AN INVESTIGATION OF SOME AUSTRALIAN PLANTS WHICH ACCUMULATE TRANSITION METALS

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by

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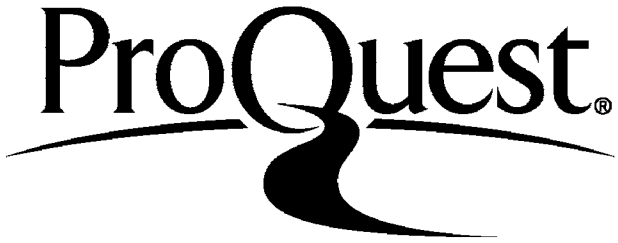

TO MY PARENTS

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ABSTRACT

Plant samples of three Australian species which grow on mineralized sites in Australia were investigated. In one of these, *Hybanthus floribundus*, extremely high nickel concentrations were found, particularly in the leaves, which were associated with pectic carbohydrates. Histochemical studies demonstrated a remarkable concentration of nickel in the epidermal layer which corresponded to those areas which gave a positive test for pectin. Mesophyll and palisade cells showed no evidence of nickel concentration, whereas the phloem elements gave a moderate staining. For the other species, *Triodia pungens*, high zinc was found in its tissues as well as high levels of the amino acid proline. Chromatographic studies show that zinc is associated with pectin and not with proline. Aerial parts of *Eriachne mucronata* had lower zinc concentrations than *Triodia pungens*, and the metal was water soluble and present apparently as zinc ion.

Chemical extraction schemes carried out on leaves of *Hybanthus floribundus* showed that 87% of the nickel was associated with pectic and similar polysaccharidic material, 3.34% was solubilized by the proteolytic enzyme pronase and 7.07% of the metal was removed with low molecular weight water soluble materials. For *Triodia pungens* the corresponding figures were: 76.37%, 3.43% and 16.13% respectively. Mechanisms by which the two plants tolerate their high metal burdens are suggested.

Complete amino acid profiles for the three species are reported and discussed.

Phenolic compounds present in *Hybanthus floribundus* were examined, these include a yellow pigment accumulated in large quantities by the plant. The probable structure of the pigment deduced from spectral measurements is suggested.

The interaction between pectin with Cu(II), Ni(II), Zn(II) and

Co(II) has been studied with the aid of ESR, NMR, electronic and IR spectroscopy.

CONTENTS

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Foreward

Many plant species and ecotypes have been found to grow and thrive on mineralized sites. They are remarkable in that they possess an ability to survive under conditions of metal contamination which would prove toxic to other plants. A large number of parameters is involved in the study of the mechanisms whereby plants tolerate and even accumulate large levels of toxic metals, and at present no clear overall picture has evolved, although some particular mechanisms seem to be established for some metals and certain plants under specified conditions. The existence of these species have caused considerable interest in the fields of mineral exploration because of the possible association of these plants with metals mineralization. The present study is an attempt to investigate the chemical forms and physical location of accumulation sites of heavy metals in certain plant species.

In the introduction a brief botanical background is given including sections on the structure of a typical plant cell, root morphology and chemical description of relevant cell structures. Further sections outline the roles played by nickel, zinc and other micronutrients in plant metabolism and the mechanism of their uptake followed by possible tolerance mechanisms and toxicity of heavy metals in plants. After this biological background the introduction continues with a survey of metal chemical bonding in plants.

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

CHAPTER I

INTRODUCTION

1.1 Cell Structure

In higher plants there are many kinds of cells with much variety in function, structure, arrangement, and with a great deal of complexity of cell wall structure.

In simple terms, however, most of the cellular organelles are contained in protoplasm, which consists of cytoplasm and, typically, one nucleus. Within the nucleus is at least one nucleolus, the chief function of which is to synthesize ribonucleic acid (R.N.A.). When the cell is not dividing, the chromosomes are dispersed in the nucleus as chromatin, which is mostly protein and deoxyribonucleic acid (D.N.A.). D.N.A. is the stuff of the genes, which are the units of heredity that control all inherited features of organisms through their ability to help form different kinds of R.N.A. molecules.

Within the cytoplasm, there are ribosomes, consisting partially of R.N.A. made in the nucleolus. These are the sites of protein synthesis, responding both to messenger R.N.A. arriving from the nucleus and to transfer R.N.A. The messenger R.N.A. carries the genetic information from the genes, and the transfer R.N.A. carries the amino acid that will form protein. Also, imbedded in the cytoplasm are mitochondria, where cellular respiration takes place. A principal function of mitochondria is to produce adenosine triphosphate (A.T.P.), which is the energy source for many of the processes going on in cells. There is an intricate network of membranes within the cytoplasm called the endoplasmic reticulum (E.R.). Some of the cellular metabolic activities are associated with enzymes located on the E.R. membranes, and these membranes are also involved in transport processes within the cell.

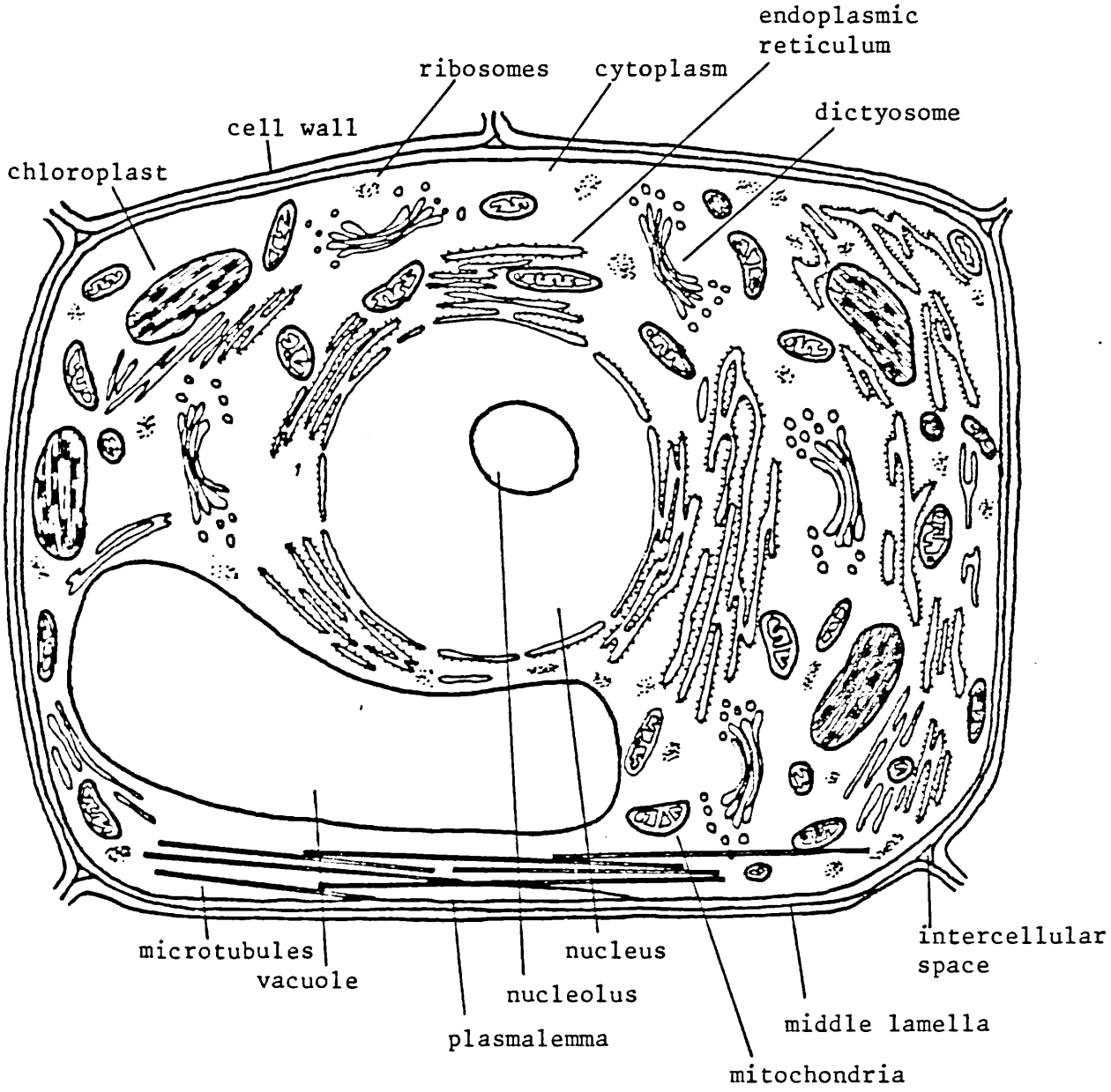

Figure 1. Cell wall and organelles of a plant cell

Cells have dictyosomes, which may be referred to collectively as the Golgi apparatus, and are concerned with cell wall synthesis and other important cellular functions. There are microtubules in the cytoplasm of virtually all cells, and these participate in the separation of chromosomes during mitosis as well as in cell wall formation and probably in other processes. Other organelles include plastids, which are round, oval or disc shaped bodies and are divided into: leucoplasts, which are colourless, and chromoplasts, which contain pigments. An example of a leucoplast is an amyloplast, which contains one or more starch grains. The most important example of a chromoplast is the chloroplast which contains chlorophyll and which is the site of photosynthesis.

The cell and all the organelles except for ribosomes are surrounded by membranes. In mature plant cells, there is a large membrane-bound volume of a dilute aqueous solution called the vacuole. It lies in the centre of the cell, interior to the cytoplasm, and usually occupies some 90% of the volume of a mature cell. The vacuoles are separated from the cytoplasm by the vacuolar membrane or tonoplast. The nucleus is separated from the surrounding cytoplasm by the nuclear membrane whereas the cell itself is bounded by a "cellulose" cell wall. Immediately within this latter wall lies a membrane, the plasmalemma, pressed against the cell wall by the turgor pressure of the cell's contents. Between the cell walls of adjacent cells there is a layer, rich in pectins, called the middle lamella.

Finally, all cells of the higher plants are interconnected by specialized structures of the plasmalemma called plasmodesmata. These are canals that penetrate through cell walls and appear to provide for intercellular movement of substances as well as to help equilibrate membrane potentials.

1.2 The Structure and Chemical Composition of Cell Walls

On the basis of development and structure, three fundamental parts may be recognized in plant cell walls: the intercellular substance or middle lamella, the primary wall and the secondary wall. The intercellular substance cements together the primary walls of two adjacent cells. Secondary walls, not present in all cells, are laid over the primary walls next to the lumen (the central cavity). Primary walls are laid down by young, undifferentiated cells that are still growing and the primary walls are transformed into secondary walls after the cell has stopped growing.

Early chemical analysis of primary walls, based primarily on relative solubilities in acids and bases, indicated that three major fractions existed. These fractions were identified as pectins (especially abundant in the middle lamella), hemicelluloses, and cellulose. Recently, it has been found that only cellulose is a fairly discrete chemical entity, while the other fractions contain rather complex mixtures of polymers of sugars and sugar derivatives.

In all primary walls of angiosperms and gymnosperms, polymers of only five sugars and five sugar derivatives occur in appreciable quantities. The five sugars are the three hexoses D-glucose, D-mannose, and D-galactose and two pentoses, D-xylose and L-arabinose. The sugar derivatives include two uronic acids, D-glucuronic acid and D-galacturonic acid, and two deoxy sugars, L-rhamnose and L-fucose, as well as the methyl ester of galacturonic acid. The nonesterified carboxyl groups of D-glucuronic acid and D-galacturonic acid are largely ionized in the walls and are associated with cations, especially calcium and magnesium. Except for glucose, these sugars and the derivatives are almost never found in the free or uncombined form in plant cells.

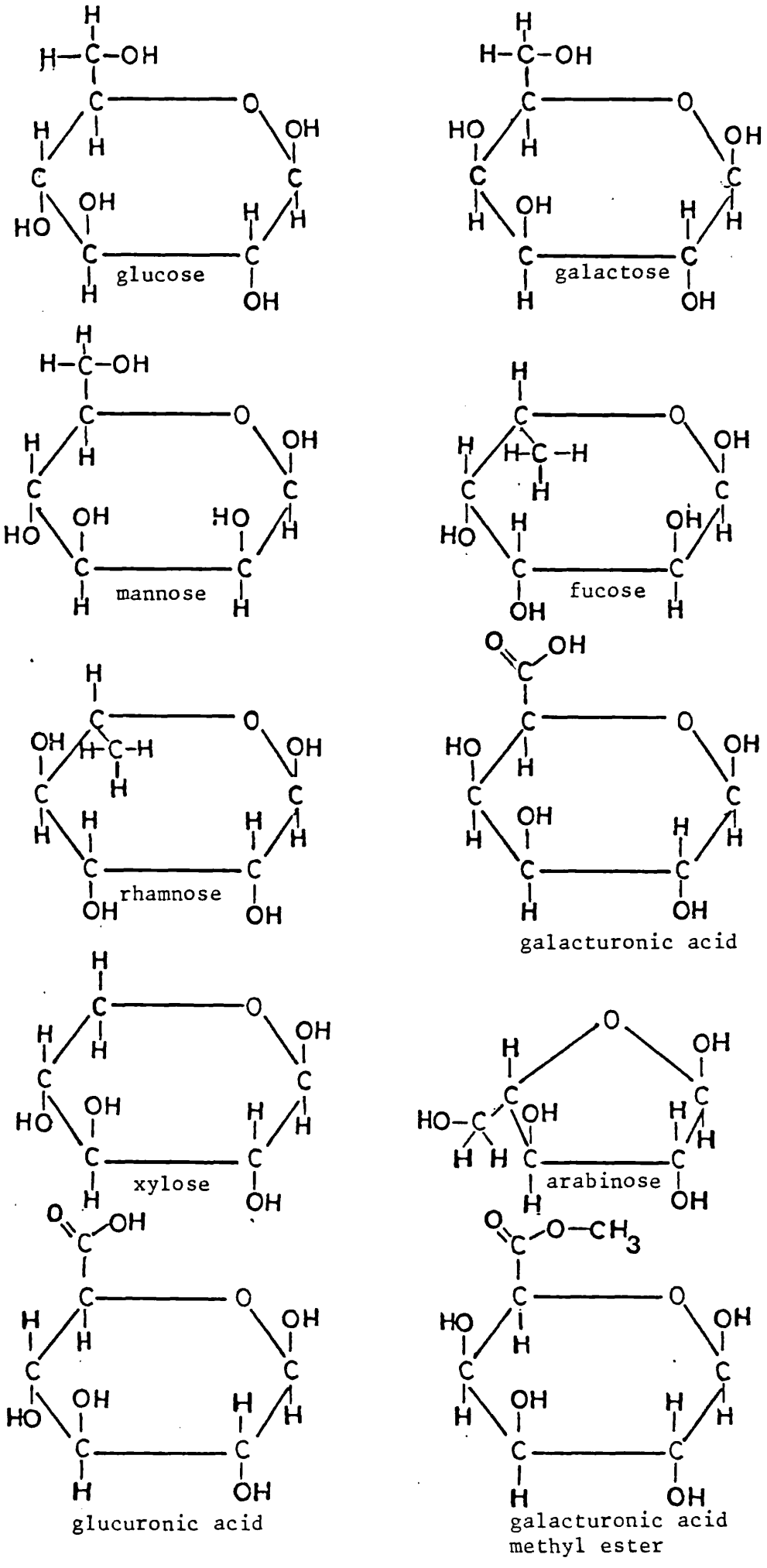

Figure 2. The sugars and sugar derivatives found in primary wall polysaccharides

Although formerly proteins were not considered to be part of primary cell walls, recently it has been found that glycoproteins are always present. Proteins comprise at least 10% of the weight of most primary walls. The functions of these wall proteins are not yet understood. They might represent structural proteins or enzymes involved in assembling polysaccharides into the wall framework.

The least complex wall polysaccharide is cellulose. Cellulose molecules are assembled from a variable number of D-glucose units, frequently 2,000 or more in primary walls (and at least 14,000 in some secondary walls). Approximately 40 cellulose chains, are held to each other by hydrogen bonds and packed together along their long axes to form microfibrils. These microfibrils are composed of long chains of β (1-4) linked glucose residues. Embedded between the microfibrils and forming a more nearly amorphous matrix lie most of the other polysaccharides, the classical pectin and hemicellulose fractions, and the wall protein.

Albersheim (1973) found that the walls of the dicotyledon *Acer pseudoplatanus* (sometimes called sycamore-maple) consist of 23% cellulose, 21% xyloglucan (a xylose-glucose hemicellulose polymer), 20% arabinogalactan (an arabinose-galactose hemicellulose polymer), 16% rhamnogalacturonan (a rhamnose-galacturonic acid pectin polymer) and 10% of a protein rich in the unusual amino acid hydroxyproline. Albersheim (1976) suggested a model to explain how the various constituents of primary walls are assembled to form the whole wall. The xyloglucan appears to be hydrogen bonded to hydroxyl groups along the cellulose chains, while all other constituents are covalently bonded to each other:

-cellulose-xyloglucan-arabinogalactan-rhamnogalacturonan-
arabinogalactan-protein-arabinogalactan-rhamnogalacturonan-
arabinogalactan-xyloglucan-cellulose-

Each cellulose microfibril is therefore hydrogen bonded on either side to a xyloglucan, so an entire network or "giant molecule" is produced,

which suggests that the cell walls of plants could be composed of a limited number of well-defined structural components and not just a relatively large number of complex polymers.

1.3 The Root Structure

The roots of plants are engaged in many physiological and metabolic activities that are common to all plant tissues. They grow and develop, they respire, they synthesize and degrade various compounds. Figure 3 shows a longitudinal section of a root tip. The layer of somewhat flattened cells on the outside of the cortex is called the epidermis. Some of the cells of the epidermis develop long projections called root hairs, which usually have a pectic coating, allowing them to adhere to soil particles. Water and inorganic salts can pass easily through the outer portion of the root, the cortex, where the cellulose walls and intercellular spaces are freely available to aqueous solutions. The cortex is bounded on the inner surface by the endodermis, which usually comprises a single annular layer of cells. These are distinguished by a layer of suberin impregnating the radial walls of each cell (the Casparian strip) which is believed to prevent movement of water and solutes within the cell wall towards the stele. The centre portion of the root, the stele, contains relatively undifferentiated tissue, the pericycle, and two systems of connecting vessels; the xylem which conducts water and mineral salts and the phloem which conducts organic substances. The xylem and phloem together form a continuous vascular system throughout all parts of the plant. Xylem elements are formed from specialized elongated cells which lie adjacent to each other in single files. The walls between the cells break down, and a continuous vessel is formed through which solute flow can take place unimpeded.

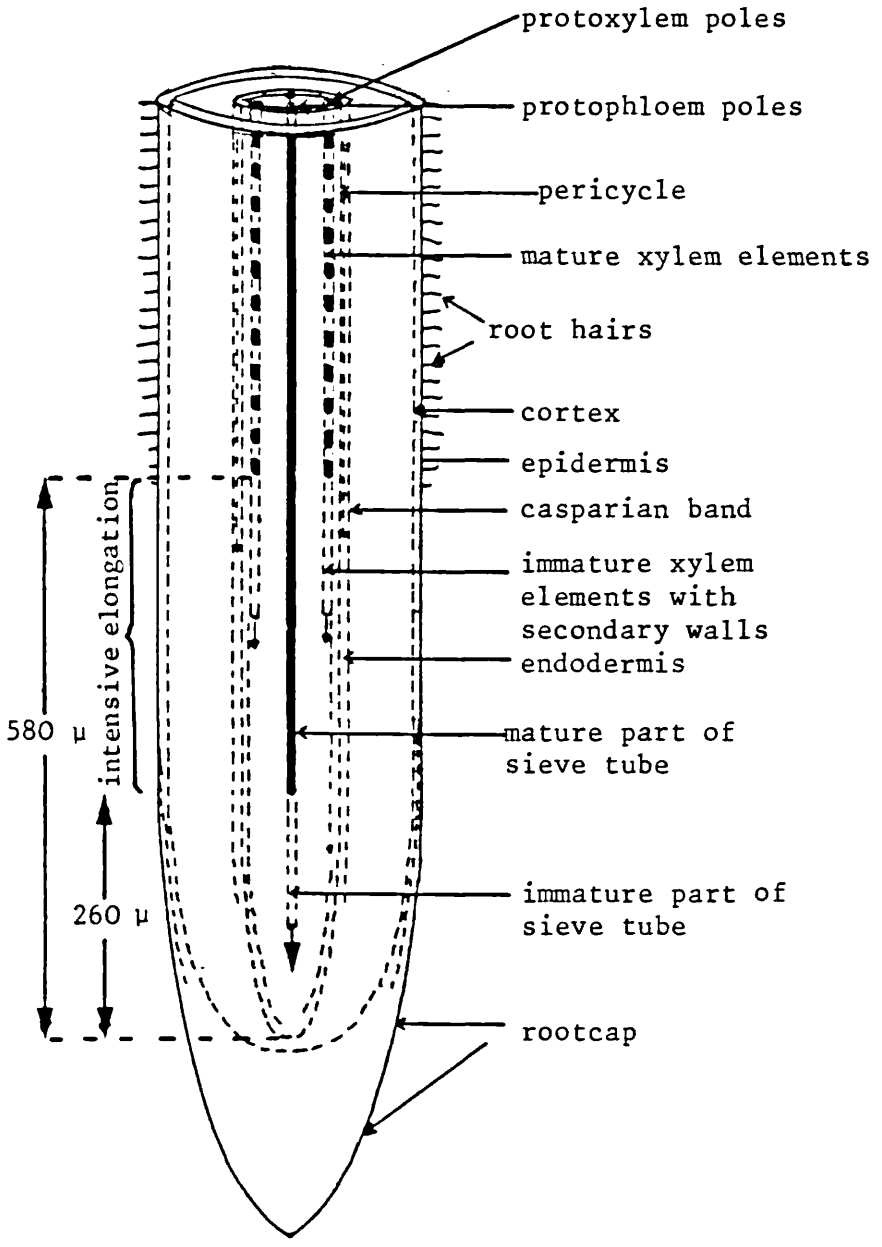

Figure 3. Longitudinal section of a root tip (Esau, 1960)

1.4 Essentiality

There are two principal criteria by which an element may be judged essential or nonessential to any plant (Epstein, 1972). Firstly, an element is essential if the plant cannot complete its life cycle in the total absence of the element. Secondly, an element is essential if one can show that it forms part of any molecule or constituent of the plant that is itself essential (for example: N in proteins, Mg in chlorophyll).

The elements so far found to be essential by these criteria are molybdenum, copper, zinc, manganese, boron, iron, chlorine, sulphur, phosphorus, magnesium, calcium, potassium, nitrogen, oxygen, carbon and hydrogen. The first seven elements are often referred to as the trace elements, minor elements or micronutrients (needed in tissue concentrations equal to or less than 100 µg/g dry matter), and the last nine as macronutrients or major elements (needed in concentrations of 1,000 µg/g dry matter or more).

Besides the above 16 essential elements which may be required by all plants, some species require others. For example, Brownell and Wood (1957) showed that sodium is required by certain desert species such as *Atriplex vesicaria*, common to dry inland pastures of Australia.

Silicon is another element that enhances growth of some plants when added to the culture solution (Lewin, 1969). Corn and several other members of the grass family accumulate the element to the extent of 1 to 4% of their dry weight, while rice and *Equisetum arvense* contain up to 16% silicon. Cobalt is also believed to be required for nitrogen fixation by micro-organisms in root nodules on legumes (Evans *et al.*, 1965). Some plants may also prove to require nickel, because this metal is a tightly bound part of the enzyme urease (Dixon *et al.*, 1976), but it is not yet certain that urease itself is essential to plants. Another element perhaps essential for a few species is selenium.

1.5 Nickel and Zinc Metabolism

The functions of essential elements have sometimes been classified into two groups:

(1) a role in the structure of an important compound;

(2) a requirement to stimulate the rate at which a chemical reaction occurs (enzyme-activating role) (Rains, 1976).

Frequently, however, there is no sharp distinction between these functions, because several elements form structural parts of enzymes and help catalyze the chemical reaction in which the enzyme participates. An example of an element with both roles is magnesium; it is an essential part of chlorophyll molecules and it also activates many enzymes.

Some ions perform still another function by contributing to the osmotic potentials of plant cells, thus allowing the build-up of turgor pressure necessary to maintain form and to allow certain pressure-dependent movements (i.e. stomatal opening).

Since this thesis is concerned mainly with the tolerance of plants to nickel and zinc, the functional role of these elements will be considered briefly here.

Nickel is not considered to be an essential element in plant metabolism, although most plants probably contain small amounts which are taken up during the course of metal absorption. An excess of nickel in plants will produce chlorosis and necrosis. Iron metabolism is upset if the concentration of nickel is too high, as is the case with toxic amounts of other heavy metals. The iron is replaced by the toxic metal and this results in an iron deficiency. Crooke *et al.* (1954) showed that both uptake and symptoms of toxicity are reduced if there is a high concentration of iron in a nutrient solution. Nickel will also interfere with the uptake of major nutrients and has been found to lower the yield of dry matter. In the presence of nickel, calcium absorption is increased and it has been found to reduce toxicity symptoms produced by nickel (Hunter and

Vergnano, 1952). For some plants nickel is possibly an essential element, since urease from jack beans has been found to be a nickel metalloenzyme (Dixon *et al.*, 1975).

Zinc is a trace element which is essential for proper plant growth and development. It is probably related to the formation of chlorophyll, because a fairly high proportion may be found in the chloroplasts. Zinc is essential for activity of the enzymes alcohol dehydrogenase and lactic acid dehydrogenase (function in anaerobic respiration). Glutamic acid dehydrogenase (crucial to nitrogen metabolism) seems to contain essential zinc; and it is present in carbonic anhydrase, a chloroplast enzyme that catalyzes the reaction between CO_2 and H_2O to form H_2CO_3. Zinc also has a possible role in the synthesis of the amino acid tryptophan (Tsui, 1948) and is essential in the biosynthesis of indoleacetic acid (auxin), so that zinc deficiency has a drastic effect on normal growth of a plant, giving rise to 'little leaf' and 'rosetting'. An effect of zinc not duplicated by magnesium (although magnesium also participates) or other divalent cations (such as manganese, copper or calcium) is to maintain the structure of ribosomes, thus allowing protein synthesis.

1.6 Uptake of Ions by Plants

Amongst the functions that a root must perform in plants is the absorption of nutrients and water from the soil solution. That is accomplished by the cells at or near the root surface: the outermost epidermal cells and the cortical cells. Nutrient ions in the soil solution can diffuse right into the porous cell-wall spaces of the epidermis and cortex. Further diffusion into the root is blocked at the endodermis where the Casparian strip acts as an effective barrier. In addition, the ions cannot diffuse into the cells of the cortex due to the presence of the plasmalemma which is a permeability barrier that does not allow charged particles such as mineral ions to diffuse through it. However, ions can

be taken up into useful metabolic sites within the plant, by active transport via a carrier across the membrane. The mechanism by which active ion transport takes place is not fully understood. But the most widely accepted theory is that subunits of the membrane called carriers bind the ion at the external face of the membrane, forming a carrier-ion complex. This complex then traverses the membrane, rotates, or undergoes some other spatial rearrangement whereby the ion is brought to the inner surface of the membrane. The complex then breaks down as a result of a change in molecular configuration of the carrier so that the ion is released and the carrier then repeats the cycle (Epstein, 1952).

Once inside the cell cytoplasm, some of these ions are further absorbed into the vacuole, where they contribute greatly to the negative osmotic potentials of roots, facilitating water uptake, turgor pressure, and growth of the roots through the soil. But the vast majority of them move, via cytoplasmic connections called plasmodesmata (tubular structures formed across adjacent cell walls) from one cell to another towards the stele. This system is called the symplasm. To reach a xylem vessel the travelling ions must cross the stelar cell plasmalemma. The latest theories on this subject suggest that the membranes of stelar cells possess "reverse" carrier mechanisms, thus transporting ions from the cytoplasm into the porous cell-wall space beyond. Once there the ions are free to move into the xylem vessels by simple diffusion, via the transpiration stream, to other plant cells (Yu, 1967).

