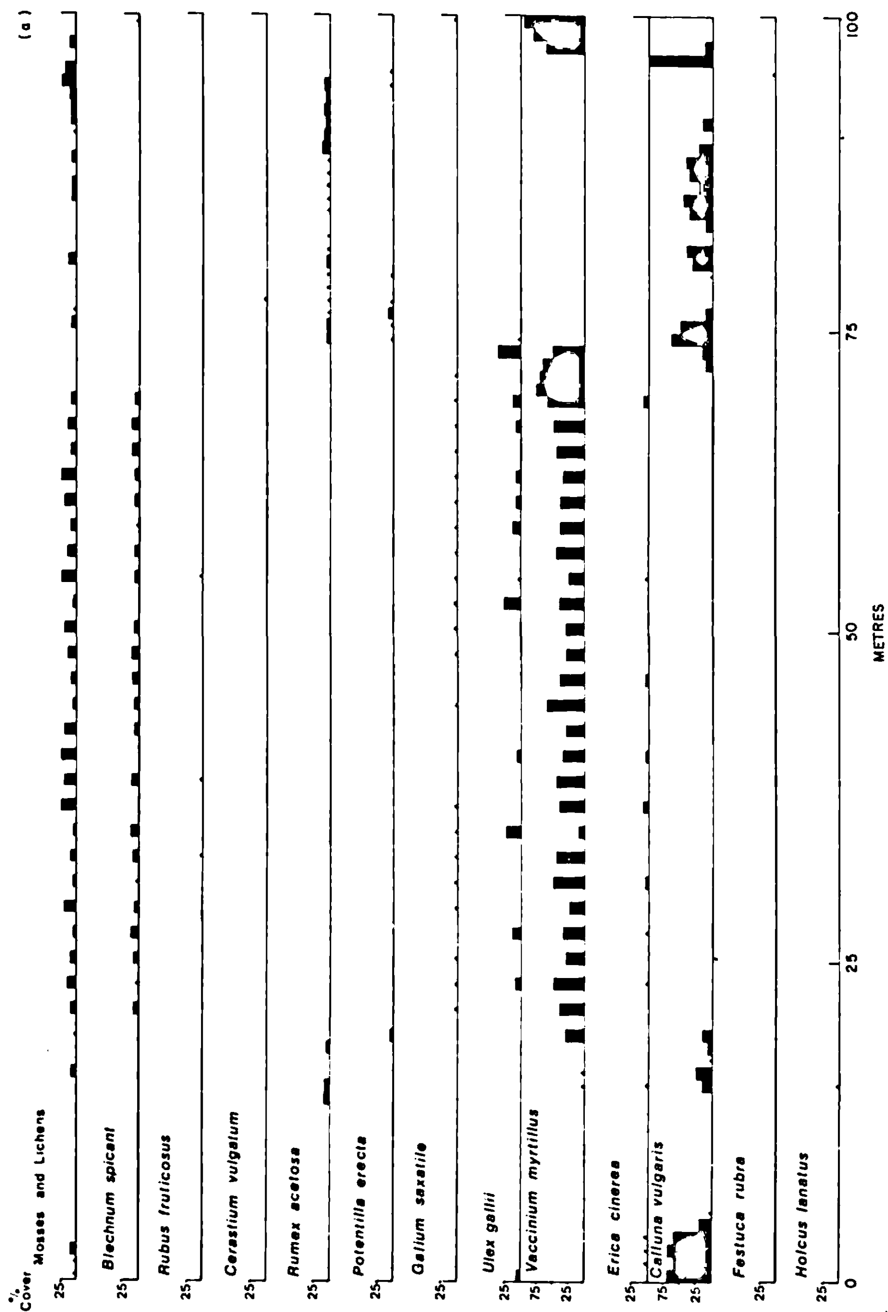

Fig. 2. Dolfrwynog Bog: Transect 5, showing (a) geobotany, (b) geochemistry and (c) weight loss on ashing.