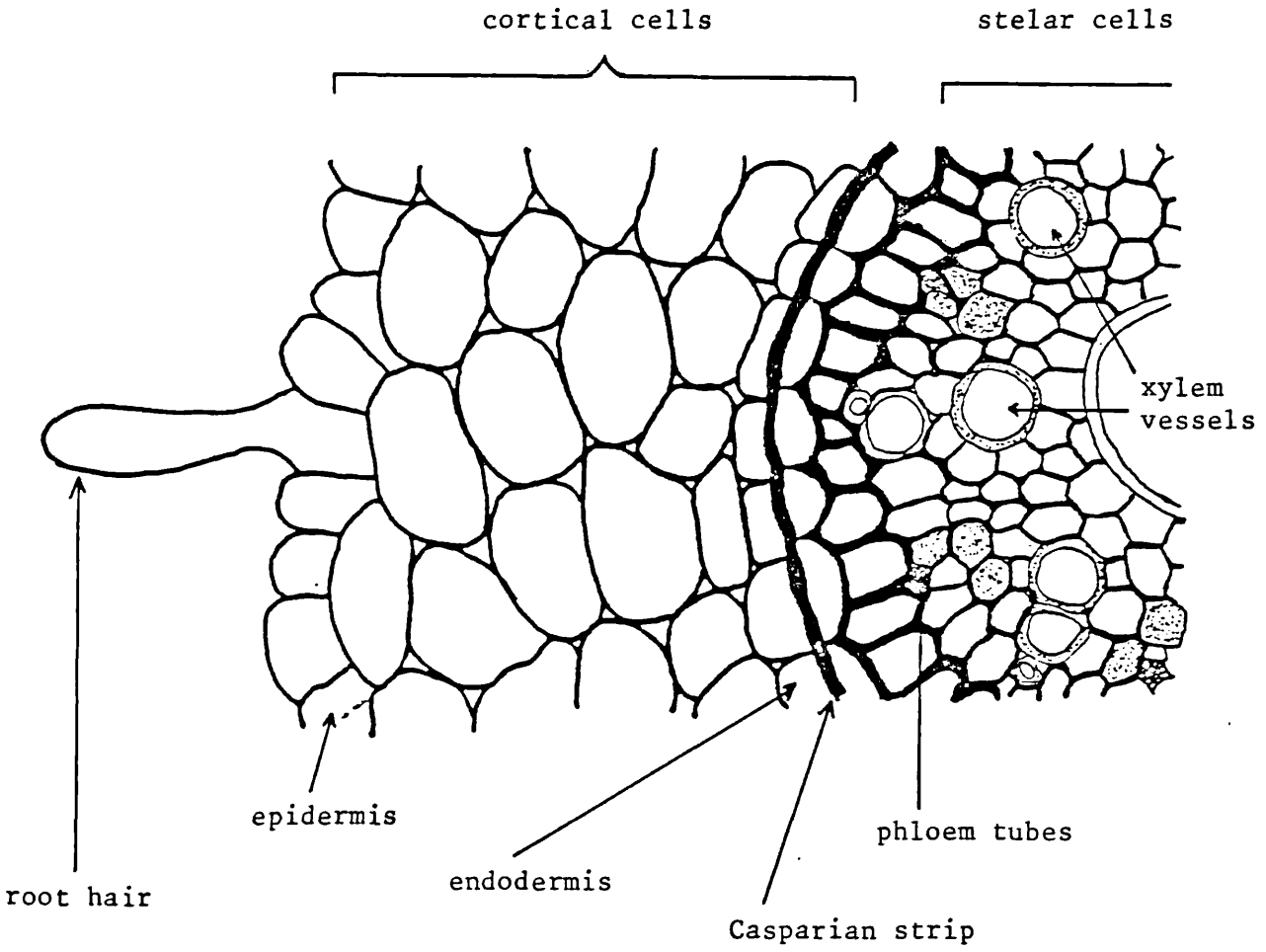

Figure 4. Root cross section

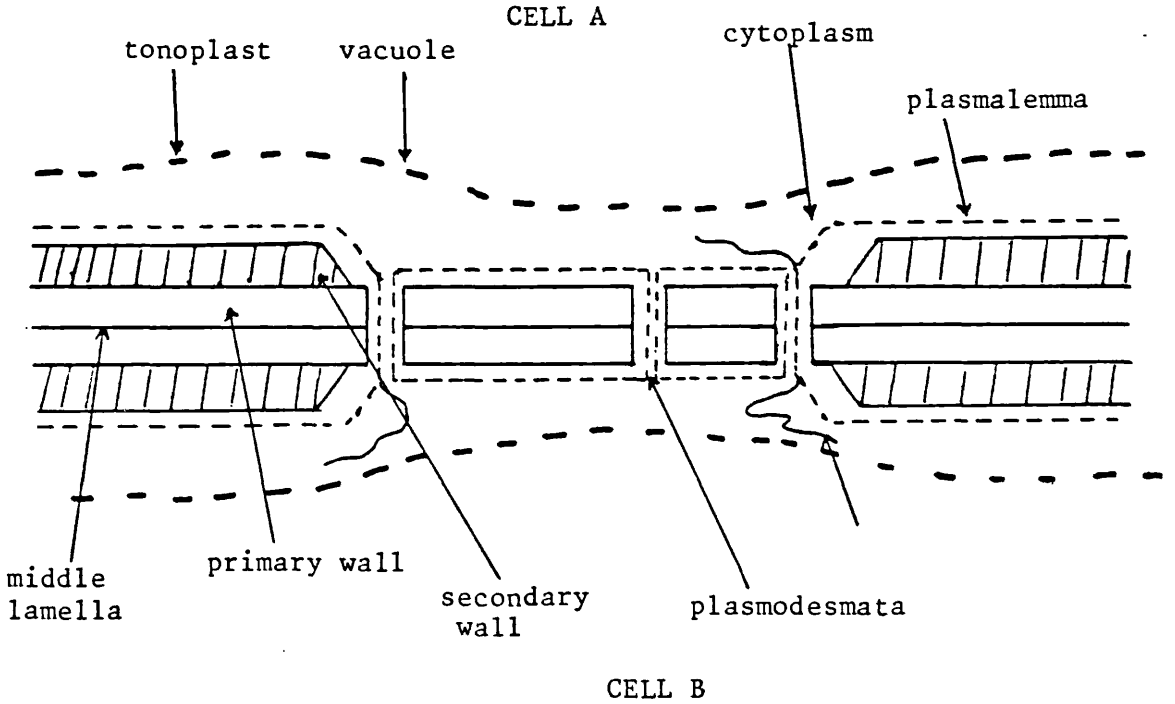

Figure 5. Diagram showing the cytoplasmic connections between cells

1.7 The Kinetics of the Uptake of Ions

The active transport mechanism is highly selective and irreversible and it obeys the Michaelis-Menten equation, i.e.

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

where V_{max} is the maximum rate of absorption, [C] is ionic concentration and K_m is the Michaelis constant (the concentration of the ion giving half the maximum rate of absorption).

The above equation indicates that the rate of ion uptake is a function of the concentration of the ion and is dependent on there being no counterflow. Many experiments have yielded results which conform to Michaelis-Menten kinetics (Epstein, 1972). At relatively low ionic concentrations the change of rate of absorption decreases with increasing ion concentration until V_{max} is reached. The carrier sites at this point could be considered saturated and further increase of concentration of metal in the solution produces no further increase in rate of metal uptake. However, on substantially increasing the ionic concentration beyond this point, a further increase in the absorption rate takes place. Epstein (1972) suggested that there are two separate uptake mechanisms, one of which is effective at low concentrations and the other at high concentrations. (Figure 6). Barber (1972) has suggested that mechanism 2 (at high concentration) is a passive diffusion of ions. There has been considerable controversy over the location of these two processes. Laties (1969) claims they are located at the plasmalemma and the tonoplast. Epstein (1972) suggested that the processes proceeding by these two mechanisms are sited at the plasmalemma. From the Michaelis-Menten equation it can be seen that the rate of absorption is dependent on three main factors: [C], V_{max} and K_m. In strictly controlled hydroponic conditions the ionic concentration in solution is invariable, but the other factors vary

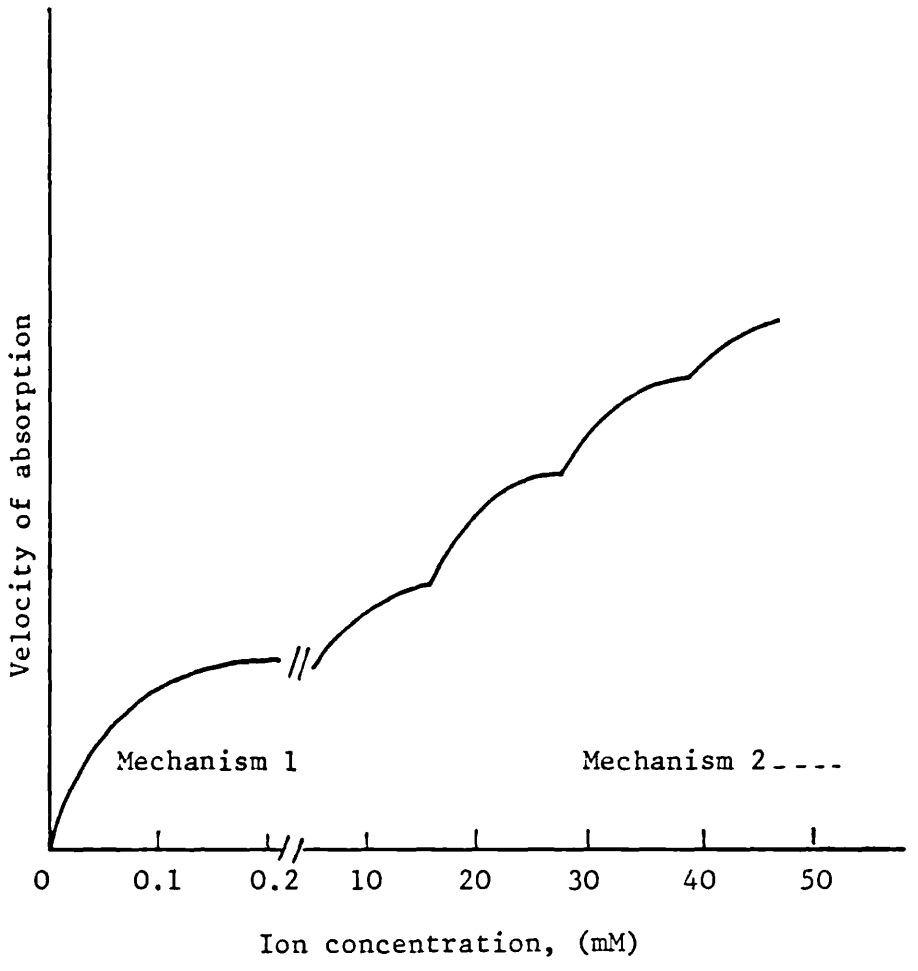

Figure 6. Generalized uptake isotherm for ions into roots, showing mechanisms 1 and 2 (after Hodges, 1973)

according to root radius and length, temperature, competing ions, oxygen availability, effects of other plants, transpiration, water stress and light.

1.8 Toxicity

The heavy metals, defined by Passow *et al.*, (1961) as those metals having a density greater than five gcm^{-3}, include about thirty-eight elements. Their common feature in relation to biological life is that in excessive quantities they are poisonous and can cause death of most living organisms. Excessive concentration of some metals in soils, producing toxic symptoms, may come about in a variety of ways. They

may be the result of natural mineralization caused by the presence of undisturbed ore bodies near the surface, known as geochemical anomalies. High concentrations of metals may be the result of the exploitation of mineral resources, e.g. mining activities, ore tailings, tips, smokes and dust. Lastly high concentrations can result from agricultural and waste disposal practices.

Somers (1960) showed that there is an approximate relationship between the toxicity of an element and its electronegativity. For divalent metals the order of electronegativity decreases in the sequence: mercury, copper, tin, lead, nickel, cobalt, cadmium, zinc, manganese, magnesium, calcium, strontium, and barium. The more electronegative metals such as copper, mercury and silver have a great affinity for sulphydryl groups, which are often the important reactive sites for enzymes. In the presence of such metals the enzyme is therefore unable to function and toxicity results.

Although any of the heavy metals can be toxic to plants at some level of solubility, only a few have been generally observed to cause phytotoxicity in soils. In soils, most heavy metals occur as simple inorganic compounds or are bound to organic matter, clays, or hydrous oxides of iron, manganese, and aluminium. Because of this precipitation and sorption of most metals by soils, only zinc, copper, and nickel toxicities have occurred frequently. Toxicities of lead, cobalt, beryllium, arsenic, and cadmium occur only under very unusual conditions. Lead and cadmium are of interest not because of phytotoxicity, but because their uptake by plants moves them into the food chain (Vallee and Ulmer, 1972).

Figure 7 shows an idealized plot of rate of growth of plants as a function of the concentration of any particular element in the tissue. In the range of concentrations called the deficient zone, as we provide more of the element and increase its concentration in the plant, the

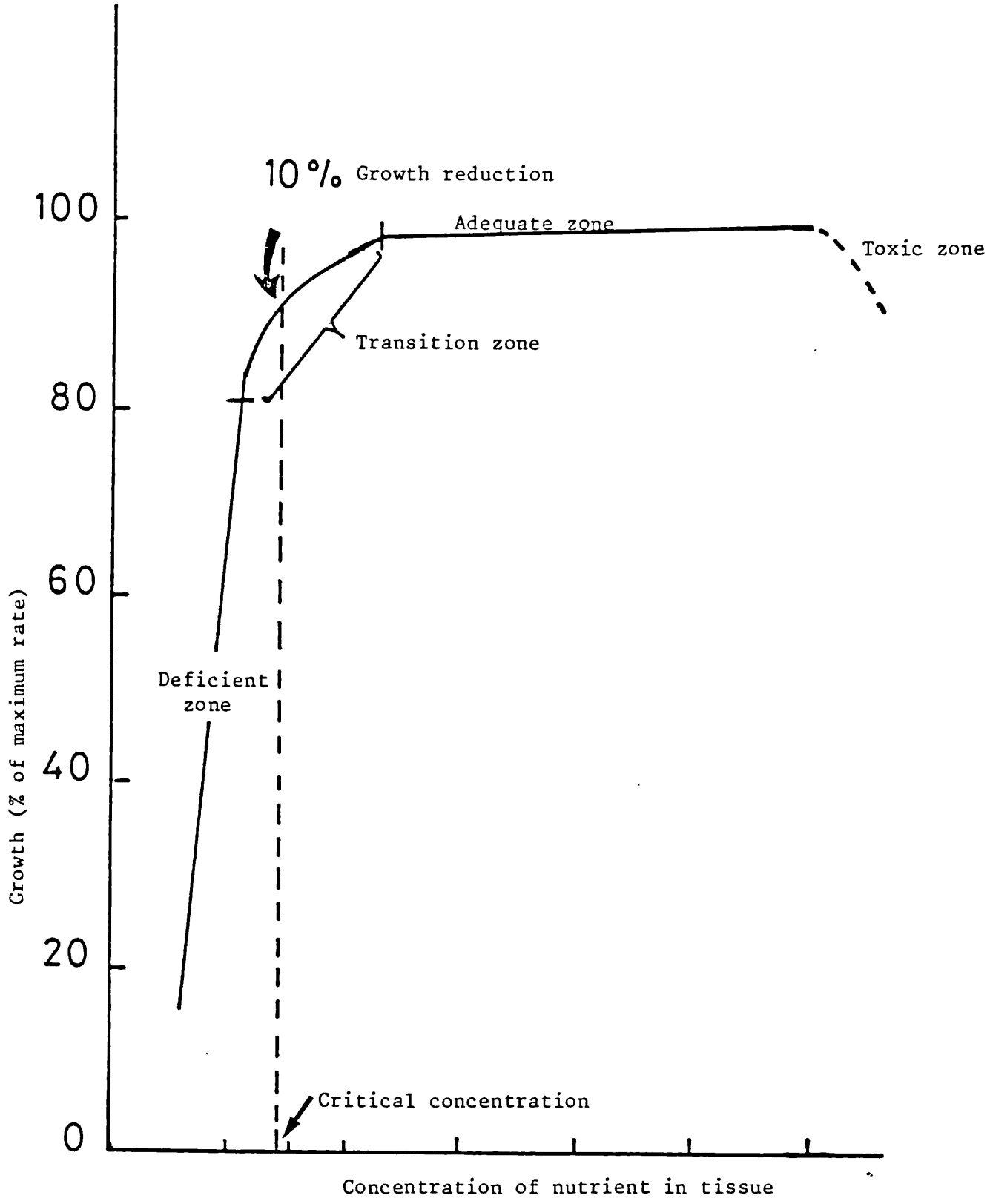

Figure 7. Generalized plot of growth as a function of the concentration of a nutrient in plant tissue (after Epstein, 1972)

growth rate is stimulated dramatically. After the critical concentration (minimal tissue concentration giving almost maximal growth), increase in concentration does not appreciably affect the growth rate (adequate zone). Further increases in concentration usually leads to toxicity and a reduced growth rate (toxic zone).

Most morphological and mutational effects in plants may be said to be due to the toxic effects of minerals in the substrate. Such morphological changes are very varied and include such effects as dwarfism, gigantism, mottling or chlorosis of leaves, abnormally shaped fruits, changes of colour in the flowers, disturbances in the rhythm of the flowering period, changes in growth form, and a large number of other indications.

Bowen (1966) has suggested that elements may be divided into three classes according to their toxicities.

1 - Very toxic: toxicity symptoms appear at concentrations of less than 1 ppm in the substrate. Such elements include beryllium, copper, mercury, silver, tin, and possibly also cobalt, nickel and lead.

2 - Moderately toxic: toxicity symptoms appear at concentrations between 1 - 100 ppm in the substrate. Examples of this are many of the transition elements and most of the elements of Groups III, IV, V and VI of the periodic table (with obvious exceptions of some of the macro-nutrients).

3 - Scarcely toxic: toxicity symptoms rarely appear at concentrations normally encountered in the substrate. Examples of such elements are the halogens, nitrogen, phosphorus, sulphur, titanium, the alkali metals, and the alkaline earths.

The above classification applies to vascular plants rather than to bryophytes. However, where plants are found under natural conditions, substantially higher concentrations of these elements can be tolerated in the soil, provided that availability is low.

Nickel is usually absorbed by plants in the ionic form, but is presumably chelated by organic carriers before translocation takes place. Nickel stimulates, and is also toxic to, the germination of some seeds. Low concentrations of nickel are toxic to a wide variety of plants. For example, in nutrient solutions a concentration of 2 - 15 p.p.m. nickel is toxic to beans and barley (Brenchley, 1938), 88 p.p.m. to tomato (Nicholas, 1952), 5 p.p.m. to oats (Hewitt, 1953), 1 p.p.m. to hop plants (*Humulus lupulus L.*) (Legg and Ormerod, 1958).

High concentrations of nickel lead to severe chlorosis caused by induced iron deficiency. It also has a specific effect of the metal itself (Hewitt, 1948b; Vergnano and Hunter, 1953). One of the toxic symptoms produced by nickel is the chlorosis or yellowing of leaves usually followed by necrosis. It also causes stunted growth of both root and shoot, deformation of various plant parts, unusual spotting and a host of other growth abnormalities. In extreme cases nickel may cause the death of the whole plant (Mishra and Kar, 1974).

Although nickel cannot normally be ranked under the essential elements, nevertheless, it is necessary for the healthy development of some plants, for example, *Alyssum sp.* (Malyuga, 1964) and *Hybanthus floribundus* (Severne and Brooks, 1972) where it is considered as an essential element.

Zinc on the other hand is highly toxic to plants except in very low concentrations, but traces must be present if normal plant metabolism is to be maintained. It is probable that zinc toxicity occurs in a number of ways. In many plant species an increase in zinc concentration in the tissues induces chlorosis due to iron deficiency. Painting chlorotic leaves with $FeSO_4$ alleviates the chlorosis (Guest, 1944). White *et al.* (1974) observed that increased soil zinc greatly increased translocation of manganese to soybean tips, and they reported that a zinc-induced-manganese-toxicity symptom (crinkle leaf) was seen in soybean.

1.9 Tolerance

Of the known micronutrients all but two, chlorine and boron, are transition metals. These transition metals are commonly referred to as heavy metals and are defined as those metals having a density greater than five (Passow *et al.*, 1961). Their common feature in relation to biological life is that in excessive quantities they are poisonous and can cause death of most living organisms. However, some plant species and ecotypes possess an ability to survive under conditions of metal contamination which would prove toxic to other living things by possession or evolution of a tolerance mechanism.

There is much evidence in the literature to suggest that plants growing on toxic metalliferous soils cannot prevent metal uptake but only restrict it and hence accumulate metals in their tissues to varying degrees (Peterson, 1971, 1975). The strategies of survival are thus tolerance and not avoidance of metal toxicity. Species differ considerably in their metal uptake characteristics and for any species these may vary for different metals.

Information on species response can be gained from an examination of metal levels in plant tissues sampled over a wide range of soil metal status. The data of Nicolls *et al.*, (1965) (Figure 8) for the grass *Triodia pungens* suggest different uptake, transport and accumulation mechanisms for zinc, lead and copper. Lead and copper are excluded from the aerial parts of the plant over much of the soil range, whereas zinc is accumulated in relation to total soil content. Barry and Clark (1978) found similar relationship for zinc and lead in *Minuartia verra* and the grasses *Festuca ovina* and *Agrostis tenuis* on metalliferous wastes in the United Kingdom. The plant/soil accumulation ratios for two closely-related *Nothofagus* species (Figure 8) indicate very different patterns of uptake for an essential (copper) and a non-essential element, although such a fundamental distinction may not always hold in other species.

For copper, physiological requirement, and hence relative accumulation at low soil levels, coupled with exclusion at the higher soil values, can account for the hyperbolic form of the curve. For nickel, a line parallel to the soil axis suggests a constant relationship between plant and soil levels.

On the basis of these and other examples, three types of plant-soil relationship are proposed (Figure 9), characteristic of:

1. Accumulators, where metals are concentrated in above-ground plant parts from low or high soil levels.

2. Excluders, where metal concentration in the shoot are maintained constant and low over a wide range of soil concentration, up to a critical soil value above which the mechanism breaks down and unrestricted transport results, and

3. Indicators, where uptake and transport of metals to the shoot are regulated so that internal concentration reflects external levels. These can be divided into two main classes according to their distribution. The first group comprises, universal indicators which will not grow in non-mineralized substrates. Examples of these include *Viola calaminaria* for zinc (Schwickerath, 1931), *Silene cobalticola* for cobalt, *Acrocephalus robertti* for copper (Duvigneaud, 1959), and *Aster venusta* for selenium (Trelease, 1949). Another group of plants comprise the local indicators. These are species adapted to tolerating mineralized ground but which will also grow elsewhere provided that competition from other species is not too great. Examples are *Alyssum bertolonii* (Minguzzi, 1948), *Asplenium adulterium* (Vogt, 1942a), and *Pulsatilla patens* (Storozheva, 1954) which all indicate nickel. These plants (whether accumulator or indicator) are all metal tolerant.

Metal tolerance is not a simple physiological attribute but a syndrome of adaptations at the cellular and biochemical level (Turner, 1969; Wainwright and Woolhouse, 1975; Ernst, 1975, 1976). In both

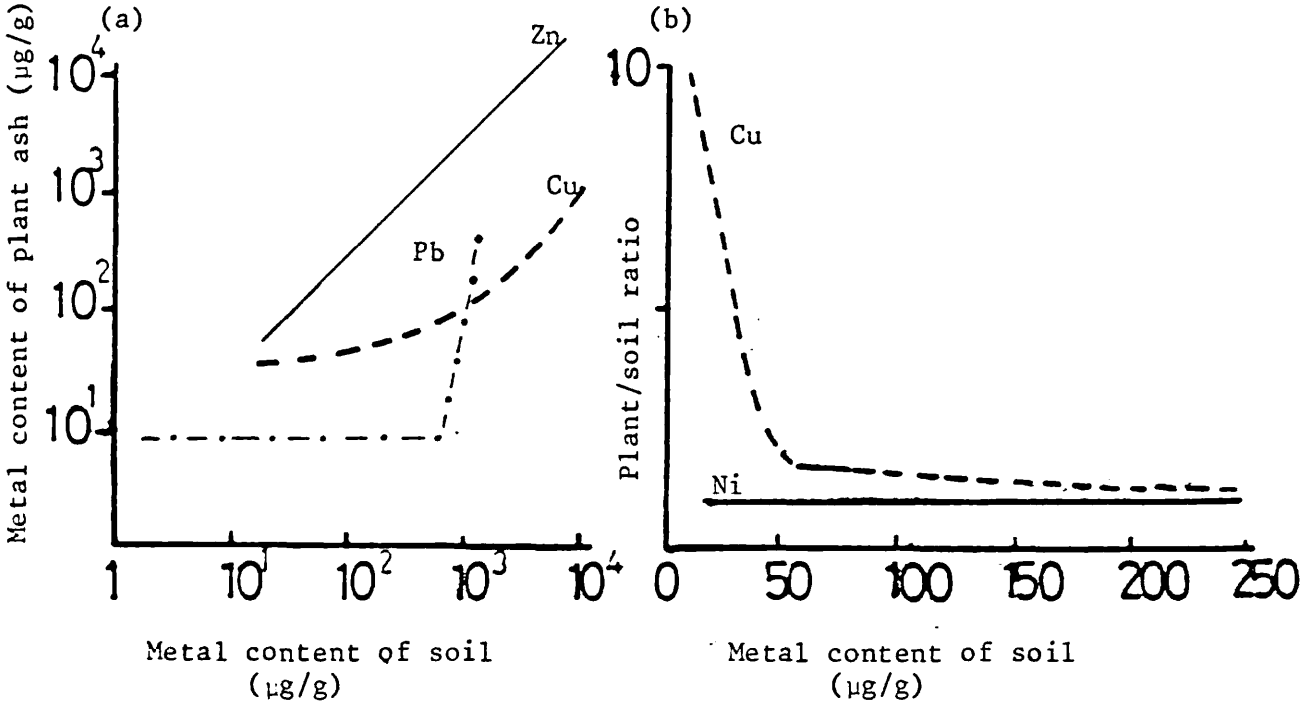

Figure 8. Plant-soil relationship for (a) zinc, lead and copper in the plant ash of *Triodia pungens* (Nicolls *et al.*, 1965) and (b) relative accumulation of nickel by *Nothofagus fusca* and copper by *N. menziesii* (Timperley *et al.*, 1970)

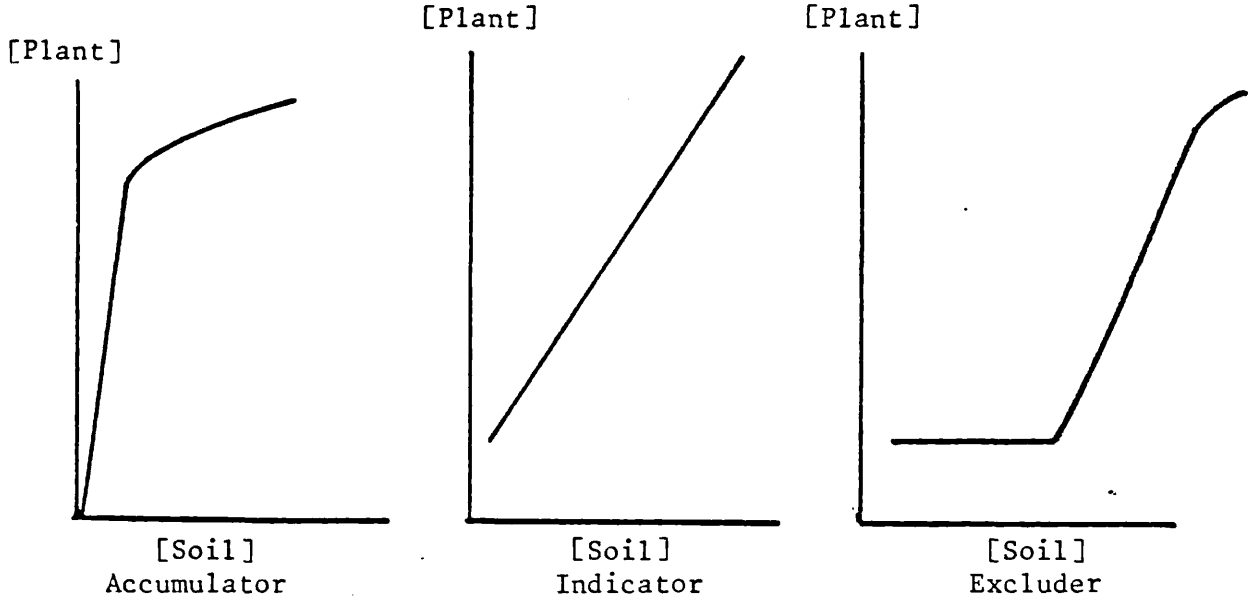

Figure 9. Three ways in which the response of plants to increasing soil metal levels may be reflected by the metal concentrations in aerial plant parts.

excluder and accumulator species the mechanisms of tolerance are largely 'internal' in that there is active detoxification of metal ions - it is the sites of detoxification which differ, being largely within the root in excluders and in the shoots in accumulators. 'External' mechanisms may also be important in both types. Accumulator species such as *Becium homblei* (Reilly and Stone, 1971) and *Thlaspi cepeaefolium* (Rascio, 1977) concentrate copper and zinc respectively in their deciduous aerial parts, thus effectively detoxifying the overwintering plant. In the case of *Becium homblei*, frequent bush fires may also remove any remaining stems and the copper accumulated therein. Rascio (1977) found that the excluders *Anthyllis vulneraria* and *Biscutella laevigata* concentrated the highest levels of zinc in the roots in the spring but the metal was then removed and translocated to the shoots in the autumn.

The mechanisms of metal tolerance have been reviewed by Turner (1968), Antonovics *et al.*, (1971), Wainwright and Woolhouse (1975), Cook and Wood, (1976), Ernst, (1976) and Woolhouse (1980). Table I gives some of the possible mechanisms of tolerance of plants to metals. The external mechanisms of tolerance represent those circumstances which prevent entry of the metal ions to the plant, thus they will not be considered here since they are not concerned directly with the active metabolic processes of the plant, but caused rather by specific soil conditions.

As a large number of parameters is involved in the study of the mechanisms of metal tolerance in plants, no clear overall picture has yet been evolved. However some particular mechanisms seem to be established for some metals and for certain plants under specified conditions. Thus copper tolerant yeast cells (*Saccharomyces cerevisiae*) deposit excess copper as sulphide at the cell periphery (Ashida, Higashi and Kikuchi, 1963). This is apparently due to a change in the normal

Table I. Possible mechanisms of metal tolerance in plants (Farago, 1980)

A. External mechanisms

(1) Metal is not available to plant root

(a) metal is present in water-insoluble form

(b) metal is present in soluble-but chelated form, not available to plant root

(c) concentration of freely diffusable metal ions is small

B. Internal mechanisms

(1) Metal is available to plant root but is not taken up

(a) alteration of cell wall membrane of roots giving decreased permeability to toxic metal ion

(b) alteration of surface enzymes of roots

(c) excretion of substance by root, rendering toxic metal insoluble or unavailable

(2) Metal is taken up but rendered harmless to metabolism within the plant

(a) metal bound in insoluble precipitate or complex

(b) metal bound in soluble, innocuous, complex of high thermodynamic stability or low kinetic lability

(c) metal removed by spatial separation, e.g. in cell walls or vacuoles

(3) Metal is taken up but excreted

(a) by loss of collecting organ e.g. shedding of leaf

(b) by guttation

(c) by leaching of soluble metal by rain

(4) Metal ion is taken up but metabolism is altered to accommodate increased concentration of metal ions

(a) increase of enzymes inhibited by metal

(b) inhibited enzyme systems by-passed

(c) alternative metabolic pathway not requiring products of inhibited enzyme system

(d) metal required for metabolism

sulphur metabolic pathways resulting in the production of hydrogen sulphide and thus copper sulphide. Nakamura (1962) investigating cadmium, cobalt, nickel and silver tolerances in yeast concluded that these are not based upon alteration in sulphur metabolism. Ashworth and Amin (1964) showed that mercury tolerance in *Aspergillus niger* is due to a pool of non-protein sulphydryl groups which complexes excess mercury, thus protecting sensitive sites within the cell. In higher plants there are only indications that sulphur may be of importance in heavy metal tolerance. Brenchley (1938), who examined the effects of cobalt and nickel in barley, and Url (1956), who examined the effects of copper, chromium and vanadium in several plant species, found that low concentrations of these metals as sulphates are innocuous to plant growth whilst equivalent concentrations of other metal salts are toxic.

Subsequent studies involving cell fractionation and differential centrifugation (Turner and Gregory, 1967; Turner, 1969, 1970; Turner and Marshall, 1971) showed the importance of the cell wall as an accumulation site for toxic metals in the roots of the species *Agrostis tenuis*. Turner (1967) also showed that in *A. tenuis* zinc tolerance may be associated with alterations in the carbohydrate composition of the cell. Significant correlations were found between zinc accumulation in the cell wall and the index of tolerance of the populations, and Turner (1972) suggested that the greater the tolerance of the population, the greater is the capacity for accumulation in the cell wall site. Thus a possible mechanism of zinc tolerance in *A. tenuis* is accumulation by a passive process not requiring metabolic energy, and storage in sites which bind cations in the cell walls of the root (Broda, 1967). Peterson (1969) suggests that in this way large amounts of zinc would be inactivated in the root cell walls.

Gambi (1967) showed histochemically that nickel was concentrated in the epidermis and in sclerenchymatous areas between vascular bundles in *Alyssum bertoloni*, a nickel accumulator found on serpentine soils.

Farago *et al.* (1975) have also found that in the nickel accumulating plant, *Hybanthus floribundus*, the pectate containing parts of the cell walls are involved in nickel storage and suggested that nickel takes the place of calcium.

Hammet (1928) and Cannon (1960) have also demonstrated the importance of the cell wall as an accumulation site for lead, uranium and vanadium.

Tolerance may also be achieved by transport to a compartment where toxic metals are removed from cell cytoplasm. Nickel accumulates to 25% of the dry weight of the latex of a New Caledonian nickel accumulator *Sebertia acuminata* (Jaffre *et al.* 1976). Ernst (1977) and Mathys (1973, 1975, 1977) favour the compartmentalization hypothesis and suggest that malate may chelate zinc in the vacuoles of tolerant plants.

Another example of metal tolerance is the restriction of the metal to a particular part of the plant (rather than cell structure). In a study of grasses from metal rich African soils, Reilly and Reilly (1973) showed that copper is either excluded from the roots or taken into the root but not translocated to the shoots, whereas in the case of herbaceous plants high levels of copper may be found in the leaves. *Dicoma nicollifera* which is generally confined to serpetine soils with high concentrations of nickel, translocates less nickel to the shoots than is found with other species of *Dicoma* (Wild, 1970) (Wiltshire, 1974). Ernst (1972) suggested that one significant difference between monocotyledons and dicotyledons is that the former can replace their roots continuously without harm to the whole plant. This specialization may explain the dominance of monocotyledons (grasses and sedges) on metalliferous soils. Shedding of leaves is a phenomenon suggested by Dykeman and De Souza (1966) in the copper tolerance of a species in a swamp forest, thus decreasing the general level of the toxic metal available to metabolic sites in the plant cells.

Differential metal tolerance of enzymes has also been studied and there is evidence that tolerant plants are metabolically different from normal plants at low metal levels. The effect of lead ion concentration on the activity of certain root cell wall enzymes of *Agrostis tenuis* has been investigated by Woolhouse (1970). He found that the rate of hydrolysis of p-nitrophenyl-phosphate by root tips of non-tolerant material was affected at very low concentrations of lead whereas the corresponding rate observed with lead-tolerant material was less affected by quite high lead levels. Cox *et al.* (1976) revealed that in zinc tolerant and non-tolerant clones of *Anthoxanthum odoratum*, the cell wall-bound acid phosphatase enzymes did possess differential sensitivity to zinc. Yet they were unable to distinguish between soluble acid phosphatases from similar sources. Recent studies on metal tolerance of enzymes from tolerant and non-tolerant ecotypes revealed that tolerant plants were able to maintain normal activities when subjected to metal stress which severely interfered with the metabolism of non-tolerant plants (Ernst, 1977). Bollard and Butler (1966) investigating nickel tolerant species suggested that mechanisms have evolved in these species so that nickel is largely excluded from the sites of activity of enzymes; this being accomplished by biosynthesis of unusual compounds or organometallic complexes, or by spatial segregation of the enzymes and nickel within the cells.

Another example of tolerance whereby plants resist heavy metal's toxicity is by inert complexation. Matsumoto *et al.* (1976) and Sivasubramaniam (1972) have reported that in the tea plant aluminium is found in the epidermal cells of old leaves and flavonoid type complexes have been suggested. Reilly (1969) has reported the presence of copper-amino acid or copper-peptide complexes in the cell sap of roots of *Becium homblei*.

In *Polycarpaea glabra* and *Triodia pungens*, zinc was found to be complexing with pectin (Farago and Pitt, 1977a) (this thesis). Pectin is also found to be the complexing agent for nickel in tolerant *Hybanthus floribundus* (this thesis). Farago and Mullen (1979) have found a copper-proline complex in tolerant *Armeria maritima*.

The index of tolerance of plants to heavy metal ions can be measured by a root elongation technique which was devised by Wilkins (1957). Using such methods, tolerance of various species has been demonstrated, and this is usually correlated with the available metals in the plant habitat. Jowett (1958) demonstrated that populations of *Agrostis tenuis* growing on sites where one heavy metal predominated were tolerant to that one metal and to no others. That is, tolerance to one metal does not confer tolerance to other metals. It was later demonstrated that tolerance of *Agrostis* is specific for copper, zinc and lead separately (Gregory *et al.* 1965).

Two exceptions to the general rule that tolerance is normally specific to the metals found at the site of origin have been reported for nickel: Populations of *Agrostis tenuis* from zinc contaminated areas showed co-tolerance to nickel without prior exposure to nickel (Gregory *et al.* 1965) and a clone of *Agrostis gigantea* was shown to be tolerant to nickel (Hogan, 1979). The nickel tolerance clone was from an area not known to contain any deposits of nickel and this seemed to suggest that the two metal tolerances are physiologically linked. However, there is no zinc tolerance in nickel tolerant populations collected from nickel mines in South West Germany (Gregory and Bradshaw, 1965). Zinc (0.69Å) and nickel (0.68 Å) are closely related in terms of ionic size and it was believed that specificity could be achieved through a control mechanism which was restricted to ions of the same size as zinc. However, magnesium (0.65 Å), lithium (0.68 Å) and cobalt (0.70 Å) have ions of similar dimensions to

those of zinc and nickel, and cobalt is also like nickel in its chemical and physical properties, but none of these ions could substitute for zinc or nickel in the tolerance mechanism (Gregory, 1964).

1.10 The Cell Wall as an Accumulation Site

Plants growing on mineralized soils containing heavy metals which are toxic at high concentrations, must possess tolerance mechanisms in order to restrict access of these metals to their metabolic reaction sites. Some plants restrict their uptake of metal from the soil, so that little or no metal is found in their tissues. Other plants effect tolerance by storage of metal at a particular site, where it may be sequestered by complexation with naturally occuring ligands. Thus it seems that in tolerant plants an internal adaptation is established to withstand the toxicity of heavy metals, and many workers have suggested that this is in part achieved by the binding of the metal to the cell wall.

As the root represents the point of entry of heavy metals into the plant's metabolic system, many workers have confined their investigations about the mechanism of tolerance to the root tissues. Turner (1970) investigated heavy metal tolerance in *Agrostis tenuis* by comparing the zinc and copper distribution within the roots of metal-tolerant and non-tolerant clones. Analysis of root subcellular fractions showed that at normal nutrient levels the root cell wall from metal-tolerant clones contained more copper and zinc than that from non-tolerant material. High levels of metal nutrition also demonstrated the importance of the cell wall in metal binding in root cells of metal-tolerant clones. Furthermore, Nevins, English and Albershiem (1967) have demonstrated that the composition of the plant cell wall is genetically controlled. The differences in cell wall composition between zinc tolerant and non-tolerant ecotypes of *A. tenuis* could thus be genetically controlled and such differences provide the basis for the mechanism of heavy metal tolerance

within this grass species. The accumulation of large amounts of heavy metals at the cell wall is known in other higher plants.

Cartwright (1966) found that 64% of the total copper within subterranean clover nodules was located in the plant cell wall fraction. Diez-Altares and Boroughs (1961) and Diez-Altares and Bornemisza (1967) showed that even at normal levels of zinc nutrition up to 44% of the total zinc in germinating corn tissues was localized in the cell wall. The cell wall fraction is therefore likely to be the most important fraction in metal accumulation and may well exert a protective action in preventing excess metals from reaching susceptible metabolic sites.

Ernst (1972) found that, in several African species tolerant to heavy metals, large amounts of nickel, lead, zinc and copper were not removed from cortex material by sequential extraction. After extraction of dried plant matter using an organic solvent, water and dilute acid the material remaining is thought to be largely cell wall material, so that the residual metal after sequential extraction is usually considered to be largely bound to cell walls.

Peterson (1969) using *Agrostis tenuis* gave ^{65}Zn to both zinc tolerant and copper tolerant plants. Subsequently, he found that the zinc tolerant plants contained more ^{65}Zn in the pectate extract of root cell wall than either the copper tolerant or non-tolerant plants. In a further study of this species Turner and Marshall (1972) suggested that the tolerance mechanism is associated with an altered carbohydrate content of the cell wall coupled with metabolic adaptations. Farago and Pitt (1977a) showed that in *Polycarpaea glabra* most of the zinc present was firmly held in the pectate fraction. Similarly it has been shown that soluble nickel in *Hybanthus floribundus* leaves is associated with pectic carbohydrates (Farago *et al*, 1975) (this thesis).

Dainty and Hope (1959) demonstrated the ability of pectin and uronic

acids in cell walls to bind metal ions by cation exchange.

Mattson *et al.* (1969) also noted that uronic acids of the pectin in cell wall possessed such a cation exchange capacity. Knight, Crook and Inkson (1961) further showed that the cation exchange capacity was proportional to the uronic acid concentration of the plant material. The functional groups present in pectic substances which are responsible for cationic binding are the carboxy and hydroxy groups of the uronic acid units. Several workers have presented evidence of the binding of heavy metals to these and other groups (Broda,1965) (Ennis, 1962). It seems certain, therefore, that part of the tolerance mechanism of some plants depends upon their ability to bind large quantities of toxic metals at pectic cation sites in the cell wall.

However as noted in section 1.9, tolerance to one heavy metal does not confer tolerance to another heavy metal, and a characteristic feature of metal tolerant plants is their rigid specificity for individual heavy metals (Gregory and Bradshaw, 1965), (Bradshaw *et al.*, 1965),(Turner, 1970). Turner and Marshall (1971), Dainty and Hope (1959), Findenegg and Broda (1965) emphasized that uptake of a heavy metal by cell walls is a passive ion exchange process. This is quite non-specific and quite inconsistent with the completely specific character of heavy metal tolerance. It would seem that the binding of heavy metals to cell wall pectate is an important part of the overall picture of tolerance but the cause of absolute specificity must be sought elsewhere.

Turner and Marshall (1972) investigating zinc tolerance in the grass *Agrostis tenuis* found that there is a significant correlation between the uptake of zinc by the cell wall fraction and the index of zinc tolerance. In addition they found that there was no correlation between the nitrogen content of the cell wall fraction and zinc tolerance. Since, therefore, proteins do not seem to be involved they concluded that tolerance was associated with an altered carbohydrate composition of

the cell wall, even though carbohydrates have not been reported as specific metal binding agents.

However, if the binding of heavy metals at cell walls is non-specific then the mechanism responsible for specific ion accumulation must be sited at the plasmalemma. Rathore *et al.* (1972) found a decreased rate of uptake in metal insensitive *Phaseolus vulgaris* compared with that by the metal sensitive strain. This sort of relatively low rate of uptake for toxic metals might, depending on nutritional levels, result in a relative build up of the metal in the cell wall, not primarily because the cell wall has a specific affinity for the metal but because there is a greater concentration of the metal at the cell wall than for the non-tolerant plant.

Work on two Australian species has shown that, although zinc is associated with the cell wall pectic material, these plants show a greater affinity for copper than for zinc (Farago and Pitt, 1977b). Thus they display the 'normal' affinity of polygalacturonic acids for metal ions, i.e. copper > calcium > zinc > nickel (Jellinek and Sangal, 1972). It seems unlikely that pectic acids would selectively complex with zinc or nickel rather than with calcium. It appears (Farago and Pitt, 1977a,b) that, although the zinc ions are stored in ion exchange sites in the carbohydrate of the cell wall, this may 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 and Marshall (1972) for *Agrostis*. In *Hybanthus floribundus*, Farago *et al.*, (1975) have suggested that nickel occurs in pectinates in the plant tissues and that it takes the place of calcium. In view of the high magnesium to calcium ratio in serpentinites and other ultra-basic rocks this appears to be a likely explanation. Questions arise, however, as to why *H. floribundus* alone of the species occurring in the greenstone belts of Western Australia accumulates nickel in this fashion.

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CHAPTER II

PRACTICAL TECHNIQUES

2.1 Elemental Analysis

In order to investigate the role of metals in plant growth and metabolism it is necessary to determine quantitatively the amount of metals in plant tissues. Methods have been available by which small amounts of metals can be determined with a reasonable degree of accuracy, of which the atomic absorption spectrophotometer is the most widely employed. Metal determination almost always commences by the ashing of the plant material. The object of ashing is to destroy all organic material which might otherwise interfere with the analytical determination of trace elements. After destruction of organic matter, some metals can be determined directly in the solution of the ash, provided the metal is of sufficient concentration to be detected.

Metals in soluble form may also be determined colorimetrically, gravimetrically, polarographically, by anodic stripping or by one of the spectroscopic methods. Today, however, more trace determinations are made possible by atomic absorption spectrophotometry than by any other method, due to the generally good sensitivity for many elements, the frequent lack of serious interferences, rapidity, and satisfactory precision.

2.2 Atomic Absorption Spectrophotometry

Atomic absorption spectroscopy can be simply defined as the absorption of radiant energy by atoms. This absorption and its quantitative correlation with the concentration of metal ions originally present in a sample solution serves as the basis of analytical atomic absorption spectroscopy. The technique consists of the following steps: sample solution nebulization, atomization, atomic absorption and detection.

A sample solution is drawn by a capillary into a nebulizer by the low pressure created around the end of the capillary by the flow of the carrier or oxidant gas. The resulting droplets are ejected with the carrier gas into a spray chamber. The fuel gas is introduced into the chamber, and also auxiliary carrier gas or oxidant, so that an intimate mixture of sample mist, fuel and oxidant leave the spray chamber and enter the burner through a narrow slot where it is ignited.

In the ignition process a large percentage of the metal ions in the sample solution is converted into atoms in their ground state.

Resonance radiation (i.e. radiation emitted when an excited atom returns to its ground-state from its closest excited level) of the element to be determined is passed from a source lamp 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. Hollow cathode lamps are the most common types of light source used in spectroscopy, where the cathode consists of a hollow cup made from the material of the element to be determined and the anode is a tungsten wire. The two electrodes are housed in a glass envelope containing an inert gas at low pressure.

However, the latest techniques in analytical chemistry regarding plant metal analysis is that of atomic fluorescence. In this technique, unlike atomic absorption, up to seven elements may be examined at one time, each element to a far greater sensitivity than in absorption and all suitable elements may be assayed on the one machine (i.e. no specific lamps must be purchased for each new element to be analyzed).

Interferences

When atomic absorption spectrophotometry was first developed, it was believed that the method was specific and free from interferences encountered in other spectroscopic forms of analysis. In actuality, however, atomic absorption has almost as many interferences as other

flame methods of analysis have, the reason being that we are measuring the atomic population in the ground state and anything that affects this, be it chemical or physical, will affect the resultant absorbance.

Spectral interference (also known as radiation interference), although it is relatively rare in atomic absorption analysis, occurs if an element other than the analyte is absorbing the radiant energy of the source. Although the occurrence of spectral interference is quite rare, there have been some reports of this effect. Koirtyohann and Pickett (1965,1966) observed molecular absorption by alkali halides in an oxygen-hydrogen flame. Jawarowski and Weberling (1966) reported a spectral interference in the determination of nickel with a multielement hollow cathode lamp. The interference was eliminated when they used a single-element, nickel lamp, which suggested that some lines which were active in absorption for other elements had been causing the interference.

Since we are measuring the neutral atomic concentration in atomic absorption, any ionization that takes place will remove a number of atoms from the ground state. If another element is present that will decrease or increase this ionization, this will lead to an ionization interference. This interference may be eliminated by matching standards and samples to the concentration of the interfering element or to add a large excess of the interfering element to all the solutions, thus creating a "radiation buffer" that will cause the same effects for standards and samples.

Chemical interference, or the chemical combination of the element of interest with other elements in the sample or the flame, is probably the most important interference in atomic absorption. Methods of eliminating chemical interferences depend on the specific type of interferences. For example, phosphate interference on calcium may be removed by the addition of excess lanthanum or strontium (Willis, 1960, 1961). The mechanism of this type is probably the formation of a more

stable compound with the interfering substance, namely, the phosphate, by the added element, than is formed by the analyte. Chemical interference when it occurs because of the formation of refractory compounds, can be eliminated by the use of higher temperature flames such as nitrous oxide-acetylene or oxyacetylene flame. The mechanism here is just the breakup of the refractory compound by the higher temperatures and/or reducing zones in the flame.

Light scattering has also been reported as the cause of interference by several workers (Willis 1962)(David 1961). If a flame contains solid particles or solvent drops that are vaporized, these particles can scatter the light beam that impinges on them, giving rise to false absorption signals. One of the methods that can be used to account for this effect is the preparation of a good blank solution. Koirtyohann and Pickett (1966) postulated that some other mechanism, such as molecular absorption, variation of the refractive index within the flame caused by vaporization of the particles, or continuous absorption owing to atom ionization, may give rise to the observed light loss.

2.3 Preparation of Plant Material for Metal Content Analysis

The most time-consuming stage in the analysis of plant material by atomic absorption spectrophotometry is undoubtedly that of sample preparation. The procedures often involve the use of hazardous chemicals and may constitute the major source of contamination in trace analysis. The destruction of organic matter for trace element determination was first described by Fresenius and Von Babo (1844). Since that time many procedures have been described for this purpose. Most of these fall into two classes: dry ashing and wet digestion.

Dry ashing is usually accomplished by heating the sample in a silica crucible to a relatively high temperature, generally between 400

and 700°C, with atmospheric oxygen serving as the oxidant. Other chemical compounds may be added to aid the process. In wet digestion, liquid conditions are maintained throughout and the oxidant is carried out by various oxidizing agents in solution, usually oxidizing acids. Dry ashing has been recommended for elemental analysis for its simplicity and relative freedom from positive errors and because large samples can be handled. The drawbacks of dry oxidation are volatilization of elements and losses by retention on the walls of the vessel. Wet digestion is considered superior in terms of rapidity, the low temperature maintained, and the freedom from loss by retention.

Wet digestion methods

Gorsuch (1959) studied ashing procedures in approximately 250 investigations reported in the literature. He found that dry ashing was the most common method, followed by wet digestion with nitric and sulphuric acids. Other methods included wet digestion with nitric, sulphuric, and perchloric acid, and with nitric and perchloric acid, and various combinations incorporating hydrogen peroxide. Lately 3:1:1, nitric, perchloric, sulphuric acid mixtures has become a standard method (Kirkbright and Sargent, 1974)(Christian and Feldman, 1970). The addition of sulphuric acid to the ashing mixture reduces the risk of the sample drying and exploding.

Another method of wet ashing was carried by Middleton and Stuckey (1954), which is reported to possess several advantages. This consists essentially of heating the sample with nitric acid to dryness on a hot plate at 350°C and repeating until organic matter is sufficiently destroyed.

Wet oxidation procedures in particular can lead to excessively acidic sample solutions and ultimately to corrosion of nebulizers and sometimes of other instrument components. Murphy *et al.* (1977) have

suggested a simple extraction with 2M HCl for the determination of calcium, copper, potassium, magnesium, manganese, sodium and zinc in pasture samples, which avoids most of these problems.

Numerous suggestions have also been made for saving time in routine analysis. Hoening and Vanderstappen (1978) suggested the use of $HNO_3/H_2SO_4/H_2O_2$ as a rapid ashing method. The use of 50% hydrogen peroxide with sufficient sulphuric acid has been suggested by Johnson (1967). Premi and Cornfield (1968) suggested heavy metal extraction from plant material by boiling for fifteen minutes with 6M hydrochloric acid.

A relatively new method of oxidation which should prove to be very useful for the preparation of biological samples for trace metal analysis is the low temperature ashing technique developed by Gleit and Holland (1962,1963,1965), where a low temperature radio frequency induced oxygen plasma efficiently destroys organic matter.

The technique used throughout this thesis is that of wet ashing the plant material or extract with concentrated nitric acid until the solution is clear and colourless, diluting to a volume and analyzing on the atomic absorption spectrophotometer. The flame used was exclusively air-acetylene.

Contamination and losses in trace analysis

Since here we are generally concerned with trace analysis, contamination errors are always a danger. This unfortunately takes on many varied forms and causes great problems in trace metal analysis.

The purity of water used for preparing solutions and for dissolving samples is the first obvious concern of the analyst. Distillation and ion-exchange chromatography are the common means employed to purify water. Double or triple distillation of tap water is required to minimize trace element contamination with an efficient reflux column. The spray trap for such a column is important to minimize fine spray from

being carried over with the vapours. Silicate is the most significant inorganic contaminant of demineralized natural waters. Distillation of the water prior to demineralization is the most effective method of insuring freedom from any contamination of silicate or even borate. Throughout the present work, the water used at all stages, even for rinsing purposes, was distilled, deionized and then glass distilled.

Acids used for digestions or acidification of samples are another common source of contamination. However most reagent grade acids are sufficiently pure and many of these can be obtained in purified states commercially. Top grade analytical materials are used in this work whenever possible while 'ultra' grade concentrated nitric acid was used for ashing.

Cleanliness of glassware is of the utmost importance in trace analysis. Throughout the course of these studies all equipment was carefully washed and acid washed before use.

Unfiltered laboratory air may contain enough suspended material to be a hazard in trace metal determinations. Airborne contaminants can be a serious source of high and irregular blanks in refined trace analysis. Frequent and thorough cleaning of the laboratory and its equipment will aid in reducing contaminants. Metal apparatus should be avoided in trace analysis and all apparatus must be covered and well stoppered. Beside fulfiling the above precautions blank extracts and blank ashing were always used in an attempt to reduce airborne contamination and the plant samples analysed were ground using an agate ball mill.

Finally, the analyst himself may be a source of trace elements, conveyed to the sample or the utensils by way of the atmosphere or in other ways. Source of contamination may be the varied powders, plasters ointments, lotions, antiperspirants and antiseptics applied to the epidermis of Homo sapiens. P.V.C. gloves were worn as a precaution to reduce contamination as frequently as possible throughout the present work.

However, the goal in purification is not necessarily the reduction of foreign elements to the lowest possible level, but rather to a level that is reasonably lower than that of the elements being determined. Blanks must always be run, and if water and reagents introduce, for example, 0.1% of an element being determined, accuracy is not impaired.

On the other hand, in separations and determination, trace elements can be lost by adsorption, volatilization, coprecipitation, and coextraction. Errors can also result from adsorption of the constituents of standard solutions on the walls of containers. There is also the possibility of mechanical losses when solutions are boiled or gases are evolved, when extractions are made etc.

Losses during wet ashing, was mostly overcome by employing gentle conditions particularly at the beginning of the ashing. Vigorous frothing and bumping of the solutions were prevented by the addition of a few fragments of acid washed "porous pot" while vapour losses are circumvented by the use of short-neck glass funnels placed in the necks of the ashing flasks.

All analysis of the metals were made as quickly as possible and when stored, samples were always kept in polyethene bottles in the refrigerator. Extreme care was taken in sample handling and in washing the walls of containers prior to analysis. All standard solutions were prepared in relatively concentrated stock solutions for each element from which working standards were prepared by serial dilution and the low concentration standards were always made up daily from concentrated stocks.

2.4 Extraction Schemes

The plant species examined in this work indicated relatively high concentrations of specific metals. The information obtained, however, from metal levels in plant material is limited and gives no idea of the chemical distribution of the metal within the plant tissues. The level of, say, zinc in plant leaves represents the sum of all the zinc containing compounds present in the plant, e.g. proteins, pectates, co-enzyme etc. More information of the chemical nature of metals present could be derived from a series of extraction schemes where discrete types of cellular components can be removed with as little overlap as possible.

The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated. Alcohol is a good solvent for preliminary extraction. When isolating substances from green tissue, the success of extraction with alcohol is directly related to the extent to which chlorophyll is removed into the solvent and when the tissue debris, on repeated extraction, is completely free of green colour, it can be assumed that all the low molecular weight compounds have been extracted. Organic constituents from dried plant tissue are usually extracted with a range of solvents, starting in turn with ether, petroleum and chloroform and then using alcohol and ethyl acetate (for more polar compounds). The extract obtained is then filtered and concentrated to small volumes using rotatory evaporator at temperature between 30 and 40^{o}C.

A number of different extraction processes have been attempted with great success. Bowen, Cawse and Thick (1962) fed tomato plants with radioactive ions and extracted the plant material with a series of reagents. Initially they extracted the plant material with ethanol, followed by a 0.2M hydrochloric acid treatment. The proteins and pectates

were precipitated by acetone addition to the acid fraction, whereas the nucleic acid was extracted with 0.5M perchloric acid. Boiling with 2M sodium hydroxide dissolved most of the remaining proteins and polysaccharides, leaving a residue of lignin and cellulose.

Peterson (1969) examined zinc in *Agrostis tenuis* by extracting with boiling 80% ethanol and then boiling water; the ethanolic solution was extracted with ether. The residual material after protein extraction was divided into two and one portion was shaken with 2% ammonium oxalate and further heated with 0.05M hydrochloric acid to remove pectates and protopectates respectively. The other portion was extracted successively with 0.1M and 0.44M NaOH followed by 1.5M KOH (boiling) to obtain hemicellulose, polysaccharides and lignin respectively, leaving a residue of α-cellulose. Diez-Altares (1967) employed a broadly similar scheme to that of Peterson (1969) concentrating on the fractionation of proteins as well as polysaccharides.

Reilly (1969), studied the tenacity with which copper is held by *Becium homblei* (De Wild) Duvign and Plancke, by the results of serial extractions with organic solvents and water followed by leaching with mineral acids. The organic solvents together extracted less than 50% of the total copper, while water extraction, followed by sulphuric acid treatment, removed a further 18-20% of the residual copper.

Farago *et al.*(1975) investigating the chemical character of the compounds with which the metal is associated in several plant species adapted a scheme which was a modification of that devised by Bowen *et al.* (1962). After an initial extraction with ether, the residue was further extracted with ethanol, water, and 0.5M hydrochloric acid. Ernst (1969, 1972) attempted to solubilize heavy metals from accumulator plants 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.