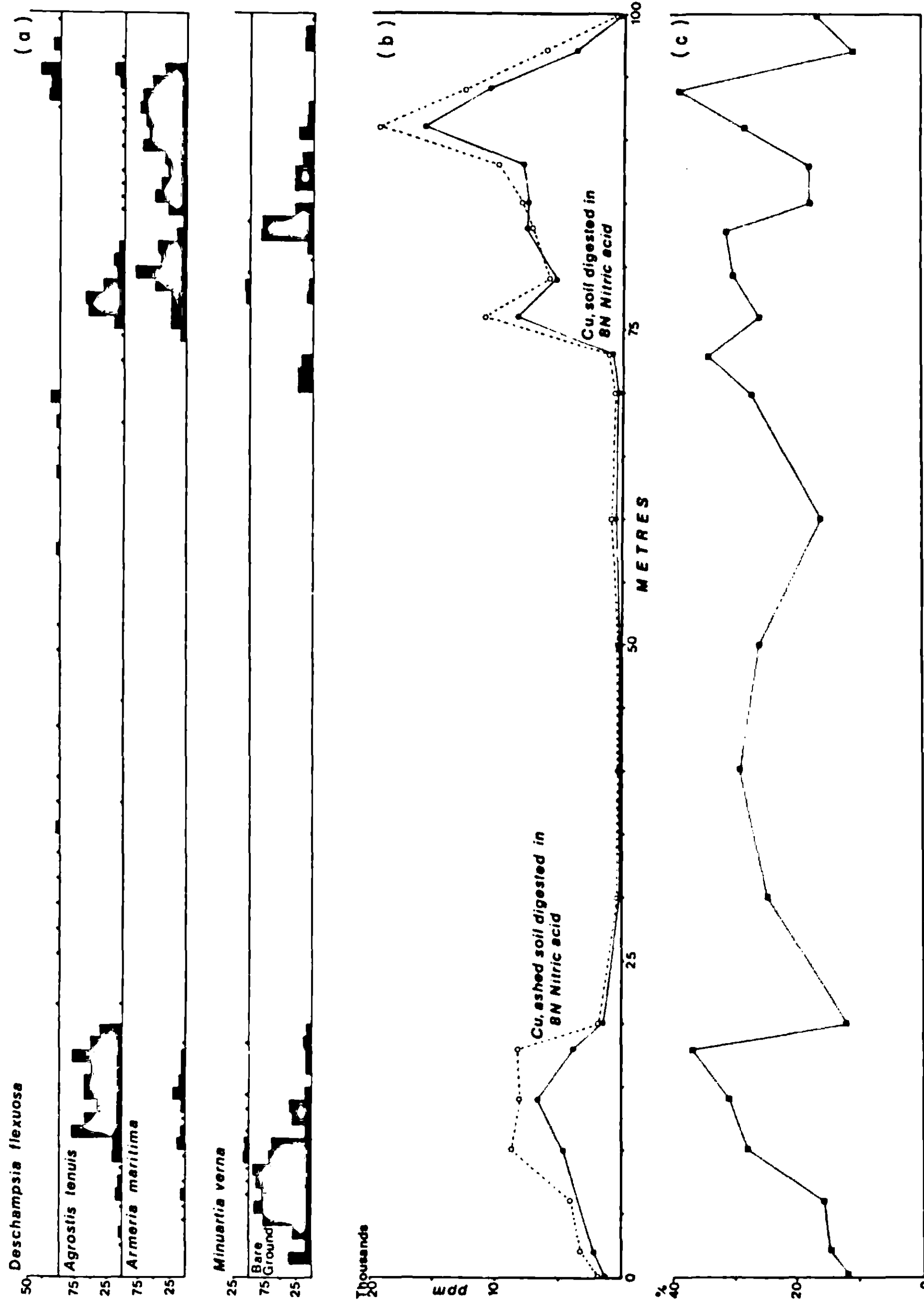

Fig. 2—*contd.*

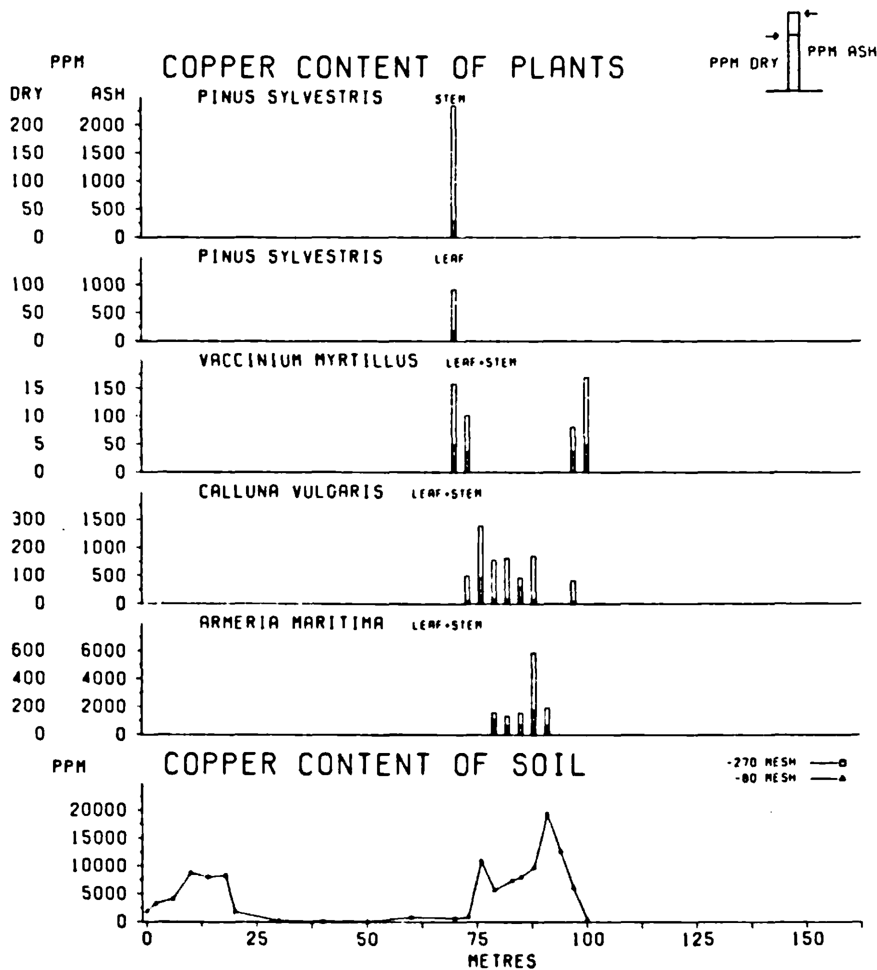

Fig. 3. Dolfrwynog Bog: Transect 5, showing analyses of plants for copper content.

The histogram for Tr. 2 (Fig. 4) reflects more complex relationships than along Tr. 5. The area crossed comprises two discrete sections: the spoil material from 0 to 30 m and that of the bog beyond.

On the relatively dry spoil heaps both *A. maritima* and *M. verna* occur but the latter is more abundant. Within the bog the distribution of these species is affected by the drainage conditions as well as by the levels of copper toxicity.

Since the bog slopes westwards, water moves obliquely across this transect line. Between 35 and 60 m, where the main community of indicator plants occurs, the bog

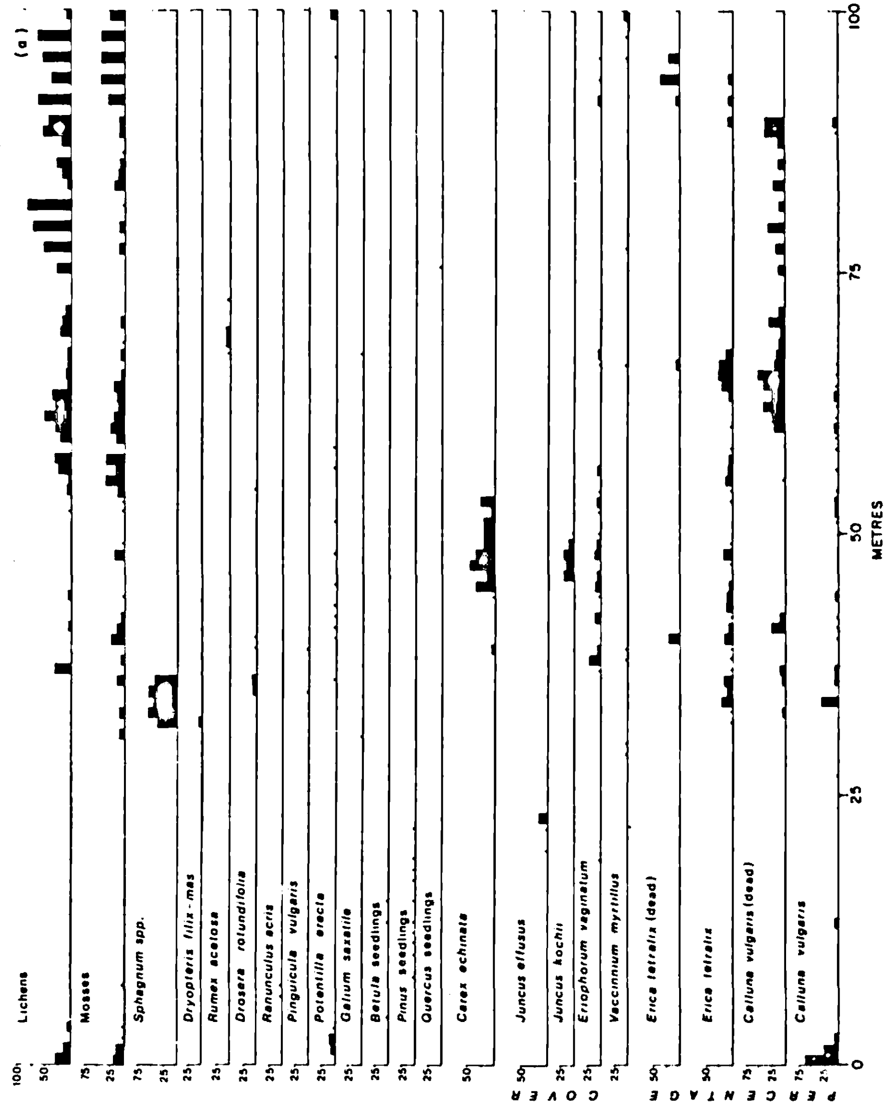

Fig. 4. Dolfrwynog Bog: Transect 2, showing (a) geobotany; (b) geochemistry; (c) weight loss on ashing and (d) relief.

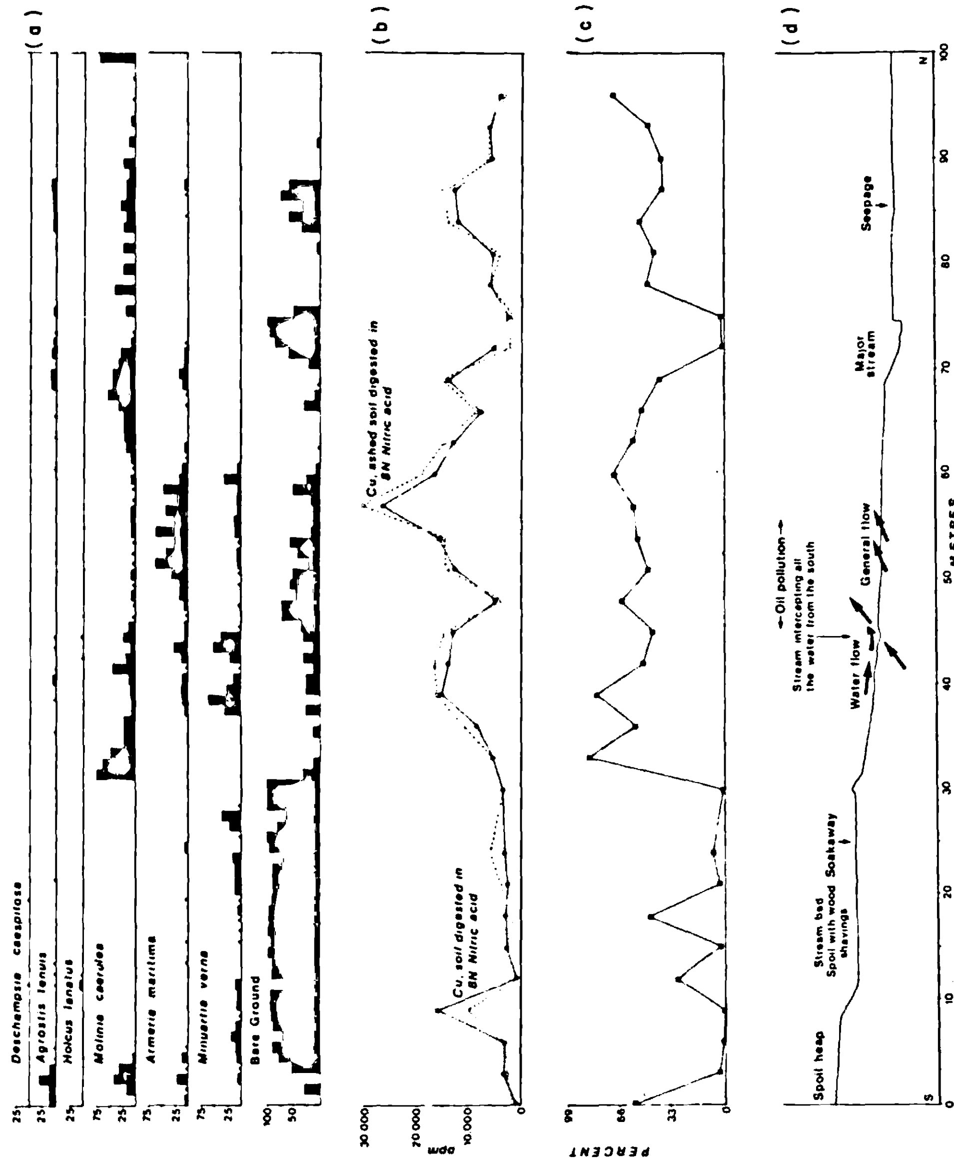

Fig. 4—*contd.*

is extremely wet and at the time of the survey the area between 50 and 55 m was covered with water to a depth of 2 cm. There were two areas of stronger water movement, one at 45 m associated with the head of a small stream and the other at 55 m. Analyses of samples from these latter sites indicated that the water was contaminated from the spoil heaps and from the mineralised veins on the southern hillside. These sites of relatively strong water flow were characterised by high copper levels in peat/soil and plant samples whereas samples collected from intervening sites (e.g. at 48 m) contained lower concentrations.