Often, particularly with plants which possess high metal concentrations in their tissues, a high percentage of metal is found to be insoluble in water. The type of compound with which the metal is associated then is probably one of the following:- proteins, pectates, nucleic acid, cellulose, lignin and other possible polysaccharides. Throughout this work, the distribution of the metals amongst these compound types was investigated using the extraction process by Bowen *et al.* (1962) and Peterson (1969). The schemes are shown in tabulated form in the relevant section.

2.5 Chromatography of Plant Extracts

The general method of investigation and identification in all the work reported involves extraction of the fresh plant material, separation normally on chromatography column, frequently using gel filtration, and examination using chromatography and electrophoresis technique. The use of paper chromatography was an obvious choice as a method of analysis since this basically simple technique allows the separation and identification of natural products on a small scale.

Both ascending and descending paper chromatography were employed with Whatman 1 and 3 MM papers and thin layer chromatography with cellulose MN300 polythene thin layer sheets and silica gel plates. Several solvent systems were employed including butanol-acetic acid-water, 12:3:5, v/v/v (upper layer), phenol-water, 4:1, m/m and n-butanol-pyridine-water, 1:1:1, v/v/v.

2.6 Electrophoresis

Differences in the mobility of charged particles (ions and colloids) in liquid media under the influence of an electric field may be used for their separation (Lederer, 1955)(Wunderly, 1961). Components are separated into zones with the aid of paper, gel, etc. as supporting or

stabilizing media, where the position of the zones is detected by means of radioactivity, colour reaction, and other properties.

Electrophoresis has fairly wide application in phytochemistry. In the first instance, this technique is only applicable to compounds which carry a charge, i.e. amino acids, some alkaloids, amines, organic acids and proteins. However, in addition, certain classes of neutral compounds (sugars, phenols) can be made to move in an electric field by converting them into metal complexes (e.g. by use of sodium borate) (Harborne, 1973). However, this technique has found only limited application in the field of flavonoid isolation and separation (Seikel, 1964) (Harborne, 1967a)(Paris, 1961). This is largely because, except for a few specific cases, it offers little or no advantage over paper chromatography. Flavonoids must be ionized or complexed with a metal ion to be mobile in an electric field and for this reason the technique is admirably suited to the isolation of flavonoids which occur naturally in these states.

In the present work, low-voltage electrophoresis paper was used to study the charge and mobility of the metal complexes in the plants investigated. A Shandon Southern low voltage electrophoresis apparatus and tank were employed with Whatman 1 and 3 MM papers. The buffer, pH and current were chosen with great care after much experimentation. The exact electrophoretic conditions used are described under the plant sample examined.

2.7 Histochemistry

The fact that deposition of specific stains in certain regions is a result of chemical or physical properties inherent in the tissue forms the basis of histochemistry. This is a field of research which has expanded rapidly and has as its goal the localization within specific areas or cell components of the chemical compounds known already by

biochemical analysis to be present. Histochemical methods are now available for many inorganic and organic substances and has been found to be exceedingly useful in the study of the actual site of metal accumulation within plant species which are tolerant to toxic metals. The plant material under investigation is first killed, then fixed and sectioned for microscopic study. The sections are stained with one or more of a variety of chemicals to render conspicuous the various components of a tissue so that they may be observed.

Ideally in histochemistry the stain chosen must be specific for the chemical species required and give strong colours with small quantities of the analyte being investigated, as is often the case with toxic metals in plants.

Having obtained a suitable stain and procedure, the section can then be mounted on a permanent slide, or drawn or colour photographed to produce a permanent record of the section.

2.8 Electron Microscope Microanalysis

High resolution electron microscopy has been widely used in biological science for many years and far-reaching deductions have been made concerning biological function on the basis of the microstructures observed. Yet attempts were made to extend the studies by securing information about the chemical composition in different parts of the cells so as to follow the biochemical processes going on. There are two basic kinds of electron microscopes, transmission (TEM) and scanning (SEM).

Transmission electron microscopes

Transmission electron microscopes involve the passage of a high-velocity homogeneous electron beam through a specimen. A relatively large area of a specimen ($10\text{-}10^6$ μm^2), which is thin enough to transmit at least 50% of the incident electrons, is illuminated. The emergent

beam is reflected by a system of imaging lenses to form a real, magnified image of the specimen on a fluorescent screen or, if a photo is desired, on a photographic emulsion.

Scanning electron microscope

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 details.

X-ray microanalysis

As a beam of electrons bombards a specimen, a myriad of events take place. Electrons may pass through some types of specimens; be absorbed by the specimen; be inelastically scattered (energy is lost); be elastically scattered (no energy is lost); or be deflected back from the specimen as secondary electrons, backscattered electrons, Auger electrons, fluorescence as visible light or X-rays.

X-ray microanalysis depends upon excitation of electrons to produce characteristic X-ray spectra for each of the elements detected. The wavelength and intensity of the X-ray emission 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 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.

The dried plant material is sectioned and then glued to a conducting base plate. The specimen is then coated with a very thin layer of an electrically conducting metal, e.g. gold which reduces changing effects in the beam.

Energy dispersive X-ray analysis

Later use of the above technique employed the secondary electron given off. The energy of these electrons is also characteristic of the particular element bombarded and the detector system may again be adjusted to record the incidence of electrons within a narrow energy band. In this technique, X-rays of all energies are simultaneously processed, and a minicomputer is commonly used to separate overlapping peaks. Elements from sodium to the transuranium elements may be simultaneously detected.

Wavelength dispersive analytical system

Wavelength dispersive spectrometers commonly contain a crystal which is bent or ground to bring a diverging beam of X-rays from the specimen to a focus at points which correspond to the respective wavelengths on a circle radius. Only one wavelength is strongly reflected at a given angle. All others are excluded. Thus, the range of wavelengths that a crystal can focus depends upon the range of angles that it can be rotated through. With this system, it is necessary to analyse for only one wavelength at a time. By the time several wavelengths are analysed to determine the presence of several elements by their characteristic X-ray spectra, biological specimens may be significantly altered. On the other hand, with energy dispersive analysis the simultaneous processing of all X-rays of all energies normally preserves the integrity of a biological specimen.

It is perhaps surprising that scanning electron microscopy and microanalysis were not applied to plant material 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 micro-nutrients 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.

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CHAPTER III

GENERAL ANALYSIS OF PLANT MATERIAL

3.1 Metal Concentration Determination - Introduction

The plant species chosen in the present investigation have been found to grow and thrive on mineralised sites. Thus they are tolerant to soils which contain high concentrations of metal ions, normally toxic to plant growth. The potential value of such plants lies in their capacity to accumulate certain mineral elements to concentrations far greater than those found in the parent rock and soils in which they grow. This in turn facilitates the detection and measurement of elements present in the rocks at very low concentration. The plant species examined in this work are: *Hybanthus floribundus*, *Triodia pungens* and *Eriachne mucronata*.

Hybanthus floribundus

Until recently the most well known nickel accumulator was undoubtedly *Alyssum bertolonii* where plant/soil ratios of up to 30 have been reported (Minguzzi and Vergnano, 1948)(Vergnano, 1958). Over the past several years an increasing number of outstanding nickel accumulators have been reported which may, depending on the species, be used as geobotanical indicators of nickel deposits. Two other *Alyssum spp.* have been recorded as outstanding nickel accumulators; *A. murale* from the U.S.S.R. (Malyuga, 1964) and *A. serpyllifolium* from Protugal (Menezes de Sequeira, 1968). *Dicoma niccolifera* is probably the best known nickel accumulator from Rhodesia (Wild, 1970,1971). With a ratio of 163, *Hybanthus floribundus* from Western Australia accumulates the greatest quantity of nickel so far recorded (Severne and Brooks, 1972)(Cole, 1973). *H. caledonicus* and *H. austro-caledonicus* from New Caledonia have recently been described as nickel accumulators with ratios of up to 16

and 54 respectively (Brooks *et al.* 1974).

Hybanthus floribundus is a shrubby member of the Violaceae. The family includes herbs and small shrubs extending beyond Australia. The leaves are alternate, with stipules present. The flowers have five sepals, five petals and five stamens. The species are often highly perfumed and the fruit is usually a one-celled capsule containing a number of fine seeds that are released when ripe. Of the Violaceae family, there are five Australian genera, one of which is the genus *Hybanthus*.

This genus extends beyond Australia, with ten to twelve species scattered throughout Australia except in Tasmania. It consists of small undershrubs or herbs with small alternate leaves. The flowers are small, white, blue, purple or yellow, axillary or solitary or in terminal racemes; there are five small sepals; of the five petals, four are inconspicuous; the lower petal is broad and spreading, giving the appearance of a one-petalled flower; the five stamens are arranged closely around the style. The fruit is a capsule that opens by three valves when ripe and releases a number of small seeds which germinates readily.

Hybanthus floribundus embraces several sub-species or ecotypes, and has an insular or disjunctive distribution across the southern part of Australia. The three nickel accumulating ecotypes, however, are restricted to a broad belt in Western Australia, which extends from Ravensthorpe near the southern coast, to Agnew, 450 miles to the north. *Hybanthus* shrubs are conspicuous by their apparent lack of xeromorphic adaptation to a climate which becomes increasingly arid towards the continental interior (Cole, 1973). The nickel concentrations in the various 'insular' populations of *H. floribundus* were generally observed to decrease southwards (from 8,000 to 1,000 p.p.m.) as the annual rainfall increased from seven inches to more than thirty inches.

Hybanthus floribundus (Lindl.) F. Muell., (Slide I), was first found in the Eastern Goldfields area of Western Australia (Elkington, 1969)(Cole, 1968,1973). Elkington reported that the species grew only over serpentinite outcrops. It was subsequently reported over a wider area of Western Australia growing over nickel toxic soils at Dordie Rocks west of Dordie Lake, east and south of Yates Knoll and on a small island in Lake Cowan in the Widgiemooltha - Mt Thirsty area, south of Coolgardie, on Kurrajong and Riverina properties in the Mt Ida area north-west of Menzies and in the Dunnsville area north of Coolgardie (Cole, 1973).

Investigation of the occurrences revealed that *H. floribundus* occurs mainly over outcropping serpentinite and at the contact of quartz feldspar porphyry and hypersthenite dykes with serpentinite or amphibolite bedrock at Widgiemooltha and Dordie Rocks and over alluvium and colluvium at Kurrajong, Riverina and Coolgardie. In some areas in each of these localities it is most abundant near the contact of ultra-basic and basic rocks. It also occupies similar sites in the Kambalda area (Elkington, 1969).

The elevated nickel content of *Hybanthus floribundus* from a metalliferous area in Western Australia poses interesting problems in plant physiology, and we have therefore undertaken some preliminary studies on the plant chemistry of this species.

Triodia pungens and Eriachne mucronata

Triodia pungens R. Br. is a shrub-like grass of the Festuceae. The family has a wide range of distribution, from moist tropical, through hygrophilous conditions, and woodland conditions to temperate meadows and mountain ranges. It includes also many types adapted to dried subtropical conditions. The species of *Triodia* examined by Burbridge (1946) are all xeromorphic tussock-forming perennial grasses, with

Slide I. *Hybanthus floribundus* plant

Slide II. *Hybanthus floribundus* flowers

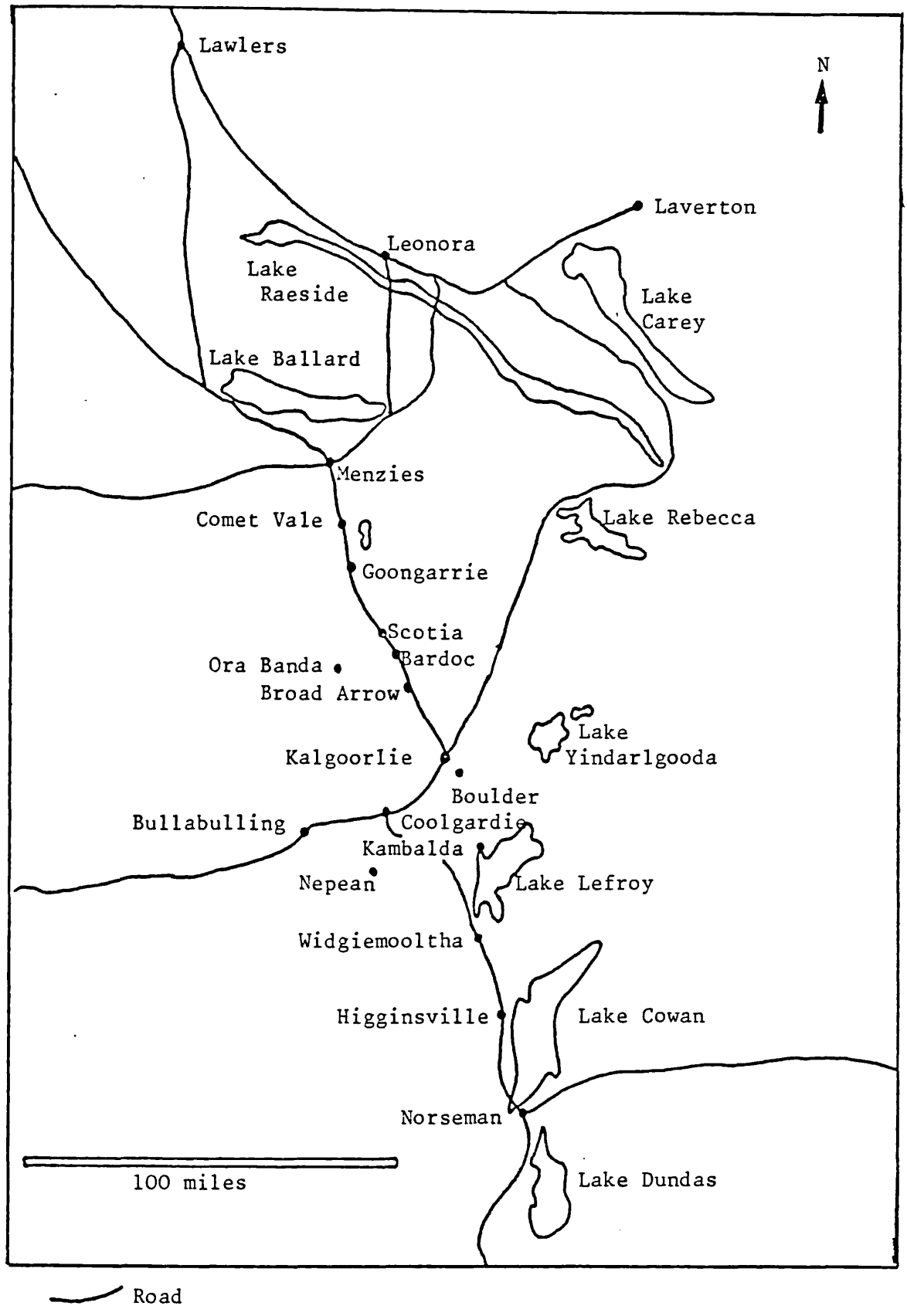

Figure 10. Locality map of the Eastern Goldfields (after Elkington, 1969).

narrow, thick-bladed leaves, occurring in the arid summer-rainfall areas of tropical Western Australia.

The species *Triodia pungens* R. Br. is widespread from Western Australia to Queensland. The coarse tussocks have projecting pungent-pointed leaves and the form may be pyramidal, flattened, ring-shaped or crescentic. The leaves are resinous and transverse section of lamina shows the midrib flanked by two small nerves on either side and the five are separated from one another by bands of motor tissue which extend across the full width of the lamina.

The grass *Eriachne mucronata* R. Br., is a member of the Aveneae. The family is almost entirely temperate in distribution in the southern hemisphere, on the mountain ranges of the tropics, in the Mediterranean region and in the Northern hemisphere. The genus *Eriachne* R. Br. includes about twenty five species, widely distributed in tropical Asia, and especially Australia, of which *E. aristidea* F. and M., *E. pallida* F. and M., *E. mucronata* R. Br., and *E. obtusa* R. Br. are the most important. The leaves in these species are characterised by having short cells, over the veins, in long rows and stomata mostly with low dome-shaped subsidiary cells. The vascular bundles, except for a few large ones, are somewhat angular in outline, and the bundle-sheaths are doubled.

The sample of the two Australian grasses, *Eriachne mucronata* R. Br. and *Triodia pungens* R. Br. investigated in this work were collected from the Dugald River area which is situated in the Mt Isa-Cloncurry mineral field of western Queensland, Australia. Under the hot, semi-arid climate of the Dugald River area low tree and shrub savanna constitutes the basic vegetation formation. An association dominated by *Eucalyptus brevifolia* and *Triodia pungens* is widely distributed over siliceous rocks, whereas one of *E. argillacea* and *T. pungens* covers the calcareous rocks.

Investigation of this area in 1962 established a hierarchical relationship between the vegetation units and environmental parameters

and disclosed the presence of an anamolous plant community composed mainly of the grass *Eriachne mucronata* R. Br. and the small shrub *Polycarpaea glabra* C.T. White et Francis. This community replaces the low tree and shrub savanna characterised by *Eucalyptus brevifolia* F. Muell and *Eucalyptus argillacea* W.V. Fitzg. trees and*Triodia pungens* R. Br. grass, where high levels of lead, zinc and copper in the soil emanating from the underlying Dugald River lode create conditions too toxic for most species (Nicholls *et al.*, 1965).

Standard biological materials

The need for standardisation of laboratory analyses over an extended period of time, together with the need of intercomparison with the work of other laboratories, has led to the development of a number of standard reference materials. These include dry plant materials (Bowen, 1965,1966,1969)(Smith and Becker, 1967)(Heinonen and Suschiny, 1972), animal tissues (Heinonen and Suschiny, 1972), clinical standards (Radin, 1967) and blood sera e.g. 'Seronorm' from British Drug Houses Ltd. Analysis of these materials has already proved valuable in confirming the reliability of established analytical techniques, in testing modifications of these techniques or entirely new ones, as well as in comparing results from different laboratories. In this work, the two standards used were the NBS (National Bureau of Standards) orchard leaves standard SRM (Standard Reference Material) 1571 and the Bowen's kale.

The NBS orchard leaves standard SRM 1571 were collected and prepared under the direction of A.L. Kenworthy of Michigan State University. These leaves were hand picked from an orchard near Lansing, Michigan, and air dried. The dried leaves were ground, dried at 85^{o}C and then thoroughly mixed. The NBS orchard leaves are certified for calcium, copper, iron, potassium, magnesium, nitrogen, sodium, nickel, phosophorus and lead. The Bowen's kale, however, is certified for aluminium,

arsenic, gold, boron, barium, bromine, calcium, cadmium, chlorine, cobalt, chromium, cesium, copper, fluorine, iron, mercury, manganese, potassium, lanthanium, magnesium, molybdenum, nitrogen, sodium, phosphorus, lead, rubidium, sulphur, antimony, scandium, silicon, strontium, uranium, vanadium, tungsten and zinc - 35 elements in all.

The fact that most biological materials can produce odours when heated shows that some chemical decomposition is taking place. It is rarely possible to dry biological samples to constant weight in as precise a manner as it is for inorganic chemicals. Nevertheless drying can usually be achieved with a reproducibility of between 0.5 and 1%. Most biological standards contain 2-5% of water, but the fresh weight may vary with the relative humidity of the atmosphere. Dry biological materials can be stored in closed containers for many years without obvious gross decomposition. Some photo-oxidation of chlorophyll occurs at surfaces exposed to light, and it is possible that microbes may slowly attack certain materials. The rate of microbial attack is presumably governed by temperature and moisture content.

In the present work we are not concerned with changes in the chemical state of elements present in the reference materials. However, if we consider only the total concentration of an element present in a standard, this is unlikely to alter on storage.

Wet ashing and sample preparation

The method described is basically that proposed by Middleton and Stuckey (1954). The plant samples were air dried and ground using a Glen-Creston agate ball mill. The powdered material was sieved through a 320 μm stainless steel sieve and stored in a desiccator over calcium chloride. Then, in duplicate, a certain mass (approximately 0.2g) was weighed accurately in a 25 cm^3 pyrex conical flask, a known volume (normally either 10 cm^3 or 15 cm^3) of Ultra grade concentrated nitric

acid was added and a short stemmed funnel inserted in the neck of the flask. This was then fitted on a thermostated sand bath and after initial frothing has ceased, the temperature of the sand bath was increased steadily during the first few hours of ashing until gentle refluxing was achieved. This set-up was left for two days or until the solution was clear and colourless, whichever was the longer. The flask was then removed from the sand bath, allowed to cool and the resultant solution transferred with washings to volumetric flasks. The solution was then diluted to a suitable volume (normally 25 cm^3 or 50 cm^3) and analysed using a Pye Unicam SP2900 atomic absorption spectrophotometer. The volume of acid required for ashing and the final volume of the solution to be analysed depends upon the weight of the material used and the concentration of elements therein, which are found by initial experiment. The readings taken were always compared with a series of standards.

Since analysis of both calcium and magnesium is severely affected by the presence of various ions especially PO_4^{3-}, SiO_3^{2-} etc., the use of strontium or lanthanum solutions was employed which effectively removed these chemical interferences.

Results

The values of metal concentrations in dried sample material are given in tables II and III. In table IV the metal concentration of Bowen's Kale and orchard leaves SRM1571 are listed as well as the values cited in the literature for typical values of mineral concentration.

3.2 Extraction - introduction

The readings given in table II shows that *Hybanthus floribundus* possess relatively high levels of nickel in the plant tissues. The concentration figures, however, represent the total concentration of metal in the tissues, it says nothing of the type of metal compounds and the

Table II Overall metal analysis of plant part samples

The readings are given on a dry weight bases and are in p.p.m. (parts per million)(μg g^{-1}).

A hyphen represents a metal concentration in solution below the limit of detection.

Sample	Sample area		Ni	Ca	Mg	Co	Cu	Zn
Hybanthus floribundus (stem)	Kurrajong		3704	4876	4216	-	-	48
		replicate analysis	3677	4845	4300	<3	-	51
		" "	3326	4577	4026	<3	-	51
		" "	3400	4835	4191	<3	-	51
		average	3527	4783	4183	<3	-	50
Hybanthus floribundus (leaves)	Kurrajong		7121	5392	2125	21	-	32
		replicate analysis	6950	5458	2329	29	-	45
		" "	7036	5311	2153	27	-	41
		average	7036	5387	2202	26	-	39
Hybanthus floribundus (leaves)	Widgiemooltha		13100	6552	5652	46	16	34
		replicate analysis	13310	6250	5613	43	16	32
		" "	13329	6429	5598	49	13	30
		average	13246	6410	5621	46	15	32
Hybanthus floribundus (roots)	Widgiemooltha		2924	4025	1006	50	21	689
		replicate analysis	2921	4155	1078	62	22	640
		" "	2934	4135	1035	53	19	650
		average	2926	4105	1040	55	21	660

Table III Overall metal analysis of plant part samples

The readings are given on a dry weight bases and are in p.p.m. (parts per million)($\mu g\ g^{-1}$).

A hyphen represents a metal concentration in solution below the limit of detection

Sample	Sample area		Ni	Co	Cu	Fe	Zn
Eriachne mucronata (stem) 1929	Dugald River lode		16	40	30	2	452
		replicate analysis	16	34	30	3	454
		" "	16	47	29	2	439
		average	16	40	30	2	448
Eriachne mucronata (stem) 1931	Dugald River lode		-	49	56	2	299
		replicate analysis	-	45	43	2	312
		average	-	47	50	2	306
Eriachne mucronata (stem) 1932	Dugald River lode		-	24	64	3	52
		replicate analysis	-	20	50	3	48
		average	-	22	57	3	50
Triodia pungens (stem) 1921	Dugald River lode		33	39	40	3	1979
		replicate analysis	37	51	46	2	1965
		" "	30	43	53	4	1996
		average	33	44	46	3	1980

Continued...

Table III continued

Sample	Sample area		Ni	Co	Cu	Fe	Zn
Triodia pungens (stem) 1945	Dugald River lode		-	-	93	2	78
		replicate analysis	-	-	77	2	89
		average	-	-	85	2	84
Triodia pungens (stem) 1946	Dugald River lode		-	-	54	3	412
		replicate analysis	-	-	60	3	409
		average	-	-	57	3	411

Table IV Metal analysis of standard biological materials

The readings are given on dry weight basis and are in p.p.m. (parts per million)($\mu g\ g^{-1}$).

A hyphen represents a metal concentration in solution below the limit of detection.

The bracket value is not certified because it is not based on the results of either a reference method or of two or more independent methods.

Sample		Ni	Ca	Mg	Co	Cu	Zn	Fe
Bowen's Kale		1	40025	1576	-	5	34	121
	replicate analysis	1	38200	1592	-	5	31	130
	average	1	39113	1584	-	5	33	126
	Reference value	0.916	41000	1580	0.0624	4.90	32.7	118.3
		±0.162	±2180	±100	±0.011	±0.65	±2.3	±17
Orchard leaves		-	20626	6484	3	13	27	282
	replicate analysis	-	20072	6572	3	13	32	293
	average	-	20349	6528	3	13	30	288
	reference value	1.3	20900	6200	(0.2)	12±1	25±3	300±20
		±0.2	±300	±200				

bonding of the metals present. Thus a simple extraction scheme was employed which is a modification of that devised by Bowen *et al.*(1962) to derive some information about the chemical character of the compounds with which the metal is associated. The extraction scheme devised is shown in Figure 11.

Method

Duplicate samples of dried and ground plant material were weighed and placed in a soxhlet thimble. The apparatus were then set up with a third set containing an empty thimble as blank. The samples and blanks were left syphoning for twenty-four hours using 150 cm^3 of solvent. The 250 cm^3 round bottomed flask in the heating mantle was then replaced with an identical flask containing 150 cm^3 of the next solvent.

After the aqueous extraction the thimble was dried at 90°C and weighed. The plug of plant material was then carefully removed from the thimble and placed in a weighed 250 cm^3 conical flask. The final mass was determined and knowing the mass of the dried thimble, the loss in mass in the transfer of the material could be calculated. 150 cm^3 of 0.5M hydrochloric acid was added to the plant material and the mixture was shaken for twenty-four hours. The mixture was allowed to settle and the clear solution was drawn off. The residues and the final residue were then wet ashed in the normal manner.

Each of the solutions after extraction were evaporated to near dryness using a rotary evaporator and then ashed with Ultra grade concentrated nitric acid on a thermostated sand bath. All samples were made up to 25 cm^3 in volumetric flasks and analysed using a Pye Unicam SP2900 atomic absorption spectrophotometer.

Results

The results obtained from the parts of the plant examined for the various chemical categories are shown in table V. The total metal

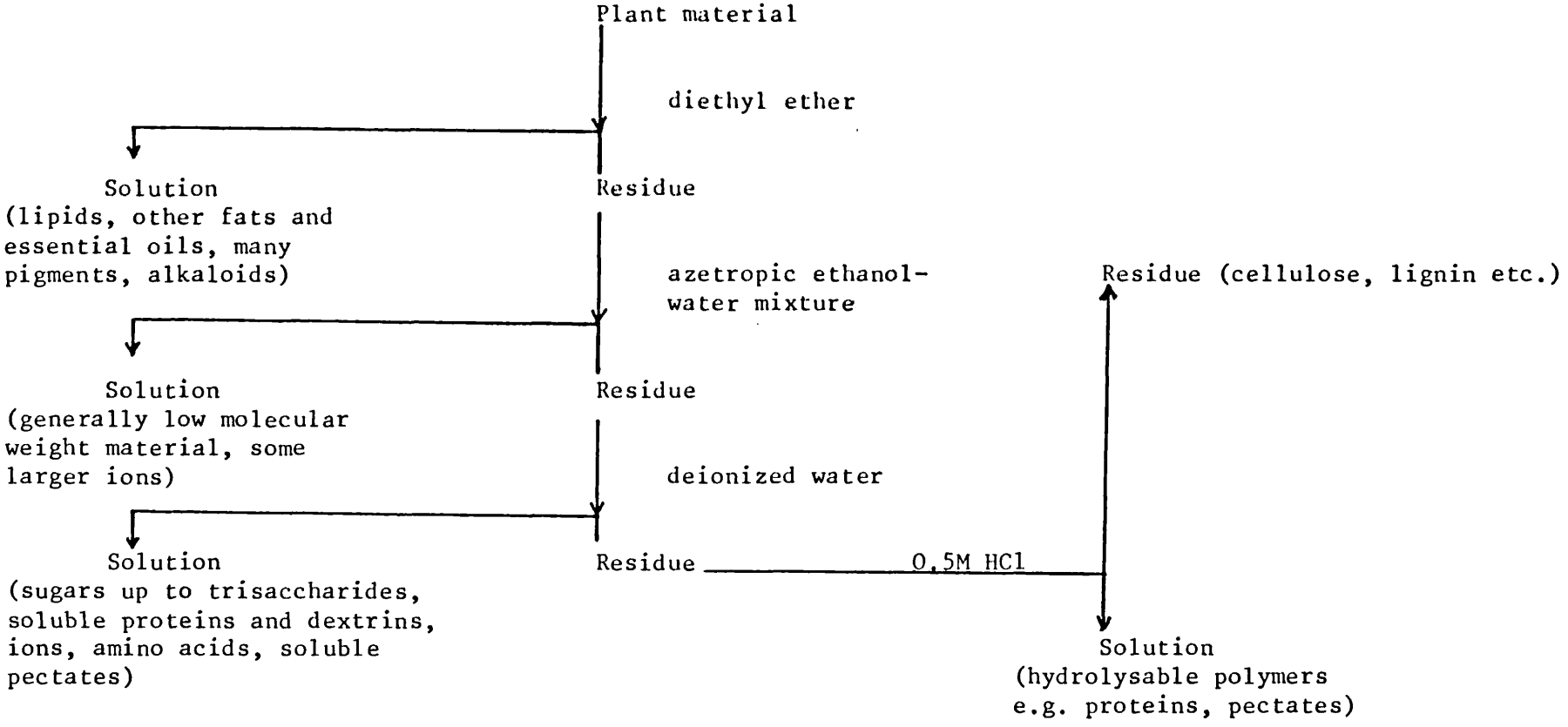

Fig.11. General extraction scheme for chemical characterization of metals present in plant material

Table V Extraction: average nickel values (p.p.m. in each extract)($\mu g\ g^{-1}$).