In the area between 38 and 46 m *M. verna* dominated the plant community whereas between 50 and 60 m. *A. maritima* was more abundant. *M. verna* occupied stony ground in the stream channel, at 40 m growing half-submerged in the flowing water. This habitat thus shows an extreme contrast with that for the same species on the dry spoil heaps. Elsewhere in the bog *A. maritima* appeared to be the more competitive species. Where the two occurred together, *A. maritima* generally dominated; thus the dominance of *M. verna* between 38 and 46 m is unusual. Here the lower frequency of *A. maritima* may be related to the wet nature of the site since its basal rosette growth means that most of its leaves are submerged. As the plant ages, the older leaves die but are not abscissed and the transition from plant to bog material is poorly defined.

At 85 m *A. maritima* occurred without *M. verna*; Fig. 4 shows that copper and organic matter contents (estimated from weight loss on ashing) in the subsoil are very similar to those in the 39–60 m region, where *M. verna* was present in most quadrats. The pH at 85 m was 4·3—i.e. lower than the value of 5·5 in the 39 60 m area. The absence of *M. verna* from the former area may be due to the greater acidity, since this species occurs over limestone in the English Pennines, or to the greater mobility and availability of copper at the lower pH. The copper content of some species along Tr. 2 is shown in Fig. 5.

Further investigations of A. maritima *from Dolfrwynog Bog*

In view of the apparent tolerance of *A. maritima* growing in Dolfrwynog Bog and its ability to take up copper into its tissues, this plant was studied in more detail.

Analysis of various organs of A. maritima: In order to ascertain where copper is located within the plant, various parts were analysed and the results are shown in Table 1. As a comparison plants from a non-metalliferous area at Aberaeron, Wales, are shown in Table 2.

Distribution of copper in the root of tolerant A. maritima: A carefully cleaned young root stock was chopped into a number of short sections. Each piece was separated into two parts, the outer part consisting of hypodermal and epidermal cells and the inner part of pith, cortex and stele. The separated parts were dried to constant weight and wet-ashed. Both samples were analysed and the results (Table 3) show that copper accumulates in the roots of the plants growing at Dolfrwynog Bog. Some copper is translocated to the aerial parts but green leaves, flower debris and

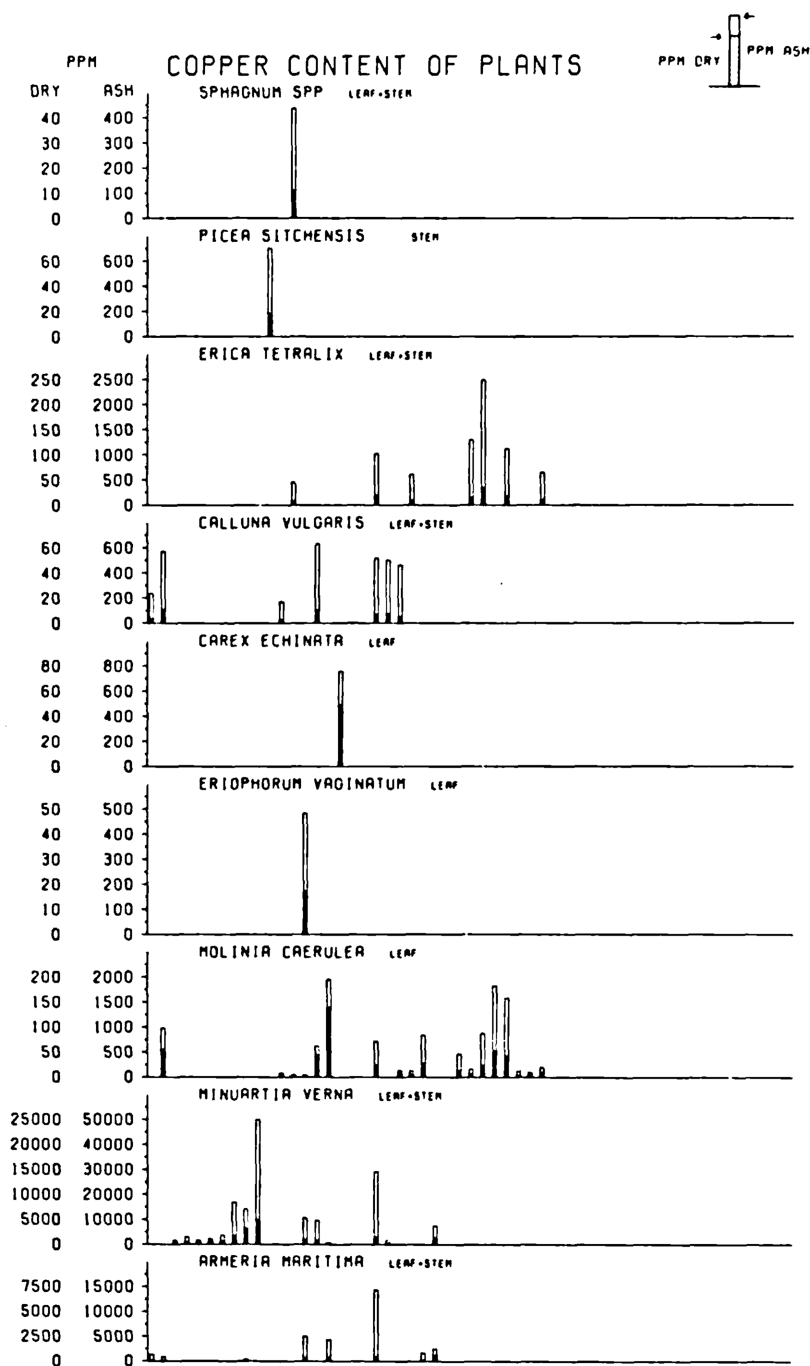

Fig. 5. Dolfrwynog Bog: Transect 2, showing analyses of plants for copper content.

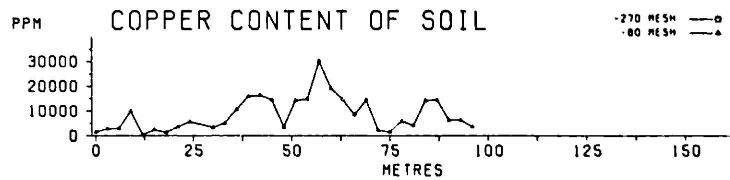

Fig. 5—*contd.*

TABLE 1
LEVELS OF COPPER IN BULK SAMPLES OF *Armeria maritima* (DRY WEIGHT BASIS) FROM DOLFRWYNOG BOG

Sample No.	*Sample details*	*Copper (ppm)*
1	Roots and leaves	15350
	Young green leaves	250
	Old leaves	4228
	Old leaves	2893
	Root	10140
2	Whole plant[a]	1134
	Whole plant[b]	2460
3	Aerial parts	747
	Green leaves	169
	Green leaves	173
	Young roots	1170
	Young roots	1180
	Flower debris	54
	Flower debris	51

[a] Dead leaves removed.
[b] Including dead leaves.

TABLE 2
LEVELS OF COPPER IN BULK SAMPLES OF *Armeria maritima* (DRY WEIGHT BASIS) FROM ABERAERON

Sample details	*Copper (ppm)*
Root	14
Leaves and flowers	9
Soil from around roots	23

TABLE 3
DISTRIBUTION OF COPPER BETWEEN OUTER AND INNER PARTS OF *Armeria maritima* ROOT

Root part	*Plant material (dry weight g)*	*Copper content (ppm)*	*Copper content (10^5 g)*	*% of total copper*
Inner	0·1350	444	5·994	13·6
Outer	0·1162	3270	37·997	86·4
Total	0·2512	1750	43·991	100·0

seed heads show progressively less copper than the roots. In contrast there was very little copper in either soil or plants collected at Aberaeron. Copper also accumulates in the old and rotting leaves. Where the old leaves were left on the plant or analysed separately much higher copper levels were obtained than for green leaves. The mechanism is probably an ion exchange process, whereby the old and rotting leaves extract the copper from the bog waters, rather than simply a translocation to old leaves.