A hyphen represents a metal concentration in solution below the limit of detection.

Sample	Sample area	Ether (non-polar compounds)	Ethanol (low M.W. compounds)	Water (remainder low M.W. compounds)	Dilute acid (hydrolysable polymers)	Residue (α-cellulose, lignin)	Total wet ashing
H. floribundus (stem)	Kurrajong	8	239	2031	1355	-	3526
H. floribundus (leaves)	Kurrajong	7	983	3251	2890	-	7035
H. floribundus (leaves)	Widgiemooltha	27	2233	5813	5272	-	13246
H. floribundus (stems)	Widgiemooltha	2	75	1048	749	-	1869
H. floribundus (twigs)	Mount Thirsty	3	30	121	1367	-	1539

concentration values are included for comparison.

3.3 Discussion

As noted in chapter II, readings of total metal concentrations in plant material are the resultants of a myriad of interacting factors and hence the information rendered by them is limited. However, one may identify which plant samples are the most heavily mineralized in their aerial parts and in which metals. In view of the fact that the plants investigated in this work seemed to have colonized the mineralized area and apparently thrived, these plants must retain some degree of tolerance to withstand the high metal concentrations in their tissues.

The species investigated in this work come from two widely differing Australian habitats. *Hybanthus floribundus* was first found in the Eastern Goldfields area of Western Australia (Elkington, 1969) (Cole, 1967,1968). Elkington reported that the species grew only over serpentenite outcrops. It was subsequently reported over a wider area of Western Australia growing over nickel toxic soils South of Coolgardie, in Widgiemooltha and in several other areas (Cole, 1973). Brooks and co-workers have recently reported data on *Hybanthus* species, particularly from New Caledonia (Brooks *et al.*,1972,1974,1975). The plant is thus well established as a nickel accumulator.

Comparing the figures given in table II with those in the standard biological materials, we can see that by far the most spectacular result is that of nickel in *Hybanthus floribundus*. Analysis of samples of *H. floribundus* collected from the areas of its occurrences revealed very high nickel levels in the plant tissue with values tending to be highest in the leaves and higher in the stems than the roots. In contrast the quantities of cobalt and copper present were very much lower.

The analysis revealed *H. floribundus* as a nickel-accumulator.

Levels of over 13,000 p.p.m. were found in the leaves of samples collected at Widgiemooltha. The highest concentration in the surface soil in this area is 8,800 p.p.m. All the plant samples collected contained excessive quantities of nickel relative to those in the supporting soils. This concentration of nickel in the plant tissues posed questions of the bedrock source of nickel and both the reason for and the mechanism by which nickel accumulates in the plant tissues.

The usefulness of a plant species in geochemical prospecting, however, depends on a linear relationship between the concentration of the element in the soil and its relative accumulation in the plant (expressed as plant concentration/soil concentration). Timperley *et al.* (1970) demonstrated that such linear relationships are found only in the case of elements which are not essential for plant nutrition. They found the relationship between relative accumulation and element concentration for essential elements was hyperbolic; high accumulation occurs at low concentration and little or no accumulation occurs at high concentration. The curve flattened at the point where the plant's requirements were satisfied, and only in this linear part of the curve was the plant of value for prospecting.

When they examined nickel accumulation in *Hybanthus floribundus*, Severne and Brooks (1972) found that the relationship between accumulation and nickel concentration was hyperbolic; suggesting that the plant has a physiological requirement for nickel. Such a requirement is difficult to explain because nickel is normally regarded as a non-essential element in plant nutrition. However the following alternatives were listed by Cole (1973) to explain the causes of nickel accumulation by *H. floribundus*; (a) internal stress as the plant adjusts to more arid areas, (b) unrestricted transpiration and (c) a requirement for large quantities of nickel. The regional correlation of increasing nickel concentrations with decreasing rainfall has been mentioned and Severne

(1974) suggested that epidermal accumulation of nickel (where concentrations probably approach 5% or more, fresh weight) reduces cuticular transpiration and that in fact, nickel accumulation by *H. floribundus* is a xeromorphic adaptation.

If a need for nickel exists in this species, however, it would seem to be restricted to the Eastern Goldfields area of Australia because populations of *Hybanthus* from elsewhere in the country do not posses this capacity for nickel accumulation. It may be that the Eastern Goldfields population of *Hybanthus* have developed this physiological adaptation as a response to a localized selection pressure.

The other significant finding is the high zinc content in the roots of *H. floribundus*. Since zinc was found to be low in the aerial parts, it seems that zinc is taken into the roots but not translocated to the shoots. The metal is accumulated and restricted to a particular part of the plant, thus preventing the zinc from reaching sensitive metabolic sites in the plant cells.

Calcium was also found at unexpected levels in the plant. The calcium levels content of *Hybanthus floribundus* appears low, but above the critical value where deficiency symptoms occur. The plant appears to be able to tolerate low calcium without significant accumulation of magnesium which is more common in the soil. The nickel, however, does not seem to replace calcium in this plant since the plant calcium concentrations rise with nickel concentrations.

The effect of calcium upon the uptake of toxic metals has been the topic of discussion for many years. Serpentine soils have a characteristic lack of fertility, which has been considered to be primarily a lack of calcium (Walker, 1954)(Procter, 1971)(Martin *et al.*,1953). The addition of calcium to serpentine soils has been shown to decrease the acidity, which in turn decreases the absorption of nickel (Procter, 1971). Conversely, the addition of ammonium nitrate reduced the pH of such soils

and increased the availability of toxic metals (Wiltshire, 1972). There may be some relationship between nickel and calcium; Wiltshire (1972) has shown that the addition of calcium to serpentine soils and vegetation does not decrease the total amount of nickel taken up, but the plants were larger and thus contained lower concentrations of nickel. Similarly Simon (1971) showed that grasses from Rhodesian serpentine soils show the ability to take up calcium from nickel/chromium toxic soils, yet yielded less than grasses from control soils.

The species of New Caledonian serpentine plants examined by Brooks *et al.* (1974) were found to have unusually high accumulation of nickel as well as higher-than-average contents of the nutrients, calcium, phosphorus and potassium, in spite of the particularly low concentrations of these elements in the New Caledonian serpentine soils. It was also found that there were strong correlations between foliar levels of nickel and those of calcium, phosphorus and potassium in the New Zealand plant *P. suteri*. It was postulated that the very high uptake of nickel may be linked to the accumulation of macronutrients and that nickel may have a stimulating effect on this uptake. On the other hand, the same effect can be explained by inhibition of growth by the high nickel content and a consequent increase of macronutrient values owing to the longer time needed for the plant or organ to attain maturity. Albersheim (1974) has suggested that a calcium-ion dependent ATPase might be activated by auxin. In this way auxin might activate a hydrogen-ion pump in the cell wall, reducing the pH giving elongation growth. If the ATPase is inhibited by nickel ions then the stunted growth of plants grown in nickel rich soils might be accounted for.

The extraction scheme employed shows more than half of the nickel in the green parts of the plant is present as a low molecular weight material soluble in the aqueous fraction, the rest of the nickel being released by acid. In the twig material considerably less nickel was

water soluble, more than 80% of the nickel appearing in the acid fraction.

The other two species, namely, *Triodia pungens* and *Eriachne mucronata* are from the Dugald River area and were found growing over the Dugald River lode which is a lead-zinc ore body. It can be seen from Table III that both species take up zinc into their tissues with the *Triodia* species accumulating up to 1900 p.p.m. zinc. It seems that these species growing on toxic metalliferous soils cannot prevent zinc uptake but only restrict it and hence accumulate the zinc in their tissues to varying degrees. The strategies of survival are thus tolerance and not avoidance of metal toxicity.

Studies have been made of the metal levels in the aerial parts of both *Triodia pungens* and *Eriachne mucronata* sampled over a range of soils (Nicholls *et al.*, 1965). In both species the relationship between zinc uptake and the zinc content of the soil is linear, even at very high soil levels. There thus appeared to be no restriction of zinc uptake. Baker (1981) has pointed out that this is typical of a metal "indicator", where uptake and transport of metals to the shoot are regulated so that the internal concentration reflects the external levels. Baker (1981) further suggests that in these species there are different uptake, transport and accumulation for zinc, lead and copper. The responses of these species to lead and copper being those of "excluders", where metal concentrations in the shoot are maintained constant and low over a wide range of soil concentration, up to a critical soil value above which the mechanism breaks down and unrestricted transport results. Similarly, Simon (1978) has suggested that different tolerance mechanisms exists for zinc and lead, where lead tolerance being an exclusion mechanism, whereas that for zinc is internal. This appears to be general for zinc-tolerance.

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CHAPTER IV

A STUDY OF WATER SOLUBLE METAL COMPOUNDS PRESENT IN *H. FLORIBUNDUS*, *T. PUNGENS* AND *E. MUCRONATA*

4.1 Complexes in Plants

It was noted in section 1.8, that concentrations of metal in the soil may create conditions which are toxic for many species. The degree of availability or toxicity will depend on a variety of factors, notably the soil pH. Different plants react to these conditions in different ways. Some may require large quantities of certain metals and can grow only where concentrations occur in the soil. Such plants usually accumulate these metals and are known as accumulators; they may be distinguished from other accumulator plants which concentrate large quantities of metals in their tissues from relatively small quantities in the soil. The accumulation of metals by these plants must involve a specific internal metabolic process to prevent metals reaching and interfering with metal sensitive sites.

As noted in section 1.9 complexing of metals by natural ligands in plants has been observed as a metal tolerance mechanism whereby heavy metals are kept from metabolically sensitive sites. Most heavy metals are capable of forming complexes with ligands containing sulphur, nitrogen, or oxygen as electron donors. In any living cell, at least the following ligands can be expected to be present:

$$-OH,\ -COOH,\ -PO_3H_2,\ -SH,\ -NH_2,\ \text{-imidazole.}$$

These ligands, which form integral parts of almost any molecule of biological significance, are frequently essential to the normal functioning of the cells.

Heavy metal binding with the above ligands is strong but not

specific with regard to either the ligands or the heavy metal. The only rules that can be established are concerned with order of affinities. For example, heavy metals are bound by amines or simple amino acids in the following order of decreasing affinity (Gurd and Wilcox, 1956):

$$Hg^{++} > Cu^{++} > Ni^{++} > Pb^{++} > Zn^{++} > Co^{++} > Cd^{++} > Mn^{++} > Mg^{++} > Ca^{++} > Ba^{++}$$

whereas the affinity towards COO^{-} groups may be represented by the following series:

$$Cu^{++} > Ni^{++} > Zn^{++} > Co^{++} > Mn^{++} > Mg^{++} > Ca^{++} > Ba^{++}$$

Precise data about the association constants for the reaction between heavy metals and sulphydryl groups of organic molecules are not available. However, as an approximate measure, Klotz (1959) presented a list of the solubility product constants for various inorganic sulphides:

$$Hg^{++} > Ag^{++} > Pb^{++} > Cd^{++} > Zn^{++} > Ca^{++} > Mg^{++}.$$

The hydroxyl group of water can as well participate in complex formation and in the formation of insoluble hydroxides. These complexes may have a very complicated structure and their electrochemical behaviour may be quite different from the behaviour of simply hydrated ions. Chloride ions, which are always present in biological materials, also form strong complexes with mercury (Passow *et al.*, 1961), and perhaps with other heavy metals. All the listed biological ligands contain dissociable protons, and heavy metal ions replace these protons in complex formation. Finally heavy metals have been shown to be involved in numerous enzymes and coenzymes and other functional constituents of normal cell activity.

In an attempt 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). In tomato exudate, nickel was found to migrate anodically at low concentrations and it was suggested it is bound to organic anion (carrier). Various crop species were also found to translocate low concentration of nickel in the same

anionic form (Tiffin, 1971). Further work by Tiffin (1966,1970) demonstrated the association between iron and citrate in exudates of sunflower and soy bean. Further to this he showed that manganese, cobalt and zinc are transported as inorganic cations in stem exudates of tomatoes (Tiffin, 1967).

Work by Reilly (1969,1973) on copper accumulating species has suggested the presence of copper amino acid complexes, in particular a copper cysteine complex which is water extractable. Reilly *et al.* suggest that copper is removed by binding as a copper peptide complex, and have further shown that much of the copper is bound in the plant in a non-extractable form.

Metal complexes as part of the structure of cell walls has also been suggested (Turner and Gregory, 1967). Peterson (1969) has found that the ^{65}zinc in the pectate extract of the metal tolerant grass, *Agrostis tenuis* is higher in zinc-tolerant than in the non-tolerant plants, and suggests that the mechanism of tolerance is the deactivation of zinc by cation binding sites in the cell wall. Farago and Pitt (1977) have also shown pectin to be the complexing agent for zinc in tolerant *Polycarpaea glabra*.

Phenolic hydroxyl and carboxylic acid groups in plant tissues have also been suggested as ligands (Timberlake, 1959). Other complexes which have been identified are: chromium as the tris(oxalato) ion in *Leptospermum scoparium* (Lyon *et al.*, 1969); zinc bound to mustard oils and oxalate ions within the vacuoles of zinc tolerant *Silene cucubalis* and *Thlaspe alpestra* (Mathys, 1977); Cobalt complexed with amino acid in *Helianthus annus* (Hoefner, 1968); iron with malate and maleate (Tiffin, 1966) and aluminium with citric and oxalic acid (Jones, 1961). Also 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. Pelosi *et al.* (1976) have shown that nickel in

an aqueous extract of *Allysum bertolonii* is associated with malic and malonic acid.

4.2 Examination of Soluble Metal Compounds

The methods used in this investigation for examination of soluble metal complexes in *Hybanthus floribundus*, *Triodia pungens* and *Eriachne mucronata* involve the extraction of dried plant material with water or aqueous alcohol, followed by initial purification stages including gel filtration and solvent extractions. This was followed by separation and examination by chromatographic and electrophoretic methods. Final identification requires synthesis of the compound for comparison with the sample compound as regards mobility to confirm the identification.

In all the steps described, chemical changes may occur which possibly lead to incorrect conclusion. For example, in a living cell, the cell membrane is the first and most important site of action of metals. Frequently, almost all of the metal applied is rapidly absorbed by the easily accessible ligands of the outer surface of the membrane. The interior of the cell, on the other hand, is protected by the membrane as a diffusion barrier and also by the many other substances in the cytoplasm that can react with and divert the metal from sensitive sites. This arrangement, however, may differ from one cell to another within the same plant. Such cellular integrity may possibly be destroyed in the crushed extracted material and any number of reactions may take place between metals and ligands in the new artificial situation. If the complexes are kinetically labile, then those which are thermodynamically stable under intact cell conditions need not be those which are stable in the extracts.

Column chromatography used in purification of the plant extract may cause complex dissociation. Silica gel, alumina, ion-exchange columns and many others have a tendency to dissociate complexes. Gel filtration and the Sephadex series, however, appears not to create too much cause for

concern in these respects.

Concentration of the eluant coming at the bottom of the column needs special precautions to avoid complex dissociation. This could be minimized by the use of rotatory evaporator at the lowest possible temperature or freeze drying where necessary.

Finally, thin layer chromatography, paper chromatography and electrophoresis may cause complex dissociation due to the adsorption process itself. The solvent used in the developing, the pH of the system, the buffer in electrophoresis are all factors which may lead to the dissociation of the complex. Thus the initial results from such studies must be treated with some caution and a discovery of uncomplexed metal in an electrophoretogram ought not lead to the automatic conclusion that the metal is present in the plant as uncomplexed metal ions.

4.3 The Chromatographic Analysis of Water Soluble Complexes of *H. floribundus*

Preparation of extract for chromatography

2g. dried milled plant material of leaves of the Australian shrub, *Hybanthus floribundus* (Lindl.) F. Muell. was shaken for 6 hours at room temperature with 150cm^3 of methanol in a stoppered 500cm^3 quickfit conical flask. The resultant solution was filtered using a "millipore" filter and the leaf material further extracted twice as above and the solution added to the original extract. The residue was dried in a stream of air, and finally extracted for 6 hours with 150cm^3 of demineralised water at room temperature. The aqueous extraction was repeated twice and the two extracts were combined. The water extract was freeze-dried, while the methanol extract was evaporated to about 1cm^3 using the rotary evaporator. Both extracts were stored in a sealed container in a refrigerator until required. Immediately before the chromatographic experiments the extracts were taken up in a minimum of solvent.

Purification of the extract was achieved by a number of methods with varying degrees of success. Attempts to use alumina column were found to be unsuccessful, since most of the nickel was found to be retained by the column and it would appear to have been bound to the alumina. Sephadex columns were used and found to be satisfactory. Also the concentrated solutions were spread along the origins of paper chromatograms and developed using different solvents. The nickel bands were then cut from the papers and were extracted with demineralised water.

Chromatography

The paper chromatography was performed in the usual manner using 300×300mm Whatman No.1 chromatography paper. A horizontal line 2cm up from the base of the paper was drawn in pencil, and a drop of the extract to be separated is placed in a marked position. When the spot has dried the papers, 5 to a frame, were placed in Shandon Glass Tanks with one end immersed in the solvent chosen as the mobile phase. When the solvent front has moved a suitable distance, or after a pre-determined time, the papers were removed from the tanks, the position of the solvent front was marked, and the papers were allowed to dry. After drying, the chromatogram was sprayed with rubeanic acid and dimethylglyoxime to locate the nickel. The following solvent systems, commonly utilised in natural product investigations, were employed:

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

(ii) butanol-acetic acid-water, 12:3:5, v/v/v;

(iii) phenol-water, 4:1, w/v.

The chromatographic results are presented in Fig.12. In both water and methanolic extracts detection for nickel with dimethylglyoxime gave a red colour. The suggestion that polyphenolic species may have been involved in chelation was investigated by spraying the papers with ferric chloride/ferricyanide or diazotised p-nitroaniline. With both detecting reagents, however, no plant phenols were found to be associated with

the nickel containing species. Although the water was found to extract much of the nickel present in the leaf, yet a test of the residue showed unextracted nickel. Ethanol 90%, v/v and acetic acid were also found capable of extracting some of the nickel left in the residue. Acetone, ether and ethyl acetate extracts found to be devoid of nickel, suggesting the fact that polar solvents were the best for nickel extraction from the leaf sample although a significant quantity of the metal remained in the residue as a more strongly bound complex. This suggestion is supported by the preliminary extraction results which revealed a water soluble and an acid soluble fraction.

In addition to the nickel spot, a yellow 'pigment' was always found with a different R_f value from that of the nickel (average R_f in butanol-acetic acid-water, 28). This yellow pigment was found to change from pale yellow to deeper yellow upon exposing the paper to ammonia vapour prior to dimethylglyoxime treatment for nickel. Under the UV it has a fluorescent yellow colour and gave a positive reaction to the reagents used for the identification of polyphenolic compounds.

From the results it would seem that nickel in the leaves of *H. floribundus* is associated with some other ligand. Spraying the developed chromatogram for acids showed no positive reaction. At this stage pectins were implied as one of the possible complexing agents involved and thus use was made of the known fact that they are soluble in 0.5% ammonium oxalate solution. Thus similar extraction procedure was carried out as before with 0.5% aqueous ammonium oxalate. Spots of the extract, nickel ions (0.03M) and nickel pectinate were applied to the sheets and the chromatograms developed in the ascending mode. The solvent systems used were:

(i) butanol-acetic acid-water, 12:3:5, v/v/v;

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

The chromatographic results are presented in Fig.13.

Figure 12. Ascending Chromatography of *H. floribundus* leaves extract using 300×300mm Whatman No.1 Chromatography paper

Spots Applied	Detecting Reagent Used	R_f values in various solvent system	
		butanol-acetic acid-water, 12:3:5, v/v/v	phenol-water 4:1, w/v
Aqueous extract	Dimethyl-glyoxime	10.35	streak 0.0 ⟷ 2.86
Methanolic extract	Dimethyl-glyoxime	7.8	streak 0.0 ⟷ 3.24

Figure 13. Ascending chromatography of *H. floribundus* leaves extract using 300×300mm Whatman No.1 Chromatography paper

Spots Applied	Detecting Reagent Used	R_f values in various solvent system	
		butanol-acetic acid-water, 12:3:5, v/v/v	butanol-pyridine-water, 1:1:1, v/v/v
Nickel pectinate	Dimethyl-glyoxime	9.5	8.3
Nickel chloride	Dimethyl-glyoxime	17.2	8.4, 7.1
Sample extract	Dimethyl-glyoxime	9.3	8.9
Sample extract	Rubeanic acid		8.9, 7.5
Nickel chloride	Rubeanic acid		8.4, 7.3

From the results it would seem that there may well be a nickel pectinate complex involved. No ionic nickel was detected in the first solvent, yet one must bear in mind that the ammonium oxalate will extract the plant pectins and may well provide a situation in which the nickel in the plant, perhaps in the form of a weak chelate, is allowed to react with the extracted species. The second solvent, however, was rejected since dissociation occurred. It is believed, that in this case, pyridine may be complexing with the metal, resulting in common R_f values for both ionic and extracted nickel.

4.4 Determination of the Charge and Mobility of the Complex by Electrophoresis

Method

Spots of the aqueous and methanolic extracts of *H. floribundus* were spotted onto Whatman Number 3 paper (10cm wide, 30cm long). The buffer used was a pyridine-acetic acid at pH6.1 and the potential difference was 150v. The apparatus was left for 1½ hours when the paper was removed. The position of nickel was determined by spraying with rubeanic acid solution which gave a characteristic blue colour with this element.

Results

The resulting electrophoretogram is drawn in Figure 14. Soluble nickel in the aqueous and alcoholic extracts can be seen to have moved towards the negative electrode. From the results it would seem that the nickel in the two extracts existed almost entirely in the form of double-charged ions or nickel complexes because the spots coincided very nearly with a Ni^{2+} stnadard. The spots were slightly retarded and this implied the existence of complexes with a greater molecular mass than free nickel ions. Electrophoresis of this complex was repeated at a pH of 7.1, with the same result.

Paper Electrophoresis

Electrophoresis	Paper	Whatman Number 3
	Locating reagent	Rubeanic acid
	Buffer	Pyridine/acetic acid pH6.1
	Potential	150 V

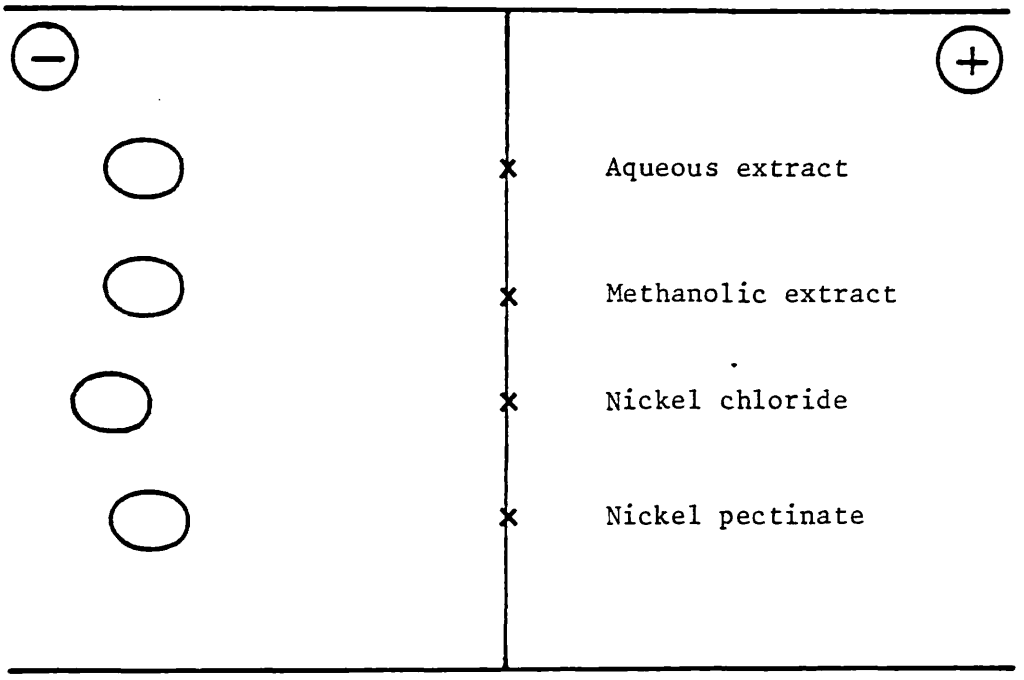


Figure 14. Nickel chloride, nickel pectinate and *Hybanthus floribundus* leaf aqueous and methanolic extracts

4.5 Investigation of the ligand of the Complex Extracted from *H. floribundus*

Extraction method

2g. of dried milled material was refluxed for 15 minutes with 150cm^3 of methanol. The residue was filtered off, washed with warm methanol, dried in a stream of air, and extracted with 150cm^3 distilled water at 75-80^{o}C for 1 hour. The residue was filtered off, and the extract was freeze dried. Further extraction of the residue with 0.5% aqueous ammonium oxalate was carried out at 70^{o}C for 1 hour. The solution was filtered and the filtrate kept. The latter two extracts were found to contain most of the nickel and were used in subsequent chromatography.

Chromatography

Two-dimensional chromatography was carried out on 300×300mm Whatman No.1 chromatography paper in Shandon Glass Tanks. Spots of concentrated water extract, nickel pectinate, nickel chloride (0.03M) and ammonium oxalate extract were applied to the paper and developed in the ascending mode. The following solvent systems were used:

(i) Butanol-acetic acid-water, 12:3:5, v/v/v

(ii) 2% acetic acid.

The chromatographic results are presented in Fig.15. The solvents systems used provided adequate separation. Similar results were obtained on spraying the papers with dimethyl-glyoxime or rubeanic acid. The aqueous extract suggested the presence of two nickel components which coincide with ionic nickel and nickel pectinate, whereas the ammonium oxalate extract gave a spot corresponding to that of nickel pectinate in R_f value.

At this stage streaks of the concentrated aqueous extract were placed on ten sheets of Whatman No.3 chromatography paper. These were developed in the descending mode with butanol-acetic acid-water, 12:3:5, v/v/v, solvent. Thin strips were cut from the sides of the papers

Figure 15. Ascending two-dimensional chromatography of *H. floribundus* leaf extract using 300×300mm Whatman No. 1 chromatography paper

Spots Applied	Detecting Reagent Used	R_f values in	
		butanol-acetic acid-water, 12:3:5, v/v/v	2% acetic acid
Aqueous extract	Dimethyl-glyoxime	8, 14.7	12.1, 22.1
Nickel pectinate	Dimethyl-glyoxime	7.2	11.3
Nickel chloride	Dimethyl-glyoxime	13.9	22.8
Ammonium oxalate extract	Dimethyl-glyoxime	7.5	12

Figure 16. Ascending chromatography of the two bands using 300×300mm Whatman No.1 chromatography paper

Spots Applied	Detecting Reagent Used	R_f values in butanol-acetic acid-water, 12:3:5 v/v/v
Band 1	Dimethyl-glyoxime	5.9
Band 2	Dimethyl-glyoxime	18.5
Nickel chloride	Dimethyl-glyoxime	19.2
Nickel pectinate	Dimethyl-glyoxime	6.1

Figure 17. Ascending chromatography of *H. floribundus* leaf extract using 300×300mm Whatman No.3 chromatography paper

Spot Applied	Detecting Reagent Used	R_f values in butanol-acetic acid-water 12:3:5, v/v/v
Nickel complex	p-anisidine hydrochloride-sodium hydrosulphite	15
D-galacturonic acid	p-anisidine hydrochloride-sodium hydrosulphite	15

and sprayed with dimethyl-glyoxime. Two bands were identified: Band 1, R_f = 11; Band 2, R_f = 21. The two bands were cut from the papers and were extracted with demineralised water. The extracts were concentrated and rechromatographed (Fig.16).