Assessment of tolerance of A. maritima *to copper:* Tolerance was tested by the series method (McNeilly & Bradshaw, 1968) using three-day observation periods.

Fully mature seedheads of *A. maritima* from Dolfrwynog Bog and Aberaeron were air-dried and the seeds removed by hand from the calyces. They were germinated in an environmentally controlled growth room with a 14-h day. The temperatures were at 20°C (day) and 15°C (night).

Seedlings were selected for uniformity of growth: root lengths between 10 and 30 mm and the absence of root branching. They were transferred to 1-litre polyethylene cartons, painted black. Each black-painted lid, 180 × 130 mm, had twelve symmetrically positioned 10 mm holes. The plants were held in the holes in a wedge of black polystyrene foam, the bottom of which was 5 mm above the surface of the solution. Aeration of each nutrient solution was by gas distribution tubes, 90 mm long, porosity 3, supported by rubber bungs placed in the corner holes of the lids.

Control boxes of twelve seedlings were grown in either 0·5 g dm^{-3} calcium nitrate solution or in full nutrient (Epstein, 1972). Further batches were grown in calcium nitrate or full nutrient with copper as copper sulphate at various concentrations as shown in Table 4.

The indices of tolerance indicate that the tolerance to copper of *A. maritima* is very high. McNeilly & Bradshaw (1968) observed indices of tolerance for *Agrostis tenuis* of between 40 and 80 for tolerant populations at 0·5 ppm copper. Non-tolerant populations showed lower tolerance indices of between 5 and 30. In calcium nitrate media *A. maritima* from the copper-rich site, with an index of 66 and 0·5 ppm copper, is thus highly tolerant. In contrast, *A. maritima* from a non-copper site had, under similar conditions, a tolerance index of only 2·4 in 0·5 ppm Cu^{2+}. At the highest level of copper applied, the initial growth was severely affected. When a full nutrient solution was applied with the copper solution at the end of the experiment, new root growth was resumed and the plants continued to grow. Davies & Snaydon (1973) have discussed the use of full nutrient solution and of calcium nitrate solution in tolerance studies. In a study of the response to aluminium of *Anthoxanthum odoratum* they found that the tolerance in terms of root length in complete culture solution was closely correlated with the dry weight yield in sand culture, whereas there was little correlation between the root length results in calcium nitrate and those in the sand culture. Our results show that the index of tolerance is much higher (Table 4) in full nutrient than in the calcium nitrate media. The apparent toxicity of

TABLE 4
TOLERANCE OF *Armeria maritima* TO COPPER

Box No.	*Number of plants in box*	*Nutrient*[a]	Cu^{2+} *(ppm)*	*Mean increase in root length*	*LSD* $p > 0.05$	*Index of tolerance*
1A	8	I	0	33·5	7·3	100
2A	10	I	0·50	22·1	7·4	66·0
3A	4	I	0·75	19·0	6·4	56·7
4A	8	I	1·00	16·7	7·7	50·0
5A	6	I	1·25	10·2	7·8	30·4
6A	7	I	1·50	8·6	7·5	25·6
7A	8	I	1·75	7·8	7·4	23·1
8A	6	I	2·00	2·8	3·5	8·4
9A	5	I	3·00	0·6	0·8	1·8
1B	4	II	0	36·8	14·6	100
2B	5	II	0·50	33·8	9·6	92·0
3B	4	II	1·50	30·8	9·0	83·7
4B	5	II	3·00	28·6	7·7	77·8
1C	8[b]	I	0	25·0	7·7	100
2C	6[b]	I	0·10	20·5	8·5	82·5
3C	8[b]	I	0·50	0·6	0·4	2·4

[a] Nutrients: I, 0·5 g dm^{-3} $Ca(NO_3)_2.4H_2O$; II, full nutrient from Epstein (1972).
[b] Plants grown from seeds from non-mineralised site, at Aberaeron.

copper is thus dependent upon the medium in which the copper is applied. It is also apparent from these studies that there is a significant difference in the tolerance to copper in plants from seeds from the copper-rich site and those from the 'normal' maritime habitat. This result is in agreement with previous results. The first comparative study of tolerance of plants grown from seeds from mine and non-mine populations was carried out as early as 1934 (Pratt, 1934). More recently the topic of metal tolerance in plants has been reviewed extensively (Antonovics, Bradshaw & Turner, 1971) and metal toxicity in plants has been discussed (Foy, Chaney & White, 1978).

Wu *et al.* (1975) studied the copper tolerance in *Agrostis stolonifera* and also found that populations on sites unpolluted by copper were not copper-tolerant. Similarly, marked differences were found by Allen & Sheppard (1971) in germination and seedling establishment between stocks of *Mimulus guttatus* arising from mine and non-mine environments.

Sequential extraction of copper from leaves and roots of A. maritima: Three grammes of dry leaf material and 8 g of dry roots were ground and passed through a sieve as previously described. The extraction procedure shown in Fig. 6 was adopted. Two 4-g root samples and one 3-g leaf sample were taken through identical procedures. All extracts were dealt with as quickly as possible.

The sample was refluxed for 15 min with 150 ml of 80% ethanol solution, the suspension was filtered using a millipore filter with a glass filter pad. The residue, *A*, was dried to constant weight at 40 °C.

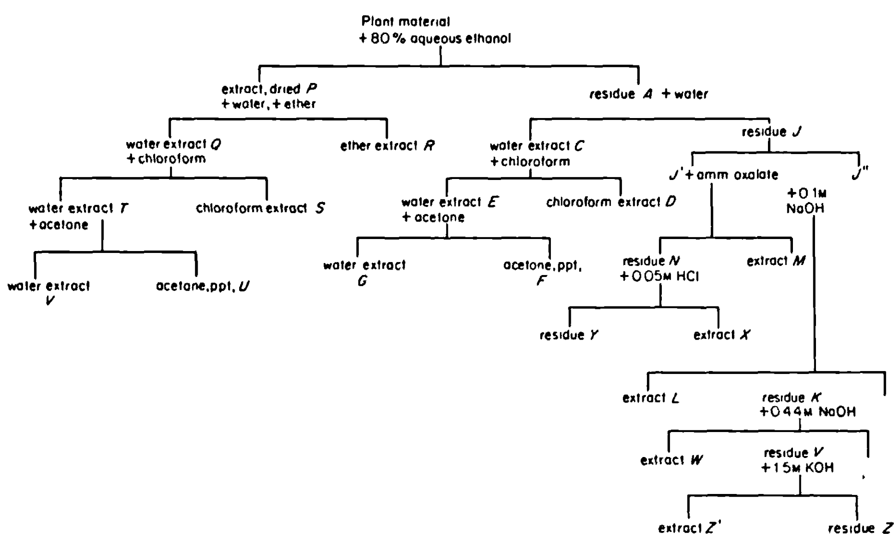

Fig. 6. Scheme for extraction procedure.

The 80% ethanol extract, *P*, was taken to dryness by rotary evaporation at 40 °C followed by freeze-drying. The dried extract was dissolved in 300 ml water and extracted with ether (125 ml). The ether extract, *R*, was taken to dryness, wet-ashed and analysed.

The water layer, *Q*, was shaken with pentan-1-ol (30 ml) and Analar trichloromethane (75 ml) for 15 min. The lower white gel, *S*, was run off, taken to dryness (rotary evaporator) and analysed. Traces of organic solvents were removed from the upper, mainly aqueous, layer, *T*, and an equal volume of acetone was added to form precipitate *U*. The mixture was centrifuged and the supernatant, *V*, was carefully decanted. Samples *U* and *V* were wet-ashed and analysed. Residue *A* from the first 80% ethanol treatment was refluxed for 10 min with 100 ml of water. The residue *B* was filtered off, using a millipore filter, and dried to constant weight. The dried extract, *C*, was dissolved in 50 ml water and the pentanol–trichloromethane–acetone treatment was carried out as before to give samples *D*, *E*, *G* and *F*.

Residue *B* was shaken with 1 g pronase, 10 mg chloroamphenicol and 200 ml phosphate buffer (pH 7·4) for 40 h. The sample was filtered off and the pronase treatment repeated. The resultant extracts, *H*, were freeze-dried and wet-ashed. The residue, *J*, was washed, dried to constant weight and accurately divided into two parts. The first portion, *J'*, was shaken with 2% ammonium oxalate (100 cm^3) for 2 h. After centrifugation, extract *M* was analysed for copper and residue *N* was heated with 0·05M hydrochloric acid to give samples *X* and *Y*. The second portion of *J*, *J"*, was extracted successively with 0·1M and 0·44M NaOH followed by 1·5M KOH

TABLE 5

COPPER (PPM DRY WEIGHT) EXTRACTED DURING SEQUENTIAL EXTRACTION PROCEDURE APPLIED TO LEAVES OF *Armeria maritima*

Samples			Classes of compounds extracted	Cu (ppm dry weight)	% of total Cu
Ether extract	*R*		Pigments	3·0	1·14
Chloroform extract	*S*		Soluble protein	15·9	6·0
Acetone, ppt	*U*		Soluble pectates	9·7	3·7
Water solution	*V*		Low molecular weight materials	30·9	11·7
Chloroform extract	*D*		Soluble protein	2·1	0·8
Acetone, ppt	*F*		Soluble pectates	11·2	4·3
Water solution	*G*		Polar low molecular weight materials	4·8	1·8
Pronase extract	*H*		Proteins, amino acids	15·0	5·7
Amm-ox extract	*M*	*J'*	Insoluble pectates	63·3	24·1
0·05M HCl extract	*X*	*J'*	Protopectates[a]	26·0	9·9
Residue	*Y*	*J'*	α-cellulose, lignin	7·0	2·7
0·1M NaOH extract	*L*	*J"*	Hemicellulose	36·6	13·9
0·44 NaOH extract	*W*	*J"*	Polysaccharides[b]	20·0	7·6
1·5M KOH extract	*Z'*	*J"*	Lignin	14·0	5·3
Residue	*Z*	*J"*	α-cellulose	3·3	1·25

[a] Including polysaccharides.
[b] Including other carbohydrates.

TABLE 6

COPPER (PPM DRY WEIGHT) EXTRACTED DURING SEQUENTIAL EXTRACTION OF *Armeria maritima* ROOTS

Sample			Classes of compounds extracted	Cu (ppm dry weight)		Average Cu	% of total Cu
Ether extract	*R*		Pigments	2·2	1·1	1·7	0·1
Chloroform extract	*S*		Soluble protein	31·1	40·8	36·0	2·2
Acetone, ppt	*U*		Soluble pectates	56·4	27·4	41·9	2·5
Water solution	*V*		Low molecular weight materials	290	325·6	307·8	18·6
Chloroform extract	*D*		Soluble protein	2·7	3·4	3·0	0·2
Acetone, ppt	*F*		Soluble pectates	27·4	11·3	19·4	1·2
Water solution	*G*		Polar low molecular weight materials	20·3	22·8	21·6	1·3
Pronase extract	*H*		Protein, amino acids	130	137·5	133·8	8·1
Amm ox extract	*M*	*J'*	Insoluble pectates	450	450	450	27·1
0·05M HCl extract	*X*	*J'*	Protopectates[a]	99·1	115·0	107	6·5
Residue *Y*	*Y*	*J'*	α-cellulose, lignin	15·3	14·4	14·9	0·9
0·1M NaOH	*L*	*J"*	Hemicellulose	456·3	447·5	451·9	27·3
0·44M NaOH extract	*W*	*J"*	Polysaccharides[b]	37·3	39·3	38·3	2·3
1·5M KOH extract	*Z'*	*J"*	Lignin	22·5	22·5	22·5	1·4
Residue	*Z*	*J"*	α-cellulose	7·6	8·3	7·9	0·5

[a] Including polysaccharides.
[b] Including other carbohydrates.

(boiling), to give *L*, *K*, *W*, *V* and *Z*. The results of the various copper analyses are shown in Tables 4 and 5.

Control experiments showed that phosphate buffer did not extract copper and that pronase did not contain significant amounts of copper.

Tables 5 and 6 show the results of sequential extraction studies. These studies show that 22·5% of the total copper in the leaves and 23·4% of that in the roots is soluble in 80% aqueous ethanol. Most of the insoluble copper appears to be associated with pectates and carbohydrates. Less than a quarter of the total copper is soluble and that appears to be associated only slightly with pectates. The major portion of this soluble copper, 52% in the leaves and 80% in the roots, is associated with low molecular weight materials.

DISCUSSION

The Dolfrwynog region contains a variety of habitats ranging from spoil heaps to apparently undisturbed vein mineralisation to the copper-rich bog. The bog itself has been greatly affected by peat cutting, mining and disturbance associated with Forestry Commission drainage operations. Nevertheless, it is a unique habitat for indicator species and will be further described in subsequent papers.

Ernst (1969) has demonstrated by pollen analysis that the indicator plants have been present in the Dolfrwynog area since the 12th century and it is probable that they have lived there for much longer. Ernst also suggested that prior to peat-cutting operations the species were much more abundant. However, the bog and its surrounding areas are still highly interesting metalliferous localities.

Within the bog itself *A. maritima* appears to be more competitive than *M. verna* and is the most generally occurring species. Where the pH is low and there is a high mobility of copper (Hawkes & Webb, 1962), there is excessive toxicity and *A. maritima* occurs alone. This very high tolerance to copper is reflected in the root elongation studies, where an exceptionally high index of tolerance is shown by plants from the bog area, which is not found in those from a non-metalliferous site.

Miller & Ohlrogge (1958*a*,*b*) have demonstrated that the chelating agents from manure produce zinc and copper in forms which are less available to plants than the simple aquo ions. Thus there is a balance in such systems between the ability of the complexing agents to solubilise the heavy metal ion on the one hand, and to sequester the ion, on the other. Bogs and mires may trap inorganic pollutants and decrease their toxicity by sequestration. Similarly, Dykeman & de Sousa (1966) observed that very high concentrations of copper in a New Brunswick bog had no visible effect on the vegetation. They suggested that the copper was chelated by organic matter in the soil.

The ion exchange capacity of bogs depends largely on the humic and fulvic acid

content which is related in turn to the degree of decomposition of the plant material (Given, 1975). The results (Table 1) show that old leaves fall back, decompose and provide sites for the uptake of copper which is in solution. Further work on the metal cycle within the Dolfrwynog Bog and the nature of the complexing agents released by the decomposing leaves is at present being undertaken.

It appears likely that *A. maritima* is able to tolerate copper in the bog because of its capacity to store the metal in the carbohydrate of the cell walls. In particular, copper is accumulated in the outer layers of the root and little is translocated to the upper parts of the plant.

The results of the extraction experiments show that the insoluble copper is associated with the pectates and other carbohydrates. In the roots about 20 % of the total copper is water-soluble and in the leaves, 12 %. Chromatographic studies (Farago & Mullen, 1979) have shown that the water-soluble copper is associated with proline and that the copper-tolerant plants had very high concentrations of proline in their tissues.

Reilly (1972) described the extraction of copper–amino acid complexes from copper-tolerant and non-tolerant *Becium homblei* and suggested that an explanation of its copper tolerance should be sought elsewhere than in sequestration as water-soluble amino acid complexes.

It has been suggested that in human serum (Sarka & Kruck, 1966) copper bound to amino acids may play a role in the transport of copper through membranes. It seems possible that as far as copper is concerned, in *A. maritima* proline complexes may form part of the transport mechanism, whereas the storage sites are in the carbohydrates of the cell walls.

The deposition of metals at cell walls is well known (Turner, 1969). Copper has been shown to be located in the cell wall fraction of subterranean clover nodules (Cartwright, 1966). The cell wall as a major site for zinc accumulation has been demonstrated in *Agrostis* spp. (Peterson, 1969; Turner & Marshall, 1972). Work on two Australian species has shown that, although zinc is associated with the cell wall pectic materials, these show a greater affinity for Cu^{2+} than for Zn^{2+} (Farago & Pitt, 1977*b*). They thus display the 'normal' affinity of polygalacturonic acids for metal ions, i.e. $Cu^{2+} > Ca^{2+} > Zn^{2+}$ (Jellinck & Sangal, 1972; Muzzarelli, 1973). It appears (Farago & Pitt, 1977*a*,*b*) that, although the zinc ions are stored in ion exchange sites in the cell wall carbohydrates, this may not constitute the specific tolerance mechanism. Thus the cell walls, although locking up large quantities of zinc, do not appear to have an altered carbohydrate composition as suggested by Turner & Marshall (1972) for *Agrostis*.

Lefèbvre (1976) has shown that strict allogamy occurs in the populations of *A. maritima* from a zinc–lead mine at Plombières, although self-fertility was shown in garden plots (Lefèbvre, 1970). The indices of tolerance showed (Lefèbvre, 1976) that the gene for hairiness of the flower stalks is not related to zinc tolerance. Baker & Baker (1975) have shown that *A. maritima* var. *maritima* from Seaford, Great

Britain, and *A. maritima* var. *californica* (monomorphic and self-fertile) have the same amino acids present in the nectar, with the addition of histidine in the latter. However, the amount of proline in the *A.m.* var. *maritima* is greater than in the *A.m.* var. *californica*. In view of these two studies of *A. maritima*, it would be interesting to analyse the nectar of both the copper- and zinc-tolerant plants.

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The Uptake of Platinum Group Metals by Tomato, Bean and Corn

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The uptake of platinum, palladium and rhodium by tomato, corn and bean plants grown hydroponically, has been investigated. Platinum, and to a lesser extent, palladium, is transported to the tops of the plants, and extensively taken up by the roots. Growth of the plants and calcium translocation is affected. Rhodium appears to have little effect on the growth of tomato and corn plants.