Finally, the band with an R_f value corresponding to that of nickel pectinate was passed through a Sephadex 25 column. The solution emerging at the end of the column was concentrated and spotted on Whatman No.3 chromatography paper and developed with butanol-acetic acid-water, 12:3:5, v/v/v. Spraying the developed chromatogram for amino acids, acids and phenols showed no positive reaction. However, the spot changed to yellow-brown colour on spraying with p-anisidine hydrochloride-sodium hydrosulphite, thus indicating the presence of a carbohydrate. The nickel complex was then hydrolysed for half an hour with dilute hydrochloric acid and rechromatographed. The chromatographic results presented in Fig.17 clearly demonstrate that soluble nickel in *Hybanthus floribundus* leaves is associated with pectic carbohydrates.

4.6 The Chromatographic Analysis of Water Soluble Complexes of *T. pungens* and *E. mucronata*

Extract preparation

5g. of the dry powdered material of each of two Australian zinc-tolerant grasses, *Triodia pungens*, sample No.1921, and *Eriachne mucronata*, sample No.1929, was extracted at room temperature for 8 hours with 200cm^3 of demineralized water. The solid material was filtered off using a millipore filter, and re-extracted. The two extracts were combined, freeze dried and stored in a sealed container in a refrigerator until required. A minimum of demineralized water was added before the chromatographic experiments.

Chromatography

One dimensional chromatography was carried out using Whatman No.1 paper in the ascending mode. A whole series of solvents were attempted before the butanol-acetic acid-water system was found to produce the least degree of dissociation and the greatest differences between R_f values of the complexed and uncomplexed metal ion.

The samples, together with zinc ions, were spotted on the paper, dried and developed with butanol/acetic acid/water present in the ratio 12/3/5 by volume. After drying, the chromatogram was sprayed with dithizone in chloroform to locate the zinc.

In the aqueous extracts of *Triodia pungens*, two zinc spots developed when the papers were treated with dithizone, one corresponded to ionic zinc and the other did not appear to correspond to any amino acid. While in *Eriachne mucronata* aqueous extract, only one zinc spot developed which corresponded to that of ionic zinc.

The chromatographic results are presented in Fig.18. Further chromatographic experiments using phenol-water, 4:1, w/v; and butanol-pyridine-water, 1:1:1, v/v/v confirmed the above results.

4.7 Determination of the Charge and Mobility of the zinc Complexes by Electrophoresis

Method

The aqueous extract of *Triodia pungens* and *Eriachne mucronata* were spotted onto Whatman Number 3 paper. The buffer used was a pyridine/acetic acid at pH6.9 and the potential difference was 150v. The apparatus was left for 45 minutes when the paper was removed, dried and sprayed with dithizone.

Results

Figure 19 shows that in *Eriachne mucronata* the soluble zinc in the

Paper Chromatography

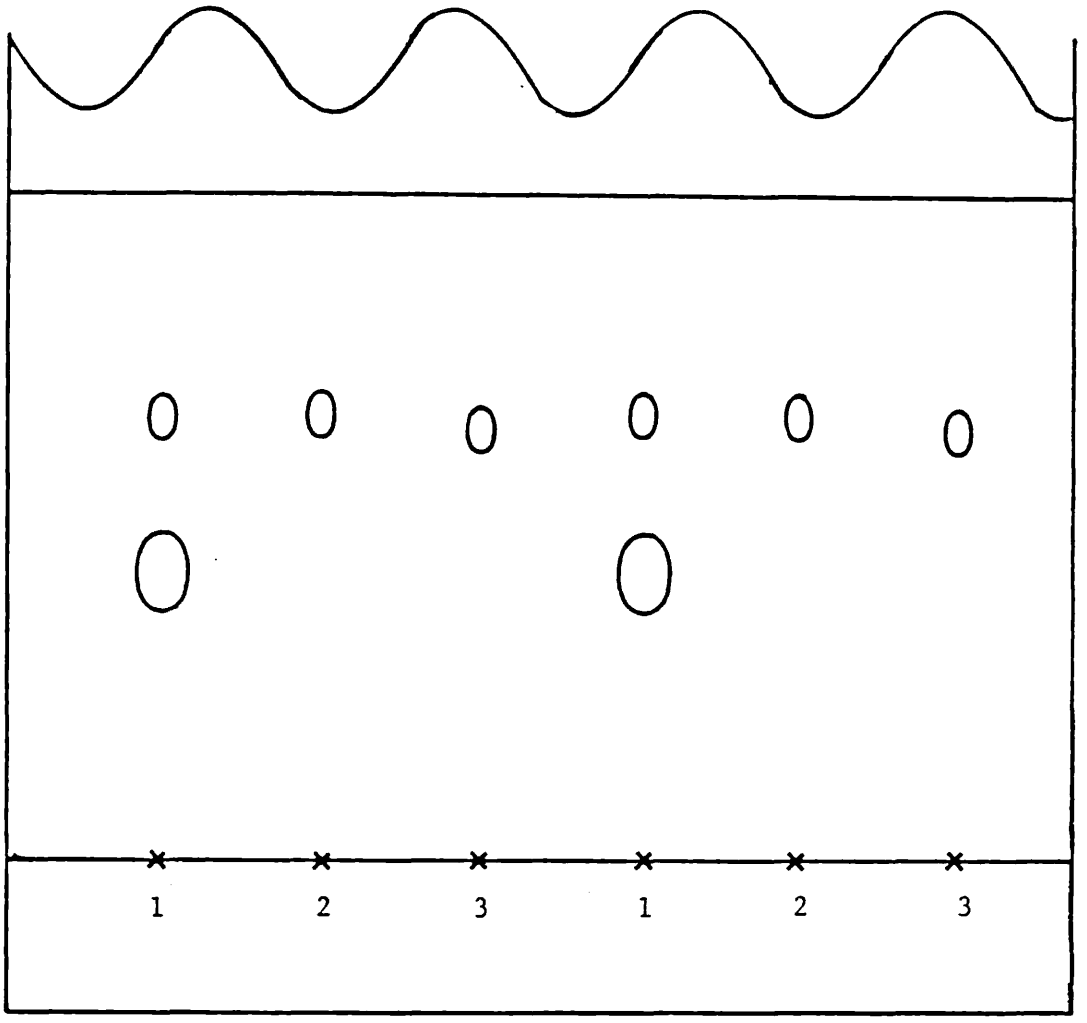


Figure 18. Aqueous extract of *Triodia pungens* and *Eriachne mucronata*. Half scale

Solvent Butanol/acetic acid/water 12/3/5, v/v/v

Indicator Dithizone

Paper Whatman 1MM

1 *Triodia pungens*, aqueous extract

2 Zinc ions

3 *Eriachne mucronata*, aqueous extract

R_f. Zn^{++} 38

Paper Electrophoresis

Electrophoresis	Paper	Whatman Number 3
	Locating reagent	Dithizone
	Buffer	Pyridine/acetic acid pH6.9
	Potential	150 V

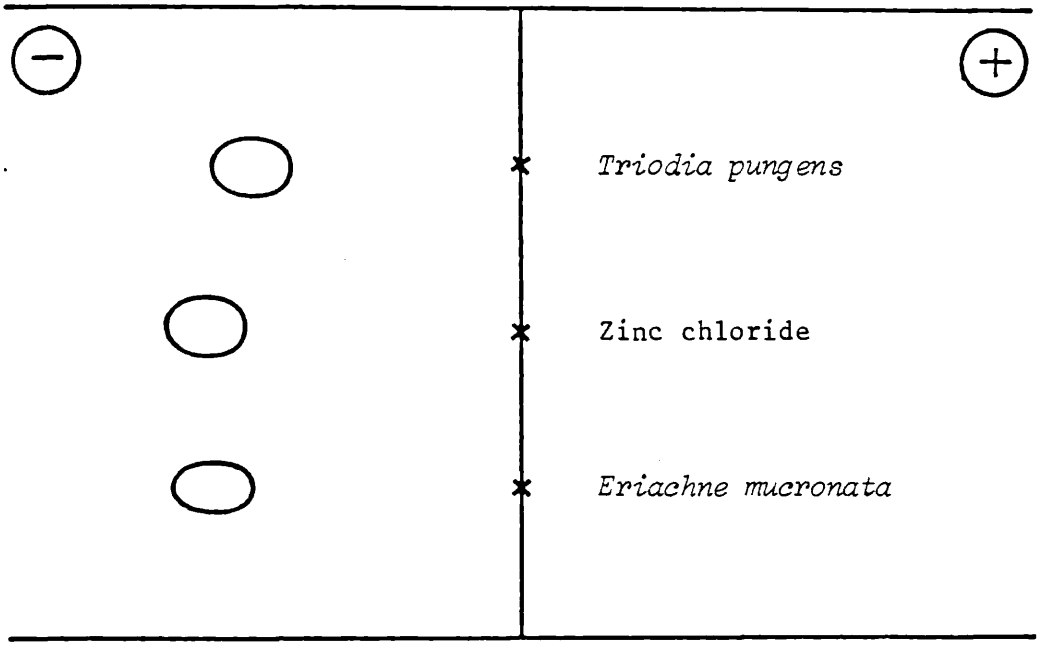


Figure 19. Zinc ions, *Triodia pungens* (aqueous extract) and *Eriachne mucronata* (aqueous extract)

aqueous extract existed almost entirely in the form of double-charged zinc ions, since the 'spot' coincided with a zinc standard. While the 'spot' in *Triodia pungens* was retarded, implying the existence of complexes with a greater molecular mass than free zinc ions.

4.8 Chromatographic Identification of Ligands in the Zinc Complex from *Triodia pungens*

~~In comparison with chromium, manganese, iron, cobalt and copper, zinc is a poor complexing metal and many of its complexes are exceedingly labile~~. As regards identification of the zinc complex in *Triodia pungens* a variety of solvent systems, paper and thin layer plates were used to study the complex, the majority of which caused the complex at least partially to dissociate. The only systems, however, found acceptable were Whatman 1 MM papers with butanol-acetic acid-water, 12:3:5, v/v/v and 12:3:10, v/v/v, for paper chromatography and the equivalent cellulose plates with the same solvents for thin layer chromatography.

Chromatography

The following methods were finally used: The extract of *Triodia pungens* was passed through a Sephadex-25 column, and the zinc-containing fraction was located by spotting the eluent onto filter paper impregnated with dithizone. The solution emerging at the end of the column was concentrated on a rotary evaporator, followed by gel-filtration to remove competing ions from the zinc complex solution.

The solution of the separated complex was chromatographed (ascending mode), together with zinc ions on Whatman No.1 paper, using butanol-acetic acid-water (12:3:10, v/v/v). Finally cellulose plates were employed with butanol-acetic acid-water (12:3:5, v/v/v) as developing agent and dithizone as identification reagent.

The chromatographic results are presented in Fig. 20 and 21.

Paper Chromatography

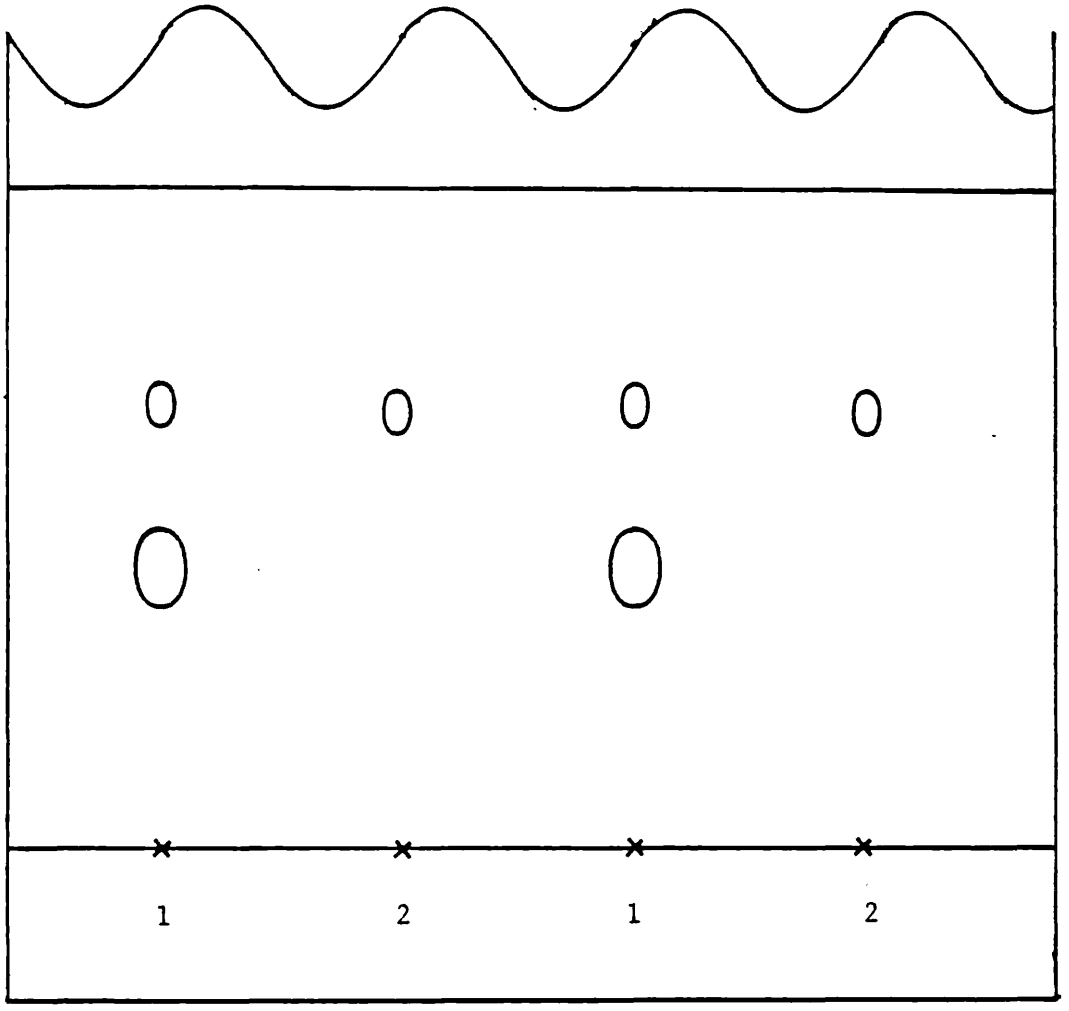


Figure 20 Purified aqueous extract of *Triodia pungens*. Half scale

Solvent Butanol-acetic acid-water, 12:3:10, v/v/v

Indicator Dithizone

Paper Whatman 1MM

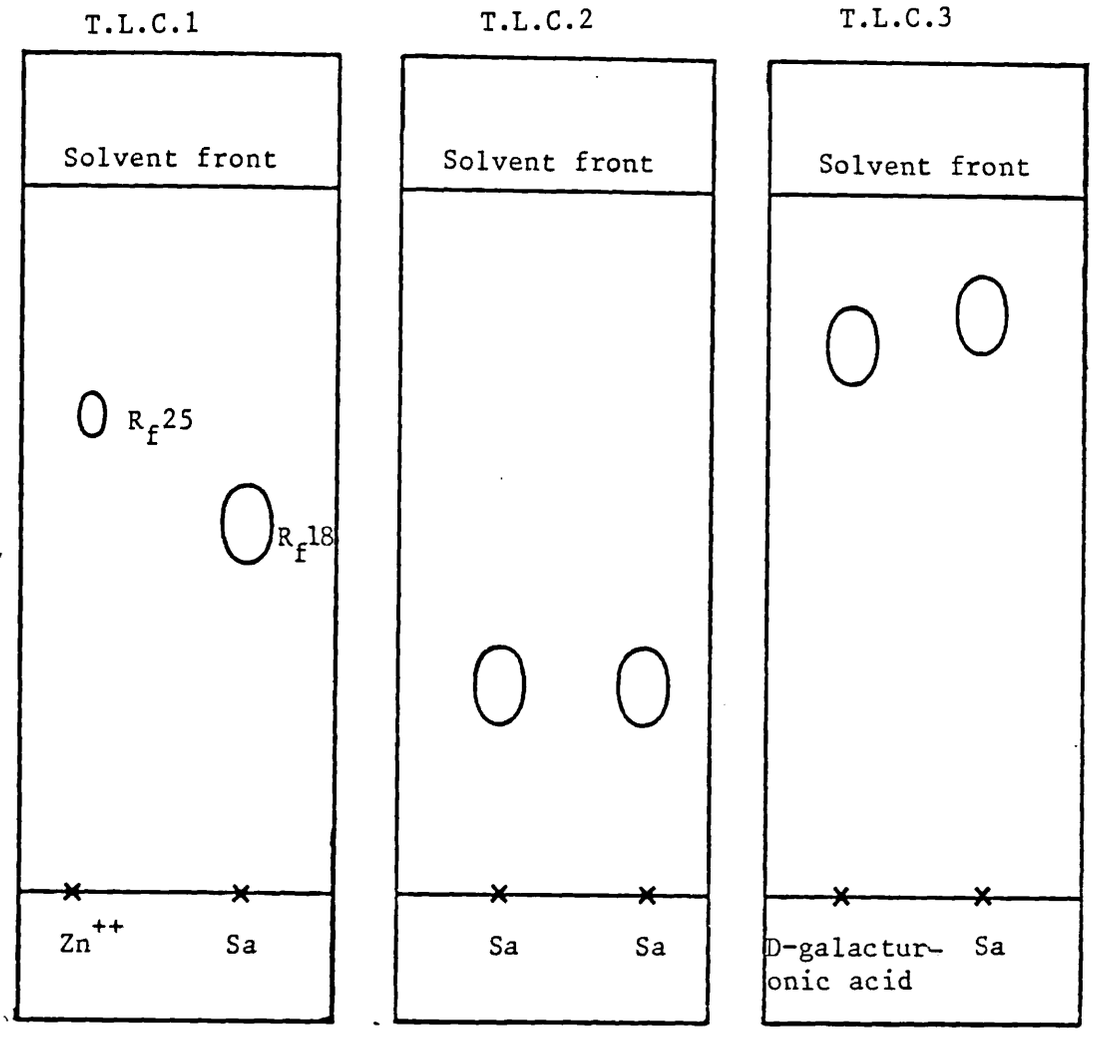
R_f *Triodia pungens* 4.5 and 33

R_f Zinc ions 35

1) *Triodia pungens*, aqueous extract

2) Zinc ions

Thin Layer Chromatography



Indicator 1. Dithizone 2. Anisidine-phthalic acid

3. p-Anisidine hydrochloride-sodium hydrosulphite

Plates 1. Cellulose 2. Silica gel 3. Silica gel

Solvent 1. Butanol-acetic acid-water, 12:3:5, v/v/v

2. MEK-acetic acid-methanol, 3:1:1, v/v/v

3. Benzene-acetic acid-methanol, 1:1:3, v/v/v

*Sa : Sample

Figure 21

At this stage bands of the concentrated extract were placed along the origin lines of ten sheets of Whatman No.1 paper, which were developed in butanol-acetic acid-water (12:3:5, v/v/v) in the ascending mode. Thin strips were cut from the sides of the papers and tested with dithizone and ninhydrin. Two zinc positive areas were located at R_f = 2.2 and R_f = 22, both were negative with ninhydrin. The two bands containing the zinc were cut from the papers and each was eluted with water.

The eluants were made acidic with dilute hydrochloric acid and digested for half an hour at 80°C. Each solution was then spotted on silica plates pretreated with 0.1M boric acid, developed with methyl ethyl ketone-acetic acid-methanol (3:1:1, v:v:v) and sprayed with p-anisidine-phthalic acid reagent. The extract of the first band (R_f = 2.2, on paper above) showed a brown colour at R_f = 12 (Fig. 21, TLC2) (D-galacturonic acid in the literature has R_f = 10 (Zweig and Sherma, 1972)), and the second extract was negative.

Finally, the following tests were carried out on the extract of the first band: using paper chromatography Whatman No.1 in the ascending mode, butanol-acetic acid-water (12:3:10, v/v/v) as solvent and spraying with p-anisidine hydrochloride-sodium hydrosulphite reagent, a brown spot was located at R_f = 28 (D-galacturonic acid, R_f = 27). With silica gel plates treated with 0.1M boric acid, developed with benzene-acetic acid-methanol (1/1/3, by volume) (Pastuska, 1961) and sprayed with p-anisidine hydrochloride-sodium hydrosulphite reagent, a carbohydrate could be seen at an R_f = 35 (Fig.21, TLC3). In the literature (Zweig and Sherma, 1972) only D-galacturonic acid has R_f value close to this, and no other carbohydrate has an R_f value with this system of less than 50.

In *E. mucronata*, however, the aqueous extract was found to contain zinc indistinguishable from Zn^{++} ions.

4.9 Discussion

The problem of the accumulation of metals by plants can be divided into three sections (a) the intake of metal through the root, (the availability of metal in the immediate vicinity of the root wall will also be involved); (b) the transport of the metal through the plant tissue and (c) the deposition of the metal in the aerial part of the plant.

Up to the present, two main methods have been used to attack the problem. One area has been the attempted elucidation of the nature of chelating agents, first proposed by Jowett (1958). Work in this connection has concentrated on plant exudates (Tiffin, 1966,1967,1971). The main area of investigation has been the localisation of the immobilised metals and suggestions have been made that metals become structural components of the cell wall (Reilly, 1969,1972) (Reilly and Reilly, 1973)(Turner and Gregory, 1967)(Turner, 1970)(Peterson, 1969) (Gambi, 1967). This process must also involve ligation of some kind. The present state of knowledge, however, does not allow us to say if a particular metal is accumulated by various plants by a similar mechanism or if plants of related species accumulate metals by similar mechanisms.

The work of Peterson (1969) using ^{65}Zn and *Agrostis tenuis* showed that zinc is extracted with the pectate fraction of the root residue which suggested that zinc accumulation is associated with the carbohydrate component of the cell wall.

Metal components of pectates are well known (U.S. Patent, 1950) (Wunsch, 1952), however their stability constants lie in a narrow range (Wunsch, 1952) and appear to bear out the objections to a simple carbohydrate mechanism of accumulation (Woolhouse, 1969). The main objection put forward was that carbohydrate binding sites would not be specific for any particular metal, and proteins were suggested as being more likely specific binding sites. However, if specificity is at the primary entry of the metal into the root, then there is no objection to

a carbohydrate mechanism of accumulation, since cell wall sites will simply store that metal ion introduced at root level.

The chromatographic results in this work have established that in both *Hybanthus floribundus* and *Triodia pungens*, the metal is associated with the pectates. In both plants the soluble metal is obtained as metal galacturonate after hydrolysis. Since galacturonic acid is the main structural component of pectic polymers, zinc is therefore established as being associated with this type of carbohydrate in the aerial parts of *Triodia pungens*. Thus the complex will not be retained on a Sephadex column for any great length of time and would show a large degree of dissociation. The presence of high concentrations of other metals would certainly dissociate the complex as would most electrophoresis and chromatographic systems.

Hence there is a general consistency in the suggestion that the water soluble complex of zinc from *Triodia pungens* aerial parts is a D-galacturonic acid containing compound, a pectinate.

Similarly it has been shown that soluble nickel in *Hybanthus floribundus* leaves is associated with pectic carbohydrates. Soluble nickel in the alcoholic extracts was found to exist almost entirely in the form of double-charged nickel ions or nickel complexes because the spots coincided very nearly with a nickel standard. The spots were slightly retarded and this implied the existence of complexes with a greater molecular mass than free nickel ions. Electrophoresis carried out on water-soluble extracts, indicated nickel spots with the same positions and intensities as those derived from the alcoholic extracts. The chromatographic experiments show that the nickel in *Hybanthus floribundus* is partially present as ionic nickel and partially coordinated to another ligand, which is not ninhydrin positive. Extraction of this latter substance, followed by hydrolysis, shows the presence of galacturonic acid.

The suggestion that calcium is associated with the cell wall pectins

was made some years ago; it seems likely that calcium forms pectate bridges, a process which limits cell wall plasticity. Calcium ions thus link together chains of polygalacturonic acids, by means of ionized carboxyl groups. It has also been shown that the polygalacturonic acids from pectin will collect other metal ions from water (Jellinek and Sangal, 1972). If the ions, copper, calcium, zinc and nickel are present in the same solution then the collection decreases in the order copper > calcium > zinc > nickel. It seems unlikely that pectic acids have a high selectivity towards zinc and nickel compared with calcium. It has been shown, however (Smimrød, 1968), that in the case of alginate, an increase in the L-guluronic content leads to an increased affinity for calcium ions and a higher gel strength of the resultant calcium alginate (Smimrød, 1972). The stereochemical modification of units in a polysaccharide chain appears to be widespread (Haug, 1974) and at least one epimerase has shown to depend on the presence of calcium. Recent work on cell wall pectic polymers (Rees, 1971) (Simmons, 1971) (Albersheim, 1974) (Talmadge *et al.*, 1973) indicates a limited number of polymeric structural components. It therefore appears possible that in plants such as *Triodia pungens* or *Hybanthus floribundus* modification of the cell wall polymers to give increased affinity for certain metals may take place.

Peterson (1969) suggested that a possible mechanism of zinc tolerance in *Agrostis tenuis* is accumulation by a passive process not requiring metabolic energy, and storage in the root cell wall cation binding sites. Peterson further suggested that in this way large amounts of zinc would be inactivated in the root cell walls.

However, the alternative mechanism that in which active metabolic processes are involved in the transport and storage of zinc in the aerial parts and where the carbohydrates are non specific cation storage sites, is attractive. The specificity of tolerance and accumulation

would then reside with specific carriers. These would transport the metal through the cell wall to the storage sites (Farago and Pitt, 1977).

Finally the aqueous extract of *Eriachne mucronata* aerial parts contained zinc indistinguishable from Zn^{2+} ions.

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CHAPTER V

THE AMINO ACID CONTENT OF THE THREE AUSTRALIAN SPECIES

5.1 Introduction

The plant amino acids are divided into two groups, the 'protein' and 'non-protein' acids, although the division between the two groups is not entirely sharp and methods of identifying and separating both groups are essentially the same. The 'protein' amino acids are found in acid hydrolysates of plant (and animal) proteins or in the free amino acid pool of plant tissue while the 'non-protein' amino acids are of more restricted occurrence and appear only in certain organisms and not as a building block for proteins.

Amino acids are colourless ionic compounds, their solubility properties and high melting points being due to the fact that they are zwitterions. They are all water-soluble, although the degree of solubility varies, the aromatic amino acids being rather sparingly soluble. Since they are basic, they form hydrochlorides with concentrated hydrochloric acid and being acids, they can be esterified.

Excluding 4-Amino-n-butyric acid, the amino acids listed in Fig.25 comprise the monomeric units of proteins together with asparagine, cysteic acid, cystine, cysteine, glutamine, methionine sulphone, methionine sulphoxide, threonine 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.

Among the 'non-protein' amino acids only 4-Amino-n-butyric acid is regularly present in plants. The remainder, of which over 200 structures are known (Fowden, 1970), are of more restricted occurrence. Their role in the plant is not entirely clear, although

their presence in seeds and their subsequent metabolism during germination suggests they may be important as nitrogen storage materials.

Amino acid analysis

Chromatographic investigation of the amino acid content of *Hybanthus floribundus*, *Triodia pungens* and *Eriachne mucronata* was undertaken. A commercial amino acid analyser was used, operating on the principle of liquid chromatography of the extract and subsequent spectroscopic ninhydrin determination of the separated components. Prior to analysis water soluble proteins were removed using Sevag's method (Sevag *et al.* 1938). By shaking the aqueous solution with trichloromethane and a foam preventing substance like pentan-1-ol, a protein gel is formed in the organic layer.

5.2 Chromatography of Aqueous Extracts of *Hybanthus floribundus*

4g. of *Hybanthus floribundus* stem material was ground and shaken with 250cm^3 deionized water for a total of 18 hours. The residue was filtered off, and the resultant solution was concentrated by evaporation on a rotary evaporator or freeze drier, thus reducing the volume to about 0.5cm^3.

The nickel-amino acid complexes were prepared after the method of Lang (1939). Equimolar (0.01M) solutions of 'Analar' $NiCl_2.6H_2O$ and amino acid each in 10% aqueous isopropanol were mixed (1:2 by volume). The amino acids used were:-

1. Glycine
2. DL-aspartic acid
3. L-glutamic acid
4. DL-serine
5. DL-threonine
6. DL-alanine
7. DL-valine
8. L-leucine
9. DL-isoleucine
10. L-histidine
11. L-argenine
12. DL-phenylalanine
13. L-tyrosine
14. DL-tryptophan
15. L-proline
16. L-hydroxyproline
17. DL-methionine

Ascending paper chromatography was carried out on Whatman No.1 paper in Shandon Glass Tanks. The following solvents were used:-

(i) S1, butanol-acetic acid-water, 12:3:5, v/v/v;

(ii) S2, phenol-water, 4:1, w/v;

(iii) S3, acetone-water, 3:2, v/v;

(iv) S4, ethylacetate-pyridine-water, 11:5:4, v/v/v;

(v) S5, n-butanol-pyridine-water, 1:1:1; v/v/v;

(vi) S6, n-butanol-ethanol-water, 2:2:1, v/v/v.

Samples of the freeze dried extracts were dissolved in a minimum of water. One dimensional as well as two-dimensional chromatography was applied using solvent S1 and S2. Marker amino acids were run at the same time for comparison.

The chromatographic results are presented in Fig.22 and 23.