Introduction

Hamner [1] investigated the effects of platinum(IV) chloride on bean and tomato plants. He found that plants grown in sand culture with added $[PtCl_6]^{2-}$ have smaller leaves, are inhibited in growth and resist wilting longer than controls. However, the uptake of platinum by the plants was not investigated. The object of this present investigation is twofold, firstly to ascertain if plants are able to extract platinum metals from solution at low levels and secondly to find species which might grow and colonise tips from mining areas. In this paper we present the results of preliminary investigations into the uptake of platinum, palladium and rhodium.

Experimental

The experiments were carried out in a Prestcold controlled environment growth room. The conditions were: illumination, 10^3 ft candles; temperature, 20 ∓ 1 °C (14 hour day), 15 ∓ 1 °C (night); relative humidity, 65%.

Uptake of Platinum Applied as $(NH_4)_2[PtCl_6]$

Seeds were placed in rows in a 355 X 217 mm seed tray of acid washed Loch Aline sand for germination. The sand was moistened with demineralised water and the tray was transferred to the growth room. During the germination period the tray was covered with polyethylene film. When the first leaves had become established, the individual seedlings, selected for uniformity of growth, were transferred to 2 dm^3 black plastic boxes. Half strength nutrient solutions [2] were added to six boxes each containing four plants. When the plants were well established the boxes were treated with full strength nutrient solutions containing the following concentrations of platinum: 0.05, 0.5, 2.5, 5.0, 10.0, 30.0 ppm.

After 14 days the plants were harvested. Wet weights of the plant tops and roots were taken. The samples were then dried and reweighed. After wet ashing with concentrated nitric acid, the mixtures were taken to dryness. 2 cm^3 concentrated HCl and 5 cm^3 lanthanum chloride solution (200 mg dm^{-3}) were added and the samples were made up to 50 cm^3 for atomic absorption analysis. Standards were made up similarly. Results at the low end of the sensitivity scale were diluted and re-analysed using the carbon furnace. Calcium was assayed similarly in the presence of strontium chloride (5 mg dm^{-3}).

Uptake of Palladium Applied as $Na_2[PdCl_4]$

The methods described above were used. When the plants were well established full nutrient solutions containing the following contractions of palladium were added: 0.05, 0.5, 2.5, 5.0 ppm. The samples were analysed as before by atomic absorption techniques [3].

Uptake of rhodium applied as $Na_3[RhCl_6]$

The method was identical to that used for palladium.

Results

The results of the growth and uptake experiments are shown in Tables I–VII.

Plants fed with platinum solutions showed normal healthy growth with 0.05 ppm platinum. At 0.5 and 2.5 ppm levels there was evidence of chlorosis and a slowing of growth. Little growth was apparent at

TABLE I. Yields and Analysis of Tomato Plants Treated with $(NH_4)_2[PtCl_6]$.

Tops		A1	B1	C1	D1	E1	F1
Applied Pt/ppm		0.05	0.50	2.50	5.00	10.00	30.00
Wet weight/g		63.15	58.91	21.44	7.27	3.53	2.80
Dry weight/g		4.060	3.630	1.473	0.752	0.433	0.353
Water present/%		93.6	93.8	~~91.9~~ 93.1	~~89.2~~ 89.7	87.7	87.4
Total Pt/μg		6.0	23	32	10	19	40
Pt (dry wt)/ppm		~~1.4~~ 1.5	6.3	21.7	13.3	43.8	113
Total Ca/mg		105	92	~~43.5~~ 34.5	12.4	7.8	4.3
Ca (dry wt)/%		2.58	2.53	~~2.40~~ 2.34	1.65	1.80	1.22
Roots	**Box**	**A1**	**B1**	**C1**	**D1**	**E1**	**F1**
Applied Pt/ppm		0.05	0.50	2.50	5.00	10.00	3.00
Wet weight/g		11.60	11.23	7.00	3.47	1.11	0.32
Dry weight/g		0.720	0.764	~~0.560~~ 0.540	0.318	0.170	0.066
Water present/%		93.8	93.2	92.3	90.8	84.7	79.2
Total Pt/μg		12	480	950	1425	1625	850
Pt (dry wt)/ppm		17	630	1760	4480	9560	12880
Total Ca/mg		6.5	5.9	4.6	2.1	1.3	0.43
Ca (dry wt)/%		0.90	0.77	0.85	0.660	0.76	0.65

TABLE II. Yields and Analysis of Corn Plants Treated with $(NH_4)_2[PtCl_6]$.

Tops	Box	A2	B2	C2	D2
Applied Pt/ppm		0.05	0.50	2.50	5.00
Wet wt/g		7.76	10.486	4.446	4.557
Dry wt/g		0.473	~~0.762~~ 0.672	0.344	0.346
Water present/%		93.9	93.6	92.3	92.4
Total Pt/μg		2.5	3.0	4.5	8.5
Pt (dry wt)/ppm		~~140~~ 5.3	~~343~~ 4.5	~~1496~~ 13.1	~~3575~~ 24.6
Roots	**Box**	**A2**	**B2**	**C2**	**D2**
Applied Pt/ppm		0.05	0.50	2.50	5.00
Wet wt/g		3.137	3.716	2.003	3.056
Dry wt/g		0.178	0.219	0.201	0.308
Water present/%		94.3	92.2	90.0	89.9
Total Pt/μg		25	100	300	1100
Pt (dry wt)/ppm		140	343	1496	3575

5.0 ppm, roots were yellow and stunted; at 30 ppm there was no growth, severe yellowing and stunting of roots, and necrotic spots on the lower leaves.

For palladium treated plants, drop-off in yield occurs between the 0.5 and 2.5 ppm levels, the effect is similar to that found with platinum treated plants but less drastic.

At the concentrations investigated, rhodium had little effect on the growth of the plants.

Discussion

The effects of platinum are largely the same as those previously described for bean and tomato [1], where it was reported that the percentage of water in the plant, the yield and the calcium content decreased with increasing concentrations of platinum applied. Tables I–III show that this behaviour is observed for tomato, bean and corn plants treated

TABLE III. Yields and Analysis of Bean Plants Treated with $(NH_4)_2[PtCl_6]$.

Tops	Box A3	B3	C3	D3	E3	F3
Applied Pt/ppm	0.05	0.50	2.50	5.00	10.00	30.00
Wet wt/g	24.16	15.08	12.03	13.22	7.72	3.61
Dry wt/g	2.214	1.279	1.386	1.578	0.986	0.511
Water present/%	90.8	91.5	88.5	88.1	87.2	85.9
Total Pt/μg	1	5	9	40	28	65
Pt (dry wt)/ppm	0.4	3.9	6.5	25.3	28.4	127
Total Ca/mg	55	23.5	21.8	20.5	11.8	4.6
Ca (dry wt)/%	2.48	1.84	1.57	1.30	1.19	0.90

(continued on facing page)

TABLE III. *(continued)*

Roots	Box	A3	B3	C3	D3	E3	F3
Applied Pt/ppm		0.05	0.50	2.50	5.00	10.00	30.00
Wet wt/g		8.53	5.40	2.71	2.38	1.63	1.21
Dry wt/g		0.527	0.301	0.336	0.303	0.221	0.121
Water present/%		93.8	94.4	87.6	87.3	86.4	89.9
Total Pt/μg		40	340	880	1650	1400	3100
Pt (dry wt)/ppm		76	1130	2620	5440	6330	25560
Total Ca/mg		2.4	1.7	1.6	1.5	0.9	1.0
Ca (dry wt)/%		0.456	0.550	0.48	0.48	0.41	0.82

TABLE IV. Yields and Analysis of Tomato Plants Treated with $Na_2[PdCl_4]$.

Tops	Box	G1	H1	J1	K1
Applied Pd/ppm		0.05	0.50	2.50	5.00
Wet wt/g		36.88	38.50	8.037	7.340
Dry wt/g		2.325	2.317	0.624	0.572
Water present/%		93.7	94.0	92.2	92.2
Total Pd/μg		1.5	2.3	2.4	3.2
Pd (dry wt)/ppm		0.64	0.99	3.8	5.5
Roots	**Box**	**G1**	**H1**	**J1**	**K1**
Applied Pd/ppm		0.05	0.50	2.50	5.00
Wet wt/g		8.515	9.897	1.765	2.142
Dry wt/g		0.607	0.670	0.210	0.184
Water present/%		92.9	93.2	88.2	91.5
Total Pd/μg		40.0	149	525	3170
Pd (dry wt)/ppm		65.9	222	2504	17230

TABLE V. Yields and Analysis of Corn Plants Treated with $Na_2[PdCl_4]$.