The results show the amino acids present (in addition a yellow 'pigment' was always found, Section 4.3). Preliminary assignments of the amino acids were as follows: leucine; isoleucine; valine; proline; (yellow pigment); glutamic acid; glycine, serine, alanine and aspartic acid. Treatment of the papers with rubeanic acid or dimethylglyoxime resulted in a streak not far from the origin and another spot which corresponded to aspartic acid.

However, great difficulties were encountered in investigating nickel amino acid complexes. These were found to dissociate during paper chromatography and behave similarly during thin layer chromatography and electrophoresis. In solvent S1, all complexes, except that of histidine which was partially dissociated, dissociated showing amino acid spots corresponding to the free amino acids. Similarly the R_f value for nickel was that for ionic nickel. In S2 only complexes of aspartic acid and histidine (R_f values 24 and 98 respectively) did not dissociate. Adjustment of the pH of S2 to 7 with ammonia did not improve the situation. The results for S4 were similar, again only the

Figure 22. One-dimensional chromatography of *H. floribundus* stem extract in butanol-acetic acid-water, 12:3:5, v/v/v and phenol-water, 4:1, w/v on Whatman No.1 paper 300×300mm.

Reagent Used	R_f Value of Spot		Colour of Spot	Assignments
	BAW	Ph/H_2O		
Ninhydrin	62	86	Purple	Leucine
"	60	85	Purple	Isoleucine
"	49	73	Purple	Valine
"	31	89	Yellow	Proline
	28	67	Yellow	Yellow pigment
Ninhydrin	26	35	Purple	Glutamic acid
"	25	39	Purple	Glycine
"	23	32	Purple	Serine
"	20	51	Purple	Alanine
"	17	20	Purple	Aspartic acid
Dimethylglyoxime	12	14	Red	Nickel

Figure 23. Two-dimensional chromatography of *H. floribundus* stem extract in butanol-acetic acid-water, 12:3:5, v/v/v and phenol-water, 4:1, w/v on Whatman No.1 paper 300×300mm.

Reagent Used	R_f Value of Spot		Colour of Spot	Assignments
	BAW	Ph/H_2O		
Ninhydrin	58	89	Purple	Leucine
"	57	84	Purple	Isoleucine
"	45	78	Purple	Valine
"	38	82	Yellow	Proline
	26	61	Yellow	Yellow pigment
Ninhydrin	27	39	Purple	Glutamic acid
"	24	42	Purple	Glycine
"	22	35	Purple	Serine
"	23	57	Purple	Alanine
"	16	19	Purple	Aspartic acid
Dimethylglyoxime	13	16	Red	Nickel

Figure 24. Thin layer chromatography of amino acids and their nickel complexes in phenol-water, 4:1, w/v, on silica gel.

Amino acid	R_f of free acid with ninhydrin	R_f nickel complex	
		ninhydrin	Dimethylglyoxime
Glycine	21	19	20
Serine	24	23	18
Alanine	29	28	28
Methionine	45	59	58
Threonine	32	30	25
Valine	39	37	35
Proline	51	53	streak
Hydroxyproline	39	40	streak
Leucine	33	31	45
Isoleucine	59	53	44
Histidine	22	65	69
Phenylalanine	48	48	45
Arginine	11	10	19
Tyrosine	52	50	51
Tryptophan	65	64	60
Aspartic acid	3	5	9
Glutamic acid	6	9	2

complexes of aspartic acid and histidine did not dissociate. The argenine complex showed partial dissociation. Solvents S3, S5 and S6 gave similar effects. Amino acids tend to run fast, with little differentiation of R_f values. Most complexes dissociated, showing long streaks when treated with the nickel reagent.

Jursik (1968) has reported thin layer chromatography of nickel amino acid complexes on silica gel using phenol-water as solvent. His procedure was applied and the results are shown in Fig.24. Again, there is evidence of extensive dissociation in all but methionine, leucine, histidine, arginine and aspartic acid.

5.3 Amino Acid Analysis

Method

The system used was a LKB4101 single column analyser. The column was 35cm long with 6mm diameter and was packed with Ultrapac II, a sulphonated polystyrene cation exchange resin. Before use the column was regenerated with 0.4M 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 *H. floribundus* stem and root material (approximately 1g.) was shaken in 100cm^3 of distilled water for 6 hours. The extract was filtered and 0.25 volume of chloroform was added together with 0.1 volume of amyl alcohol to the protein solution. This mixture was shaken for 1 hour, and upon centrifugation separated into two layers. The upper aqueous layer was carefully decanted from the lower chloroform-protein gel layer. The procedure was repeated until no more gel formation took place and the resulting solution freeze-dried.

70mg. of each sample was taken and made up to 5cm^3 with water prior to analysis. 1.5cm^3 of the sample solution was added to 0.2cm^3 of aqueous norleucine as an internal standard and this solution was then divided into two equal parts. The first was made up to 3cm^3 with a pH 2.2 citrate buffer and 0.5cm^3 of this solution was introduced on to the column for analysis. The second portion was hydrolysed with 2M hydrochloric acid at 105°C for 5 hours, evaporated and dissolved in 3cm^3 of the citrate buffer. 0.5cm^3 of this solution was also used in amino acid determination. Both hydrolysed and non-hydrolysed samples contained 16.7 nanomoles of norleucine.

Results

Amino acid analyses were carried out on the stem and root extract of *Hybanthus floribundus* from a metalliferous area in Western Australia and the results are presented in Fig.25 and 26.

Figure 25. Amino acid analysis of stem extract of *H. floribundus* expressed as percentages of total ninhydrin material excluding ammonia.

Amino acid	Quantity of amino acid present (μM)	Percentage of total ninhydrin + ve material (%)
Alanine	15.730	6.24
Arginine	4.429	1.76
Aspartic acid	23.020	9.13
4-Amino-n-butyric acid	16.610	6.59
Glutamic acid	17.400	6.90
Glycine	18.000	7.14
Histidine	7.800	3.10
Isoleucine	17.450	6.92
Leucine	11.950	4.74
Lysine	8.250	3.27
Methionine	0.680	0.27
Phenylalanine	3.940	1.56
Proline	3.875	1.54
Serine	83.880	33.28
Tyrosine	4.910	1.95
Valine	14.090	5.59

Figure 26. Amino acid analysis of root extract of *H. floribundus* expressed as percentages of total ninhydrin material excluding ammonia.

Amino acid	Quantity of amino acid present (μM)	Percentage of total ninhydrin + ve material (%)
Alanine	1.76	1.51
Arginine	0.87	0.75
Aspartic acid	10.28	8.79
4-Amino-n-butyric acid	3.47	2.97
Glutamic acid	20.89	17.87
Glycine	1.79	1.53
Histidine	5.59	4.78
Isoleucine	2.30	1.97
Leucine	1.44	1.23
Lysine	2.84	2.43
Phenylalanine	0.41	0.35
Serine	61.58	52.67
Threonine	1.74	1.48
Tyrosine	0.96	0.82
Valine	0.99	0.85

5.4 Amino Acid Analysis of Aqueous Extracts of *T. pungens* and *E. mucronata*

Extract preparation and chromatography

5g. of the dry powdered material of each plant was shaken at room temperature for 8 hours with 200cm^3 of demineralised water. The solid material was filtered off using a millipore filter, and re-extracted. The two extracts were combined, freeze dried and stored in a sealed container. A minimum of demineralised water was added before the chromatographic experiments. One dimensional chromatography was carried out using Whatman No.1 paper in the ascending mode, the solvents were phenol/water (4:1, w:v) and butanol/acetic acid/water (12:3:5, v:v:v). Amino acids were located with ninhydrin.

The following amino acids were identified in the aqueous extracts:

Triodia pungens: 4-amino-n-butyric acid, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, valine;

Eriachne mucronata: alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, valine.

Amino acid analysis

Samples for amino acid analysis were prepared by Sevag's method and carried out as described previously. The results are shown in Fig. 27 and 28.

5.5 Discussion

The amino acid analysis of stem extract of *Hybanthus floribundus* differ from those of Brooks *et al.* (1974) who investigated *H. floribundus* from Queensland. Our sample contains serine (which probably corresponds to their first unknown), proline and isoleucine. The last two amino

Figure 27. Amino acid contents of aerial parts of *Triodia pungens* (sample 1921) expressed as percentages of total ninhydrin material, excluding ammonia

Amino acid	Quantity of amino acid present (μM)	Percentage of total ninhydrin + ve material (%)
Alanine	35.71	7.42
Aspartic acid	10.48	2.17
Arginine	60.66	12.61
γ-Amino-n-butyric acid	11.27	2.34
Glutamic acid	54.87	11.41
Glycine	8.49	1.76
Histidine	24.75	5.14
Isoleucine	6.72	1.39
Leucine	7.83	1.62
Lysine	24.53	5.10
Phenylalanine	9.18	1.90
Proline	133.33	27.73
Serine	63.19	13.14
Tyrosine	7.07	1.47
Threonine	9.09	1.89
Valine	13.63	2.83

Figure 28. Amino acid contents of aerial parts of *Eriachne mucronata* (sample 1929) expressed as percentages of total ninhydrin material, excluding ammonia.

Amino acid	Quantity of amino acid present (μM)	Percentage of total ninhydrin + ve material (%)
Alanine	42.10	13.55
Aspartic acid	30.75	9.90
Arginine	12.00	3.86
γ-Amino-n-butyric acid	17.87	5.75
Glutamic acid	12.50	4.02
Glycine	30.22	9.73
Histidine	3.90	1.25
Isoleucine	9.58	3.08
Leucine	27.45	8.84
Lysine	8.41	2.70
Methionine	7.50	2.41
Phenylalanine	17.58	5.66
Proline	24.44	7.87
Serine	28.48	9.17
Tyrosine	15.90	5.12
Threonine	9.54	3.07
Valine	12.27	3.95

acids were found in other *Hybanthus* species but not in *H. floribundus* from Queensland (table VI). Proline, however was found to be absent in the roots of *H. floribundus*.

Table VI Presence of proline and isoleucine compared with nickel content of *Hybanthus**

	isoleucine	proline	%Ni
H. caledonicus A	+	+	0.88
H. caledonicus B	+	-	0.17
H. austrocaledonicus var. linearifolia	+	+	1.03
H. austrocaledonicus	+	-	1.46
H. flroibundus Queensland	-	-	0.006
H. floribundus Western Australia (stem)	+	+	0.35
H. floribundus Western Australia (root)	+	-	0.29

*Results from Brooks *et al.* (1974) except for last two entries, this work.

Work on the chromatographic identification of amino acid complexes of copper (Sarkar *et al.*, 1969) (Jursik, 1967) (Fare, 1966)(Jursik and Petrov, 1967) and nickel (Jursik, 1968) has been described. There are great difficulties involved with those of nickel, since these mostly dissociate during paper chromatography and behave similarly during thin layer chromatography and electrophoresis.

Triodia pungens, which accumulates zinc was found to contain high levels of proline in the tops. The accumulated zinc is associated with pectins as in *Agrostis tenuis* (Peterson, 1969) and *Polycarpaea glabra* (Farago, 1977) and not with proline.

Proline is one of the amino acids contained in all proteins, and plant tissues under various types of environmental stress have been shown to accumulate proline. These environmental conditions include

water stress (Stewart, 1972) (Huang, 1979) (Tulley *et al.*, 1979), high salt conditions (Stewart, 1974) and temperature stress (Chu *et al.*, 1978). High levels of organic solutes, including proline, have been found in the tissues of sodium chloride - tolerant plants (Goas, 1965) (Stewart, 1974). It has been suggested that the accumulation of these compounds provides a mechanism for the equilisation of the water potentials of the cytoplasm and the vacuole, where the sodium chloride is preferentially accumulated (Storey, 1975).

One of the inherent difficulties in searching for metal-complexes from plants sources is that many of the metal ions involved form kinetically labile complexes (Eigen, 1963) (Bennett, 1971) (Frey, 1974). Extraction procedures lead to the release of many potential complexing agents. If the metal ions involved are labile, then the complexes which are isolated from the extracts may not be those which existed in the plant, but those with the greatest thermodynamic stability in the extraction mixture. Within the plant there will be a distribution of the metal between the various complexing agents depending on many parameters. Eigen and Tamm (1962) have discussed the rates of coordination in biological systems in terms of the rate determining step being desolvation of the primary coordination sheath of the aquo-ion. The rate constants for the exchange of water molecules between bulk solvent and primary coordination sheath in the hexa-aquo ions are: Cu^{2+},$10^{9}s^{-1}$; Zn^{2+},$10^{7}s^{-1}$; Ni^{2+},$10^{4}s^{-1}$. Thus these metal centres can all be considered as very labile. However there are systems in which the exchange of ligand will be considerably slower, even in relatively simple systems, e.g. Cu^{2+} is known to bind to peptides, and the effect of increasing the number of Cu-N (peptide) bonds is, in general to decrease the chance of direct nucleophilic reaction (Margerum, 1974). In complex systems such as metallo-enzymes the metal ion will also be kinetically inert. Similarly the kinetic lability of a metal ion contained in a

polymeric network, such as pectin is likely to be low.

From the thermodynamic stability constant data collected by Martell and Smith (1974), the ratios log K_1(pro)/log K_1(mal) (where the log K_1 values for proline and malate are compared) are Cu^{2+}, 2.58; Ni^{2+}, 1.87; Zn^{2+}, 1.75. The relative stability of the simple amino acid complex is thus greater for Cu^{2+} than Zn^{2+}. It is likely therefore that where simple systems are examined, Cu^{2+} would be likely to be found complexed with amino acids, rather than simple organic acids, in particular the log K_2 value of the copper-proline complex is very high.

The tolerance to copper of *Armeria maritima* from the Dolfrwynog Bog in Wales (Farago *et al.*, 1980) has been shown to be associated with high levels of proline in the roots (Farago, 1979) which appears to be complexed with copper. Copper in the aerial parts was found to be associated with pectins. The high proline is not a result of copper stress, since it is present in plants from seed of tolerant individuals, grown with and without copper. The high proline concentrations were not observed with plants from a maritime, non-copper site, nor in the roots of plants grown from non-tolerant seed, with or without added copper. There does appear to be a relationship between copper tolerance and proline production in the roots of *Armeria maritima*. Thus a viable mechanism for copper-tolerance would be the storage of copper as the proline complex in the roots, when a critical copper level is reached it is translocated to the top as the proline complex to be stored in the pectin layers.

If this is a general mechanism item it seems possible that high proline levels are associated with a copper exclusion mechanism at low levels of copper. In this case similar proline levels might be expected for both *Eriachne mucronata* and for *Triodia pungens*. However, further examination of the data of Nicolls *et al.* (1965) shows that the response to copper of *Eriachne mucronata* and *Triodia pungens* are

somewhat different. The response of *E. mucronata* to increasing copper in the soil is typically that of an excluder, whereas that for *Triodia pungens* could be interpreted as being similar to that with zinc, i.e. the response of an indicator, but with a much lower gradient than that with zinc.

One further possibility is that high proline levels are a result of the collection and air-drying of the samples in the field. This seems unlikely since proline accumulation starts when 30% of the total tissue water is lost (Singh *et al.* 1973). However, both *Hybanthus floribundus* and *Eriachne mucronata* were subject to the same stresses and did not show very high proline levels. Root material of *Hybanthus floribundus* clearly showed the absence of proline.

The accumulation of proline comes about from inhibition of proline oxidation and/or increased proline synthesis (Stewart *et al.* 1977). Proline is oxidised by proline dehydrogenase to Δ^1-pyrroline-5-carboxylic acid which in turn produces glutamate via P5C dehydrogenase. Proline dehydrogenase has been found in peanut (Mazelis, 1971) and in chlorella (McNamer, 1974). P5C dehydrogenase has been isolated from mitochondrial preparations (Stewart, 1974) and from barley, a proline accumulating species (Bogess *et al.* 1975). Recently both proline oxidase and Δ^1-pyrroline-5-carboxylic acid dehydrogenase from *Zea mays* have been found to be associated only with the inner mitochondrial membrane (Elthon, 1981), both enzymes were found to support oxygen uptake in isolated mitochondria. Inhibition of P5C dehydrogenase from barley (Bogess *et al.* 1975) occurred in the presence of various ions, which appears significant and would account for proline accumulation in salt stressed plants. The effects of copper on these enzymes has, however, not been investigated.

Thus proline accumulation seems likely to be associated with copper-tolerance, rather than with zinc accumulation in *Triodia pungens* or nickel accumulation in *H. floribundus*.

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CHAPTER VI

INVESTIGATION OF WATER INSOLUBLE METAL COMPOUNDS

6.1 Introduction

In chapter III analysis of the metal concentrations in the three Australian species revealed high metal concentration, but this information gives no idea of how the metal is distributed chemically within the plant tissues. However, more information of the actual chemical nature of the metals present may be obtained by solvent extraction scheme.

Among the extraction techniques available for the purpose are those based on the scheme of Bowen, Cawse and Thick (1962) and Bremner and Knight (1970) or Diez-Altares and Boroughs (1961). Peterson (1969) used an extraction scheme, involving the use of the proteolytic enzyme, pronase, to determine in which insoluble fraction zinc is bonded in *Agrostis tenuis*. He found a relatively high concentration in the pectate extract.

Previous analysis showed that the zinc containing compound in the aerial parts of *Triodia pungens* was a water soluble pectinate. For *Eriachne mucronata* the zinc was present in water soluble form, apparently as unassociated zinc ions, whereas, in *Hybanthus floribundus* species the major portion of nickel, is accumulated in the leaves where it is associated with pectic carbohydrates. However, the type of compound with which the metal is associated in the above species is further investigated using the extraction scheme of Peterson (1969) involving enzyme digestion - 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 stems, twigs and leaves of *Hybanthus floribundus*, as well as the aerial parts of *Triodia pungens* and *Eriachne mucronata* and is presented in Fig.29 together with a simple breakdown of the major classes of compounds extracted.

6.2 Determination of the Compounds Associated with Insoluble Nickel in *Hybanthus floribundus* and Zinc in *Triodia pungens* and *Eriachne mucronata* Species

Method

A known mass (approximately 3g.) of ground sifted *Hybanthus floribundus* stem and leaf material from Widgiemooltha area and twigs from Mount Thirsty as well as aerial parts of *Triodia pungens*, sample No.1921, and *Eriachne mucronata*, sample No.1929, from Dugald River lode were taken through the procedure shown diagramatically in Fig.29. The following extraction procedure applied similarly to all five samples.

The first extraction solvent was an 80% absolute ethanol solution. The sample was refluxed for fifteen minutes with 150cm^3 of the solvent in a 500cm^3 flask with a condenser. The suspension was filtered using a "millipore" type Buchner filter with a fine glass fibre 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.

The 80% ethanol extract was taken to dryness firstly by rotary evapoaration at 40°C to remove the ethanol and further by freeze

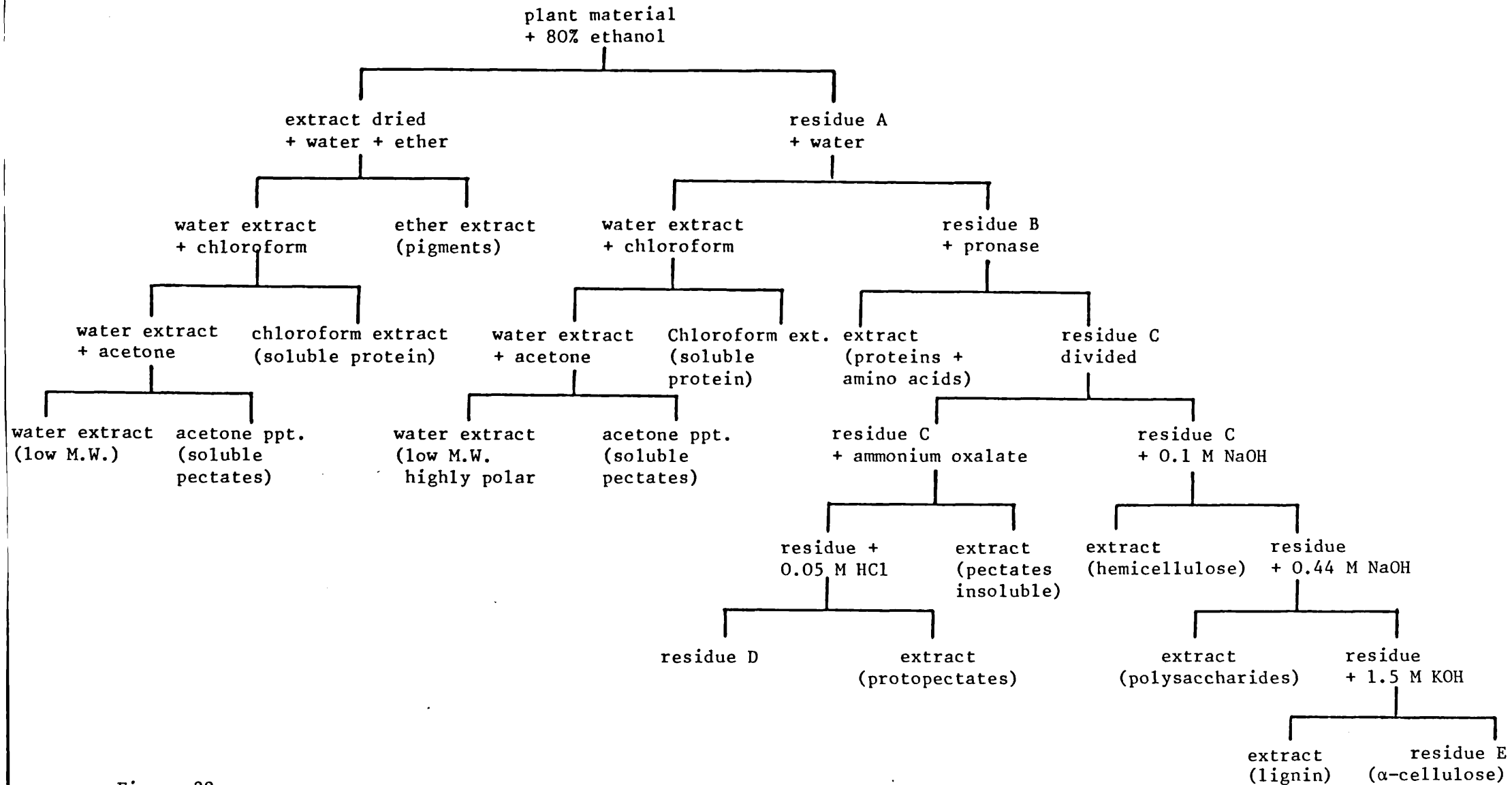


Figure 29

Sequential extraction schedule to determine metal binding sites

drying to remove the water. The dried extract was dissolved in 300cm^3 of water and extracted with 125cm^3 of sodium dried ether. Separation of the extract between the two phases was affected 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 300cm^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. Traces of organic solvents were removed from the upper, mainly aqueous layer and an equal volume (100cm^3) of analar acetone was added to form a precipitate. The mixture was centrifuged and the supernatant was 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) from the first 80% ethanol treatment was weighed and placed in a 500cm^3 flask with a condenser and refluxed for 10 minutes with 100cm^3 of water. 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 50cm^3 of water and the trichloromethane-pentanol and acetone treatments were carried out as before.

Residue (B) together with 1g. of the proteolytic preparation pronase, 10mg. of chloroamphenicol and 200cm^3 of phosphate buffer (0.01M, pH7.4) was placed in a 500cm^3 conical flask and shaken for 40hours. The sample was filtered and the pronase treatment repeated.

As the calcium containing enzyme pronase was found to possess a small proportion of zinc, a blank was run and the mass of pronase added to the sample and blanks were measured to three places of decimals and hence, the zinc added in the pronase could be eliminated. Again, 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. 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 divided into two parts, each of which were subjected to a different series of extraction procedures.

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

The second portion was extracted successively with 0.1M NaOH, 0.44M NaOH followed by 1.5M KOH (boiling). The final residue (E) was also wet ashed.

Atomic absorption analysis of the nickel and zinc levels in each extract and the final residues were made. These results are presented in Figs.30,31,32,33,34.

6.3 Results and Discussion

The figures below give the concentration of nickel in *Hybanthus floribundus* and zinc in *Triodia pungens* and *Eriachne mucronata* among the various chemical groups of compounds present in the insoluble fraction of plant material.

A general assessment of the accuracy of the extraction scheme

Figure 30

Sequential extraction schedules to determine the binding sites within twigs of *Hybanthus floribundus*

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	0.05M HCl extract	Residue D	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Proteins and amino acids	Pectates insoluble	Proto-pectates		
Ni p.p.m. (dry wt)*	2.32	1.79	212.20	254.01	1.26	91.16	347.86	344.05	211.80	122.10	7.44	1595.99
Percentage of total nickel	0.15	0.11	13.30	15.92	0.08	5.71	21.80	21.56	13.27	7.65	0.47	100%

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	0.1M NaOH extract	0.44M NaOH extract	1.5M KOH extract	Residue E	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Proteins and amino acids	Hemi-cellulose	Poly-saccharide	Lignin	α-cellulose	
Ni p.p.m. (dry wt)*	2.32	1.79	212.20	254.01	1.26	91.16	347.86	344.05	217.62	41.46	17.36	7.54	1538.63
Percentage of total nickel	0.15	0.12	13.79	16.51	0.08	5.92	22.61	22.36	14.14	2.69	1.13	0.49	99.99%

*Dry weight refers to weight of original plant sample

Figure 31

Sequential extraction schedules to determine the binding sites for nickel within stems of *Hybanthus floribundus*

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	0.5M HCl extract	Residue D	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	pectates insoluble	Proto-pectates		
Ni p.p.m. (dry wt)*	1.80	7.62	419.47	16.23	15.75	415.26	294.66	425.08	147.46	43.94	9.60	1796.87
Percentage of total nickel	0.10	0.42	23.34	0.90	0.88	23.11	16.40	23.66	8.21	2.45	0.53	100%

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	0.1M NaOH extract	0.44NaOH extract	1.5M KOH extract	Residue E	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	Hemi-cellulose	Poly-saccharide	Lignin	α-cellulose	
Ni p.p.m. (dry wt)*	1.80	7.62	419.47	16.23	15.75	415.26	294.66	425.08	51.68	45.86	32.88	43.38	1769.67
Percentage of total nickel	0.10	0.43	23.70	0.92	0.89	23.47	16.65	24.02	2.92	2.59	1.86	2.45	100%

*Dry weight refers to weight of original plant sample

Figure 32

Sequential extraction schedules to determine the binding sites for nickel within leaves of *Hybanthus floribundus*

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	0.05M HCL extract	Residue D	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	Pectates insoluble	Proto-pectates		
Ni p.p.m. (dry wt)*	14.04	77.95	4680.09	18.37	182.82	4992.20	950.86	457.98	1645.70	649.10	29.46	13698.57
Percentage of total nickel	0.10	0.57	34.16	0.13	1.33	36.44	6.94	3.34	12.01	4.74	0.22	99.98%

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	0.1M NaOH extract	0.44M NaOH extract	1.5M KOH extract	Residue E	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	Hemi-cellulose	Poly-saccharide	Lignin		
Ni p.p.m. (dry wt)*	14.04	77.95	4680.09	18.37	182.82	4992.20	950.86	457.98	921.66	422.08	112.12	52.80	12882.97
Percentage of total nickel	0.11	0.61	36.33	0.14	1.42	38.75	7.38	3.55	7.15	3.28	0.87	0.41	100%

*Dry weight refers to weight of original plant sample

Figure 33

Sequential extraction schedules to determine the binding sites for zinc within aerial parts of *Triodia pungens*

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	0.05M HCl extract	Residue D	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	Pectates insoluble	Proto-pectates		
Zn p.p.m. (dry wt) *	2.09	15.45	423.49	169.46	46.30	467.86	146.56	67.28	459.12	145.64	15.80	1959.05
Percentage of total zinc	0.11	0.79	21.62	8.65	2.36	23.88	7.48	3.43	23.44	7.43	0.81	100%

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	0.1M NaOH extract	0.44M NaOH extract	1.5M KOH extract	Residue E	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	Hemi-cellulose	Poly-saccharide	Lignin		
Zn p.p.m. (dry wt)*	2.09	15.45	423.49	169.46	46.30	467.86	146.56	67.28	412.62	196.26	28.24	10.38	1985.99
Percentage of total zinc	0.11	0.78	21.32	8.53	2.33	23.56	7.38	3.39	20.78	9.88	1.42	0.52	100%

*Dry weight refers to weight of original plant sample

Figure 34

Sequential extraction schedules to determine the binding sites for zinc within aerial parts of *Eriachne mucronata*

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	0.05M HCl extract	Residue D	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	Pectates insoluble	Proto-pectates		
Zn p.p.m. (dry wt)*	2.03	6.06	13.29	96.68	20.23	57.96	29.11	79.28	52.90	18.72	7.68	383.94
Percentage of total zinc	0.53	1.58	3.46	25.18	5.27	15.10	7.58	20.65	13.78	4.88	2.00	100%

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	0.1M NaOH extract	0.44M NaOH extract	1.5M KOH extract	Residue E	
Compounds extracted	Pigments	Soluble protein	Soluble pectates	Low M.W.	Soluble protein	Soluble pectates	Low M.W. highly polar	Protein and amino acids	Hemi-cellulose	Poly-saccharide	Lignin		
Zn p.p.m. (dry wt)*	2.03	6.06	13.29	96.68	20.23	57.96	29.11	79.28	11.40	19.40	8.06	4.64	348.14
Percentage of total zinc	0.58	1.74	3.82	27.77	5.81	16.65	8.36	22.77	3.27	5.57	2.32	1.33	99.99%

*Dry weight refers to weight of original plant sample

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 the twigs material of *Hybanthus floribundus*, taking an average value for both schemes A and B, a level of 1567 p.p.m. (dry wt) was extracted which compared well with 1539 p.p.m. (dry wt) in the plant tissue (table V). A similar correlation for stem material gives a level of 1783 p.p.m. (dry wt) compared with 1869 p.p.m. (dry wt). Also for the leaf material a level of 13290 p.p.m. (dry wt) was extracted which compared well with the average of the three replicates of 13246 p.p.m. (dry wt) (table II). For aerial parts of *Triodia pungens* a level of 1972 p.p.m. (dry wt) was extracted compared with sample 1921 (table III) of 1980 p.p.m. (dry wt). A similar correlation for aerial parts of *Eriachne mucronata* gives a level of 366 p.p.m. (dry wt) extracted compared with 448 p.p.m. (dry wt) in the plant tissue of sample 1929. It appears therefore that losses, contamination and atomic absorption interferences have been kept to a minimum.