	Tops G2	H2	Roots G2	H2
Applied Pd/ppm	2.5	5.0	2.5	5.0
Wet wt/g	4.187	5.439	2.029	3.056
Dry wt/g	0.306	0.404	0.190	0.297
Water present/%	92.7	92.6	90.7	90.3
Total Pd/μg	1.5	4.5	410	2150
Pd (dry wt)/ppm	4.9	11.1	2160	7237

with $(NH_4)_2[PtCl_6]$, in addition the platinum taken up by the plants increases as the contraction of applied platinum is increased. In all three plants platinum is translocated to the leaves. The roots appear to extract platinum from solution, and the high levels shown by tomato and bean roots at 5.0, 10.0, and 30.0 ppm Pt, when little of no root growth takes place, could be explained by the precipitation of platinum species in the free spaces of the roots. This

TABLE VI. Yields and Analysis of Tomato Plants Treated with $Na_3[RhCl_6]$.

Tops	Box	L1	M1	N1	P1
Applied Rh/ppm		0.05	0.50	2.50	5.00
Wet wt/g		12.80	34.08	36.85	37.19
Dry wt/g		0.857	2.025	2.309	2.149
Water present/%		93.0	94.0	93.7	94.2
Total Rh/μg		0.75	10.5	46.0	65.0
Rh (dry wt)/ppm		0.87	5.2	19.9	30.3
Roots	**Box**	**L1**	**M1**	**N1**	**P1**
Applied Rh/ppm		0.05	0.50	2.50	5.00
Wet wt/g		4.944	6.554	8.651	7.676
Dry wt/g		0.259	0.418	0.532	0.500
Water present/%		94.8	93.6	93.9	93.5
Total Rh/μg		10.0	142	450	385
Rh (dry wt)/ppm		38.7	339	847	771

TABLE VII. Yields and Analysis of Corn Plants Treated with $Na_3[RhCl_6]$.

	Top L2	M2	Roots L2	M2
Applied Rh/ppm	10.0	30.0	10.0	30.0
Wet wt/g	13.44	8.134	4.406	3.632
Dry wt/g	0.783	0.540	0.261	0.247
Water present/%	94.2	93.4	94.1	93.2
Total Rh/μg	22.3	45	45	108
Rh (dry wt)/ppm	28.4	83.3	173	438

precipitation would cause their yellow colour. This point is being further investigated. In the course of the experiments the percentages of the total platinum removed by the tomato plants was: A, 14%; B, 29%; C, 14%; D, 5%; E, 3%; F, 1%. They are thus reasonably efficient at the removal of platinum from dilute solution.

Tables IV and V show the results from the treatment of tomato and corn plants with $Na_2[PdCl_4]$.

The limited results indicate that there is some transfer to the leaves, and that like platinum, palladium is deposited in the roots.

Preliminary results for rhodium, applied as Na_3RhCl_6 are shown in Tables VI and VII. Tomato plants are not affected by rhodium at the concentrations studied, and the possible toxicity at higher concentrations is being investigated. There is some transfer of metal to the leaves, but much less metal is deposited in the roots than with either platinum or palladium.

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Complex Formation Between Copper and Organic Substances Extracted from a Copper Contaminated Bog

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The Dolfrwynog Bog in the Coed-y-Brenin Forest, Gwynedd, Wales is mineralised by copper from the Coed-y-Brenin porphyry copper deposit [1, 2]. The distribution of plant species in the bog and their relationships to geochemistry and other environmental factors have been discussed [2, 3]. Some species have been shown to withstand the very high, usually toxic, copper levels in the soil [3]. An important factor which has an effect on the toxicity of metals in soils is the presence of organic complexing agents. For example Dykeman and de Sousa [4] observed that although copper was present in a bog at concentrations up to 7%, it had no visible effects on vegetation. This they related to the chelation of the copper by organic matter in the soil. Humic and fulvic acids from soils and sediments have been widely investigated but their specific structures have not yet been established. It is generally accepted that these substances control the availability of metal ions to plants by chelation [5].

In this paper we report two experiments carried out on the bog material from Dolfrwynog. The first experiment investigated the influence of the bog extract on the removal of copper from other soils. The second gave an indication of the thermodynamic stability of the complex or complexes formed by Cu^{2+} and organic substances in the extract. The methods were similar to those of Miller and Ohlrogge [6].

Experimental

Preparation of the Bog Extract

A bog sample together with its attendant surface liquid was collected. The solid material was filtered off, and washed to give a total volume for the filtrate plus washings of 1100 cm^3, this had a pH value of 7.5 compared with 5.5 for the bog liquid measured *in situ.* The filtrate was concentrated using a rotary evaporator to about one third of this volume. The solid material was Soxhlet extracted with water at 70–80 °C for 5 h. The concentrated filtrate and the extract were combined to give a volume of 820 cm^3. This was used in the following experiments as the 100% bog extract.

Preparation of Copper Containing Soils

Two soils were treated with copper and the capacity of the bog extract for removing copper from these soils was tested. Two soils, a loam from Regent's Park, London, and a clay loam from Sonning Common, Berkshire, were used. Both soils had low organic and negligible copper content.

In each case 20 g soil, which had been dried at 40 °C and ground in a ball mill, was mixed with solid $CuSO_4 \cdot 5H_2O$ (400 μmol of Cu^{2+} per g soil). 250 cm^3 water was added, and the mixtures were shaken for 3 days, 6 h per day. The soils were filtered off and washed several times with 2 *M* KCl solution. After numerous washings a very low constant level of copper wash leached out at each washing, this was taken into account in the subsequent calculations. The copper fixed in the soils was calculated by subtraction of the amount removed in the combined washings from that in the original application.

All water used in the preparations was de-ionised and doubly distilled. Copper analyses were carried out by Atomic Absorption using standard methods.

Removal of Copper from the Soils by the Bog Extract

2 g samples of the copper treated soils were placed in plastic vials. To these were added 1.0 *M* KCl (10 cm^3) to maintain ionic strength and 10 cm^3 of bog extract of various dilutions. The suspensions were shaken for 3 h per day for 3 days and the solid was filtered off. The copper contained in the filtrates was determined and the results are shown in Fig. 1.

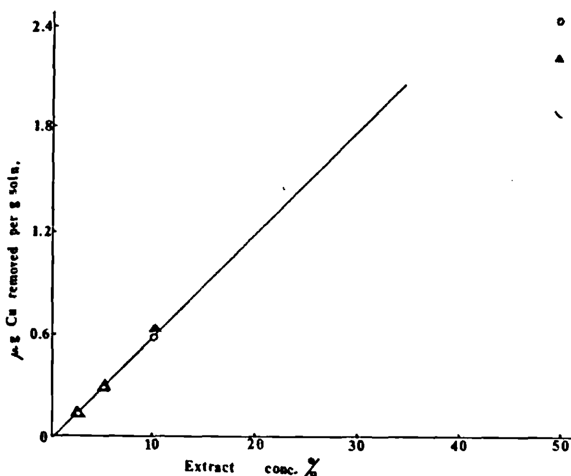

Fig. 1. Copper extracted from two soils by bog extract solutions. Δ, Regent's Park loam; O, Sonning Common clay loam.

Complex Formation by the Bog Extract

The stability of the complex formed between the constituents of the bog extract and copper was found by using an ion exchange method. The stability constant of the complex or complexes was determined using the relationship [6]:

$$\log_{10}(\lambda_0/\lambda - 1) = \log_{10}K + x\log_{10}[Ch]$$

where: λ_0 = distribution constant in the absence of complexing agent

λ = distribution constant in the presence of complexing agent

K = stability constant of complex

x = number of mol of complexing agent combining with one mol of metal

[Ch] = concentration of complexing agent in mol dm^{-3}

The distribution of copper ions between an ion exchange resin and the bog extract was determined. 1 g cation exchange resin (Zerolit 225, SRC 15 SO_3-H^+) acid and base washed and finally saturated with K^+ ions, was placed in a plastic vial together with 10 cm^3 of the test extract and 10 cm^3 1.0 *M* KCl solution. All mixtures had a pH of 6.0 and a copper content of 3.0 ppm. The vials were shaken for 4 h, the resin was allowed to settle and the supernatant was analysed for copper. The distribution of copper between the resin and solution was found for each dilution of bog extract.

Results and Discussion

Removal of Copper from Soils by the Bog Extract

The relationship between the concentration of the extract and the amount of copper removed is shown in Fig. 1. At low extract concentrations similar quantities of copper were removed from both soils and a linear relationship was obtained. Removal of the Cu^{2+} ions must involve an equilibrium process in which the metal ions are exchanged between soluble and insoluble complexing sites. The straight line also indicates that at high dilutions of the extract, all the complexing agent is in the form of its copper complex. The concentration of the complexing agent in the extract can thus be calculated. The 10% extract removed 0.61 ppm of the copper per cm^3, therefore 1 cm^3 of the 100% extract would complex with 6.1 μg of copper. The concentration of complexing agent in 1 cm^3 of the 100% extract is 0.96 × 10^{-7} mol; the 100% extract is 0.96 × 10^{-4} mol dm^{-3}. The assumption is made that the complex is 1:1 metal: ligand.

Removal of Copper by Ion Exchange

The data for removal of copper from soluble complexes of bog extract is shown in Fig. 2 and Table I. Fig. 2 shows the plot of $\log(\lambda_0/\lambda - 1)$ against the logarithm of the dilution of the bog extract. The slope and thus x in the equation above is 1.15, showing that the assumption of a 1:1 complex is reasonable. LogK values for the copper complex were

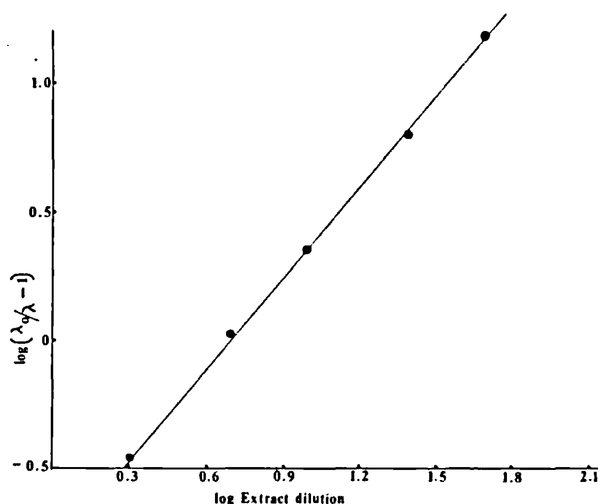

Fig. 2. Determination of the mol ratio of copper to complexing agent in bog extract.

TABLE I. Removal of Copper from Cu^{2+}/Bog Extract Mixture by Cation Exchange Resin at pH 6.0 (Total Cu, 3.0 ppm).

Dilution of Extract %	Cu removed ppm	$\log(\lambda_0/\lambda - 1)$	[Ch] mol dm^{-3}	LogK
0	2.67			
2	2.57	−0.4514	−5.717	6.12
5	2.39	0.0274	−5.319	6.14
10	2.15	0.3422	−5.018	6.11
25	1.60	0.7839	−4.620	6.09
50	1.02	1.1675	−4.319	6.13

then calculated. The values of log[Ch] were obtained for each dilution of the bog extract using the value of 0.96×10^{-4} mol dm^{-3} for the 100% extract obtained above. These experiments demonstrate that an extract of the bog material was able to extract copper from soils and that the complex formed had an apparent logK value of 6. Miller and Ohlrogge have shown that ligands from manure render zinc and copper in forms which are less available to plants than are the simple aquo ions. Thus there is a balance between the ability of complexing agents in soils to solubilise metal ions and to sequester them. Ligands in bogs and mires may trap inorganic pollutants and decrease their toxicity. The ion exchange capacity of bogs has been discussed by Given [5, 7], it depends largely on the humic and fulvic acid content which in turn is related to the degree of decomposition of the plant material. The Dolfrwynog Bog contains rotting leaves and roots [2], which decompose and provide sites for the uptake of copper which is in solution. Further work on the metal cycle in the bog and the nature of the complexing agents is being undertaken.

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Plants which Accumulate Metals. Part IV. A Possible Copper–Proline Complex from the Roots of *Armeria Maritima*

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Metal complexes from plants have been identified in a number of cases. Recently iron has been found as the citrate [1], nickel in malic and malonic acid complexes [2], and zinc as the galacturonate [3]. This paper presents a study of the copper complex of a ninhydrin positive ligand in the water extract from the roots of *Armeria maritima*. The *Armeria* plants were collected from a mineralised bog near Dolgellau, Wales, and are highly copper tolerant [4].

Experimental and Results

Detection Reagents for Copper

The water extracts from *Armeria maritima* contain both copper and zinc. The location agent PAN (α-pyridylazol β-naphthol) was used since it is very sensitive for copper and distinguishes between copper and zinc (violet for copper and pink for zinc).

Extract Preparation

Root stocks of *Armeria maritima* were cleaned, dried and powdered as before [4]. 2 g of the powdered material was shaken for 6 hours at room temperature with 200 cm^3 of demineralised water. The solid material was filtered off using a millipore filter, and re-extracted twice. The three extracts were combined, freeze dried and stored in a sealed container in the refrigerator until used. A minimum volume of demineralised water was added before the chromatographic experiments.

Chromatography

Preliminary experiments showed that a complex of copper was present and that in both strongly acid (methylethylketone–conc. HCl–water, 25:3:2 by volume) and in basic (butanol–pyridine–water, 1:1:1 by volume) media, the complex undergoes decomposition. However, in phenol–water (4:1 weight: volume) decomposition did not appear to take place. The solvent system n-propanol–water (1:1 by volume) was chosen as the second solvent (Table I). Other combinations of solvent and papers or thin layer sheets produce dissociation.

TABLE I. R_f Values of Copper in Water Extract of *Armeria maritima*, Detected by PAN.

Spots Applied	Whatman No. 1 phenol–water	MN 300 cellulose thin layer n-propanol–water
Extract	93	37
Cu^{2+} Ions	0	Streaks

TABLE II. Amino Acid Analysis of Root Extract of Copper-tolerant *Armeria maritima* Expressed as Percentage of Total of Ninhydrin Positive Material.

Alanine, 1.08; Argenine, 6.50; Asparagine, 10.80; Aspartic acid, 2.60; Glutamic acid, 7.70; Glutamine, 7.70; Histidine, 0.94; Isoleucine, 0.47; Leucine, 0.42; Lysine, 1.96; β-Phenylalanine, 1.35; Proline, 40.45; Serine, 1.16; Threonine, 2.54; Tyrosine, 0.58; Valine, 1.57 (also ammonia, 7.42; five unknowns, 4.74).

TABLE III. Comparison of the Chromatographic Behaviour of the Root Extract with that of Copper Proline/Proline Mixtures on Whatman No. 1 Paper.

Spot Applied	Phenol–Water	n-Propanol–Water	
	R_f with PAN	R_f with PAN	R_f with isatin
Cu^{2+} ions	0	Streaks	–
Proline	–	–	61
Extract	94	64	64
Cu-proline	96		
Proline/Cu-proline 2.5:1	96	71	71
Proline/Cu-proline 5:1	95	70	70
Proline/Cu-proline 10:1	94	62	62

The preliminary investigations thus indicated that a ninhydrin positive ligand was present. The uncomplexed ninhydrin positive compounds were removed as follows. Streaks of the concentrated extract were placed on five sheets of Whatman 3MM chromatography paper. These were developed in the ascending mode with phenol–water solvent. Thin strips were cut from the sides of the papers and sprayed with PAN. Three bands were identified: Band 3, R_f = 93 ± 5; Band 2, R_f = 63 ± 5; Band 1, R_f = 46 ± 1. Bands 1 and 2 were due to zinc complexes, the ratio of copper to zinc being 10:4 (metal analyses by AAS). The three bands were cut from the papers and were eluted with demineralised water,

using descending chromatography. The extracts were concentrated and rechromatographed.

The copper containing extract was further purified by Sephadex G25 gel filtration using water as eluent.

Sequential extraction [4] has already indicated that the copper was not associated with water soluble proteins. These were therefore removed [5] from a sample of the crude extract, and the remaining water soluble amino acids were analysed. The results are shown in Table II.

Solutions of marker complexes of the ten most plentiful amino acids in the extract were chromatographed. Complexes were made by mixing 0.01 *M* amino acid solution and 0.01 *M* copper chloride solution 2:1 by volume. Only the copper proline, copper valine and copper β-phenylalanine complexes had high enough R_f values to be considered. Chromatography of the purified extract indicated that the complex was copper proline, however the R_f value was below that of the marker complex. It was found that the R_f value of the copper proline complex depended on the amount of excess amino acid present (Table III).

Discussion

The results show that the sample of *Armeria maritima* investigated had very high concentrations of proline in the tissues of the roots. The results also indicate that the water soluble copper is associated with this proline. Reilly [6] has suggested that copper amino acid complexes exist in the water soluble extracts of the copper accumulator, *Becium homblei*, but the explanation of the copper tolerance of the plant must be looked for elsewhere. In the roots of *Armeria maritima* about 20% of the total copper appears in the water extract. In the leaves the figure is 12%. It has been suggested [7] that in human serum there is an equilibrium between copper bound to albumin and copper bound to amino acids, and that the biological role of this system is the transport of copper through membranes. It seems possible that copper amino acid complexes in plant systems may also play a part in copper transport. Most of the insoluble copper in *Armeria maritima* is in association with carbohydrates, and this possibly constitutes the storage mechanism. Work is at present being undertaken to find out if non-tolerant *Armeria maritima* from an unmineralised area (containing little copper) also has high concentrations of proline in the root tissues.

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