An assessment of extraction Scheme A for twigs material reveals that the water soluble proteins and pectates account for 0.19% and 19% of the nickel removed in contrast to 37.72% removed in association with low molecular weight water extractable materials. Of the water insoluble material, which can be considered largely cell wall material, 21.56% is solubilized by pronase, this material will consist of protoplasmic proteins, polypeptides and possibly cell wall protein. About 20.92% of nickel is associated with pectic and protopectic materials. The residue consisting of α-cellulose, lignin, neutral hemicellulose (polysaccharides) and less readily hydrolysed acidic hemicelluloses, appears to be nickel free.

As much as 39.9% of nickel extracted is therefore associated with materials based on uronic acid. Information from Scheme A concerning the binding sites for nickel in stems and leaves of

H. floribundus shows that 57% of the stems and 87% of the leaves nickel is associated with materials based on uronic acid, 24.9% in the stems and 5.24% in the leaves is removed with soluble and insoluble proteins or polypeptides. Nickel associated with low molecular weight material in the water extract of the stems, 17.3%, and 7.07% in the leaves, is considerably lower than that found in the twigs of the same plants.

An assessment of extraction Scheme A for aerial parts materials of *Triodia pungens* and *Eriachne mucronata* reveals that 45.5% in *T. pungens* and 18.56% in *E. mucronata* of the zinc is water soluble pectates. As much as 76.37% in *T. pungens* and 37.22% in *E. mucronata* of zinc extracted is associated with materials based on uronic acids.

Scheme B presents a broadly similar picture, the 0.1M NaOH extract releasing pectic as well as hemicellulose material. Materials extracted by 0.44M sodium hydroxide and 1.5M potassium hydroxide are likely to include also acidic hemicellulose and pectic materials.

Though the extraction scheme employed in this work was applied by Peterson (1969) to the extraction of radio-active tracers from plant material, it can be seen here that the method is applicable to large scale extraction of nickel and zinc. The results obtained here indicate that for *Hybanthus floribundus* and *Triodia pungens* a very high percentage of the metal is present associated with pectate. Further histochemical work (section 6.4) on *Hybanthus floribundus* has shown that nickel is accumulated in the pectate layer of leaf cell walls, a conclusion similar to that reached for *Alyssum bertolonii* by Gambi (1967). In the zinc accumulating plant *Crotalaria novae hollandiae* the metal has been detected in the phloem in addition to the cell wall (Farago, 1977). In the copper containing *Becium homblei* the metal has been found in association with amino acids (Reilly *et al*, 1970). Copper is similarly associated with amino acids in *Armeria*

maritima (Farago *et al.*, 1980).

Thus there is evidence that the mechanisms of accumulation of copper and of zinc and nickel are different. For both *Hybanthus floribundus* (section 6.4) and *Crotalaria* (Farago, 1977) the metal has been detected in the phloem in addition to the cell wall. It seems unlikely that nickel is travelling in the phloem in high concentrations. But it is more likely that the phloem is the repository for a significant fraction of the total nickel present. It seems likely that the phloem membrane restricts entry to nickel ions as it does to calcium ions. Thus large concentrations of the metal ions become immobile at the membrane; this mechanism perhaps becomes operational only when very large amounts of metal are accumulated, as in *Hybanthus floribundus* and *Crotalaria novae hollandiae*.

With regard to the interpretation of these results, an attractive conjecture may be cautiously proposed, that as *Hybanthus floribundus* and *Triodia pungens* plants take up higher concentrations of nickel and zinc, respectively, the pectate fraction becomes an accumulation site, storing the majority of the excess metal. This is exactly what appears to occur with *Agrostis tenuis* (Turner and Marshall, 1971).

6.4 Determination of accumulation site within *Hybanthus floribundus*

Histochemistry

Histochemical techniques have been of limited use in the past in studies of heavy metals in plants, simply because general metal concentrations in plant organs are far too low to be detected by most reagents. Nevertheless, the technique has been of enormous value in the study of the chemical composition of living material.

In the case of high metal concentration, however, the technique can be most revealing. Where accumulation of a metal occurs at a distinct site, the local concentration may well be above the sensitivity limit of the indicator.

Various stains have been used to locate nickel in the plant tissues. Gambi (1967) used dimethylglyoxime to locate nickel in the stems of *Alyssum bertolonii* which concentrates exceptionally high quantities of this element. The red nickel-dimethylglyoxime complex showed a very intense colour in the epidermis and in the sclerenchymatic areas placed between the vascular bundles, pointing out a preferential localization of this element in these tissues. Another stain used to locate nickel is dithioxamide which gives a blue colour with nickel.

As a general technique the plants were killed and fixed and in some cases preserved. The part of the plant under study was then sectioned and stained with a chemical which will locate the particular compound or element being examined. The stain chosen for metal concentration must be specific and produce vivid colours, to allow easy detection even when small quantities of the analyte are present. The section is then mounted and a permanent slide may be made, or the section drawn or colour photographed giving a permanent record of the accumulation site.

Method

Tissue sections of leaf were examined under a light microscope using the spot reagents dimethylglyoxime and dithioxamide to locate areas of nickel concentration. The leaf showed epidermal accumulation of nickel particularly at the midribs and leaf edges where large cells were found.

Leaves material were then taken from the plant sample and

immersed in equal proportions by volume of ethanol and glycerol. Sections were then made free-hand using stainless steel disposable razor blades. A microtome was not employed as extended preparation of material for sectioning may alter the distribution of the metal within the tissues. Also since exact cytological detail was not necessary, ultra-thin sections were not required.

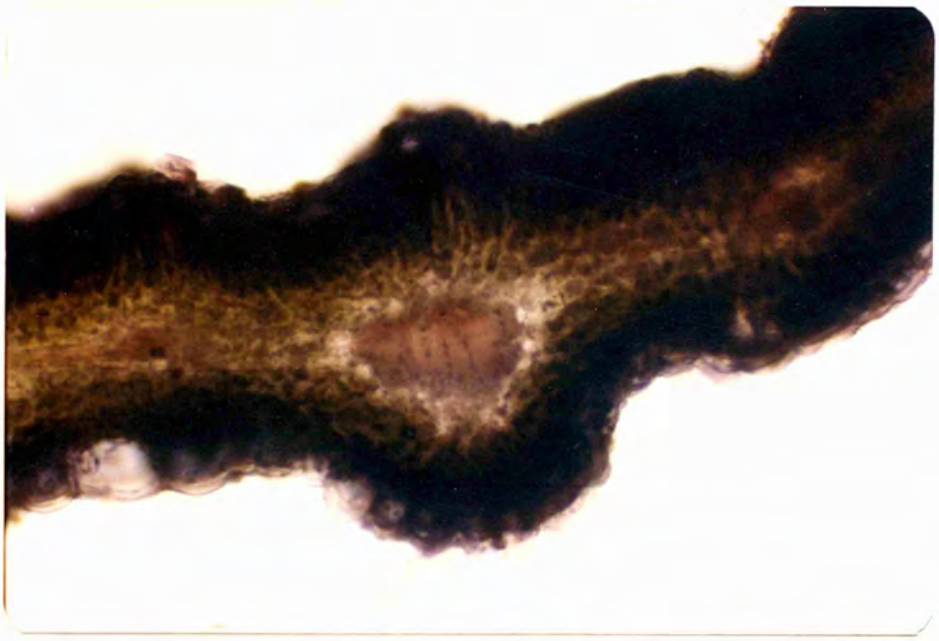
Two spot test reagents were chosen to locate the nickel in the leaf material. The two reagents were dimethylglyoxime which gives a red colour with nickel and dithioxamide which stains nickel blue. These reagents were chosen for their colour contrast, insolubility and sensitivity. They also required mild conditions and thus minimized chances of mobilization. A technique was developed for direct application of the stains to tissue cross-sections while under the microscope. This reduced chances of altering the distribution of the metal within the tissues while staining, and allowed development to be followed with time. Ruthenium red stain (Gurr, 1953) was also used to stain pectin, which took on a red colouration.

Results

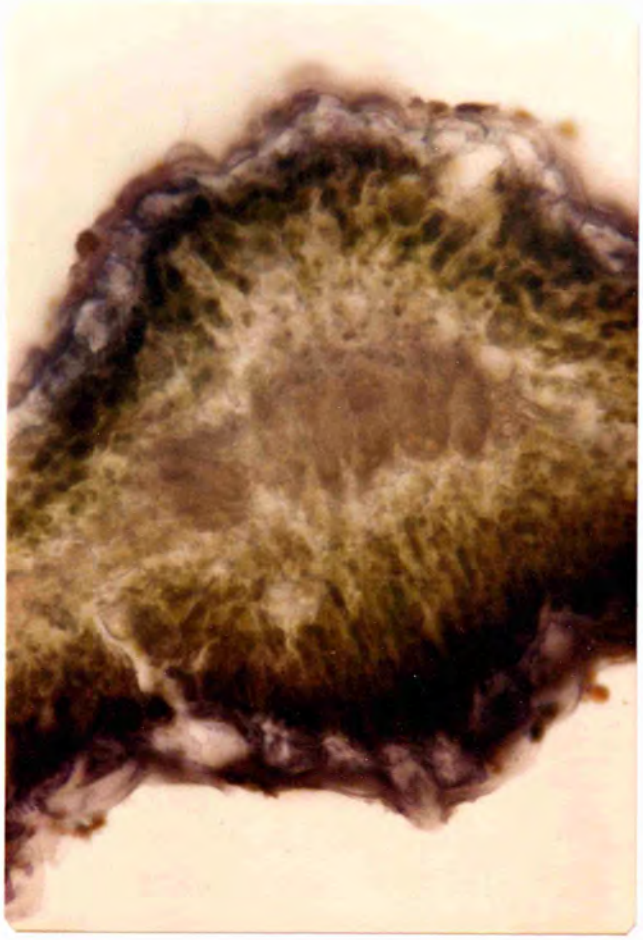
In slides III, IV and V of *Hybanthus floribundus* the leaf stain is the dithioxamide stain which gives a blue colour with nickel, while slides VI and VII are stained with dimethylglyoxime which stains nickel red. Slides VIII and IX is the ruthenium red stain which gives a red colour with pectin.

All the slides show the cross section of the leaf. Dithioxamide was found to be much more suitable as no crystal formation occurred while results were otherwise confirmed.

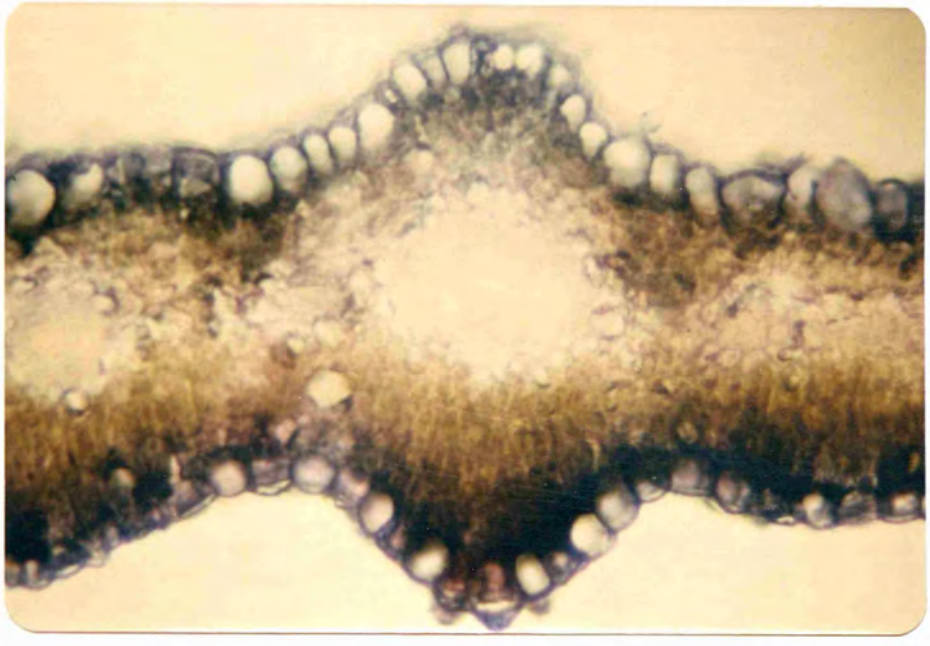
The leaves of *Hybanthus floribundus* have large epidermal cells and ridges of large cells which continue along the leaf stem and on to the main stem of bush. It was found that there is a remarkable



Slide III. *H. floribundus* leaf cross-section (×160) stained for nickel with dithioxamide

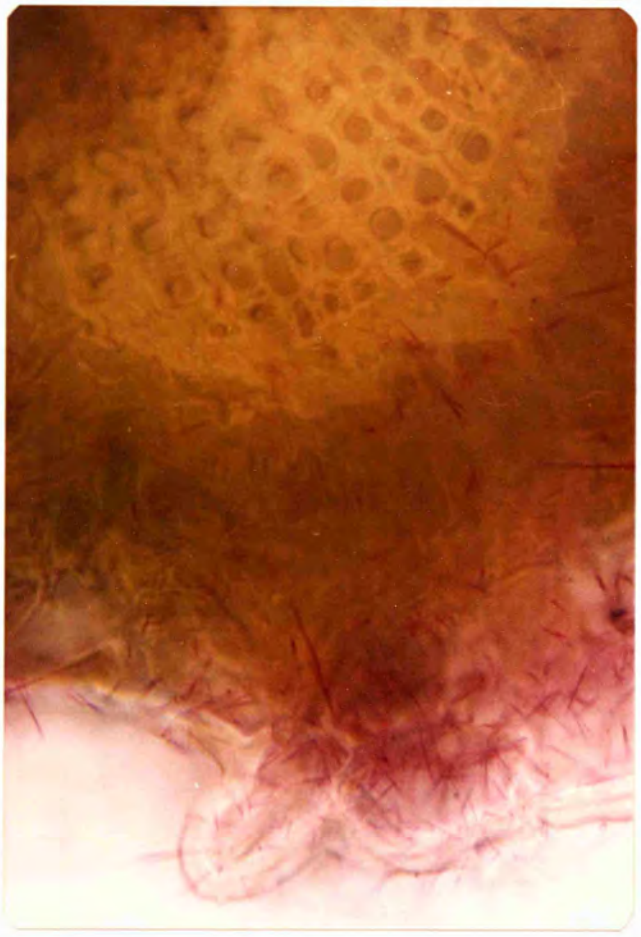


Slide IV. *H. floribundus* leaf cross-section (×800) stained for nickel with dithioxamide

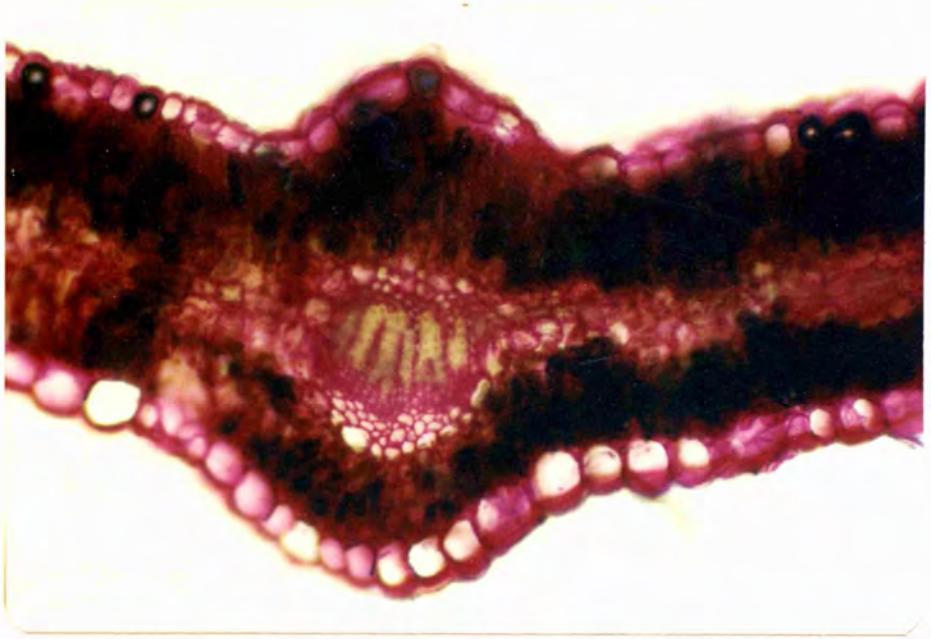


Slide V. *H. floribundus* leaf cross-section (×170) stained for nickel with dithioxamide

Slide VI. *H. floribundus* leaf cross-section (×1600) stained for nickel with dimethylglyoxime



Slide VII. *H. floribundus* leaf cross-section (×1600) stained for nickel with dimethylglyoxime



Slide VIII. *H. floribundus* leaf cross-section (×160) stained for pectin with ruthenium red

Slide IX. *H. floribundus* leaf cross-section (×800) stained for pectin with ruthenium red

concentration of nickel in these epidermal layer as shown by slides III, IV, V, VI and VII. In some cases so much nickel was present that nickel-dimethylglyoxime crystallised out under the microscope (Slide VII). Epidermal studies showed the nickel to be present in regions corresponding to those stained for pectin (Slides VIII and IX). Under highest magnification, a clear picture of the nickel location in the outer cell wall was obtained, especially for those cells of the midrib and leaf edge. The mesophyll and palisade cells showed no evidence of nickel concentration, whereas the phloem elements gave a moderate staining.

6.5 Electron Microscopy

The general theory of this technique has been presented in Chapter II. The use of electron microscope microanalysis has been of great value in the understanding of the roles played by macronutrient elements in plant metabolism. However, the instruments have a sensitivity of only about 1000 p.p.m. (dependent on the metal) which, although of very great use in potassium and calcium analyses where the concentrations may be very high, is of limited applicability to the study of heavy metals in plants. This is because the metals may be in too low concentration and thus require high energy electrons which tend to destroy the section. However, the nickel concentration in *Hybanthus floribundus* samples are sufficiently high enough for the technique to be employed.

Method

A section of the leaf of *H. floribundus* was dried and sectioned longitudinally so as to avoid any nickel displacement. The sample was cut to a suitable size (about 1cm), glued to a base plate and coated with aluminium as previously described (Chapter II). The

sample was then placed in the electron microscope and examined by scanning electron microscopy and energy disperse X-ray analysis.

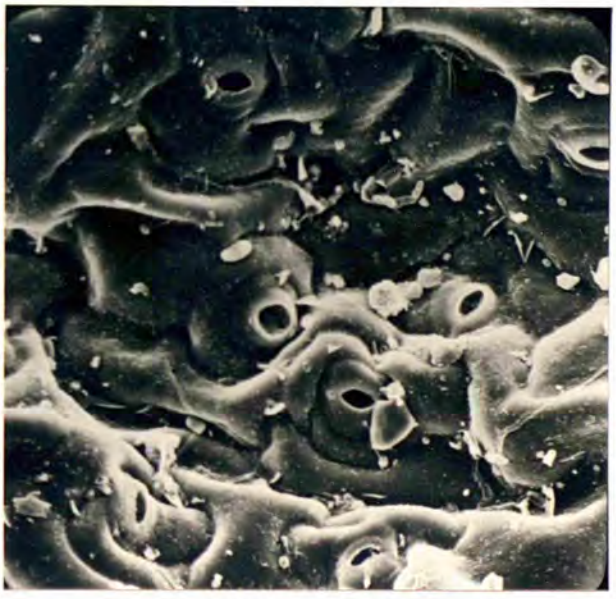
Results

Slide X shows the scan of the underside of a leaf of *Hybanthus floribundus*, the mid-rib can be seen in the centre. Slide XI shows detail of stomatal pores in the mid-rib region. Preliminary energy-dispersive X-ray analysis of the specimen shown in slide XII gave the spectrum shown in slide XIII. The peaks of interest are: (a) aluminium K_α = 1.49 k.e.v.; (b) silicon K_α = 1.74 k.e.v.; (c) phosphorus K_α = 2.02 k.e.v.; (d) sulphur K_α = 2.31 k.e.v.; (e) chlorine K_α = 2.64 k.e.v.; (f) potassium K_α = 3.31 k.e.v. and K_β = 3.59 k.e.v.; (g) calcium K_α = 3.69 k.e.v. and K_β = 4.01 k.e.v.; (h) iron K_α = 6.40 k.e.v. and K_β = 7.06 k.e.v.; (i) nickel K_α = 7.48 k.e.v. and K_β = 8.26 k.e.v.

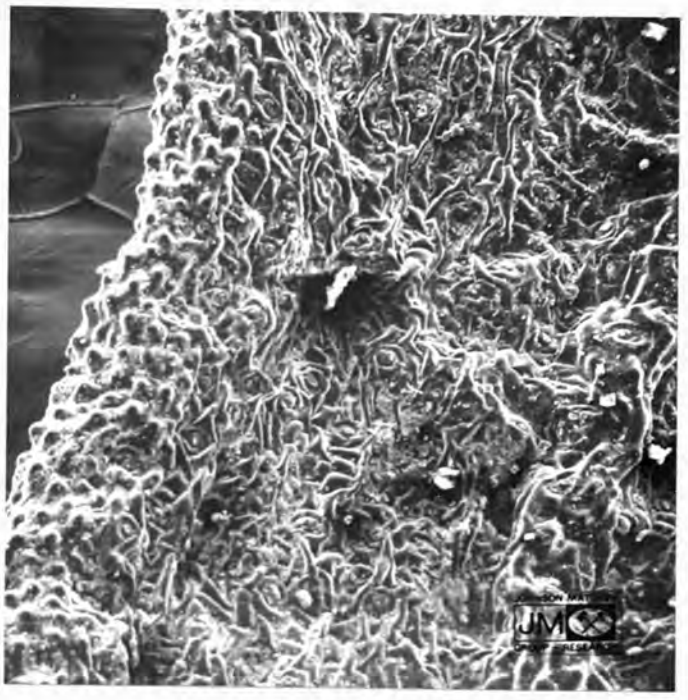
On uneven samples such as these the amount of "bounce" is very high leading to high aluminium (from the stub) and high iron (from the pole piece of the microscope) signals - such a signal was found to obscure the iron signal from the specimen. Even when the signal is distinct and not obscured by background, the topographical relief was found to make mapping with low levels of metal impossible.

In conclusion it is plain that energy dispersive X-ray mapping of elements at low levels cannot be carried out unless the elements have no interfering peaks and the specimens are completely flat. However the nickel K_α peak at 7.48 k.e.v. can be clearly distinguished and shows potential for the use in this work.

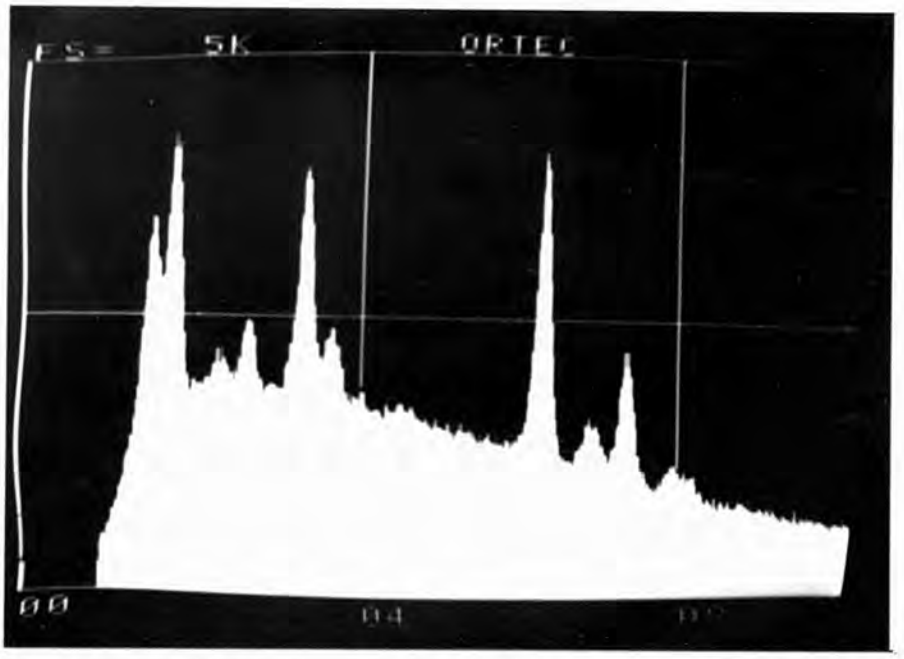
Slide X. Scanning electron micrograph across leaf (×20) of *H. floribundus* showing the mid-rib



Slide XI. Scanning electron micrograph across leaf (×500) of *H. floribundus* showing stomatal pores



Slide XII. Scanning electron micrograph across leaf (×125) of *H. floribundus*



Slide XIII. Energy dispersive X-ray mapping of leaf sample shown in slide XII, (a) aluminium K_α = 1.49 k.e.v.; (b) silicon K_α = 1.74 k.e.v.; (e) chlorine K_α = 2.64 k.e.v.; (f) potassium K_α = 3.31 k.e.v.; (g) calcium K_α = 3.69 k.e.v.; (h) iron K_α = 6.40 k.e.v.; and K_β = 7.06 k.e.v.; (i) nickel K_α = 7.48 k.e.v.

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CHAPTER VII

A STUDY OF PIGMENTS PRESENT IN LEAVES OF *HYBANTHUS FLORIBUNDUS*

7.1 Introduction

It has long been recognised that some plants accumulate certain metals without ill effects, in concentrations which would normally be toxic. A greater understanding of the factors involved may be of value in elucidating the metal tolerance mechanism in plants.

This section of the work is concerned with the study of a yellow crystalline pigment from leaves of the Australian shrub, *Hybanthus floribundus* (Lindl.) F. Muell. This pigment is of interest not only because it occurs in a plant which accumulates extraordinarily high amounts of nickel, up to 13,000 p.p.m. dry weight, but because the pigment also appeared to be present in large quantities.

The elucidation of the structure and configuration of naturally occurring organic compounds should result in an understanding of their biosynthesis which is a matter of fundamental systematic importance. The method of chemical taxonomy is basically concerned with the chemical survey of restricted groups of plant, mainly for secondary constituents but also for macromolecules and the application of the data so obtained to plant classification.

Perhaps the most useful class of compounds for such a study are the flavonoids (Harborne, 1967, 1970, 1972). The exceptional usefulness of flavonoid constituents as taxonomic guides, however, is because they have particular advantages over most other low molecular weight constituents in that they are universally distributed in vascular plants, they show considerable structural diversity, they are so chemically stable that they can be detected in herbarium tissue and, finally, they are easily and rapidly identified.

The Flavonoids

The flavonoids are 15-carbon compounds that are generally distributed throughout the plant kingdom (Harborne, 1976) (Swain, 1976). The basic flavonoid skeleton, shown in the following diagram, is usually modified in such a way that more double bonds are present, causing the compounds to absorb visible light and thus giving them colour. The two carbon rings at the left and right ends of the molecule are

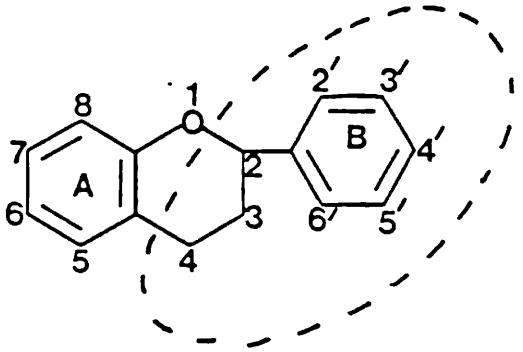


Figure 35

designated the A and B rings, respectively.

The dashed lines around the B ring and the three carbons of the central ring indicate that part of flavonoids derived from the shikimic acid pathway. Ring A and the oxygen of the central ring are derived entirely from acetate units provided by acetyl CoA. Hydroxyl groups are nearly always present in the flavonoids, especially attached to ring B in the 3' and 4' positions, to the 5 and 7 positions of ring A, or to the 3 position of the central ring. These hydroxyl groups serve as points of attachment for various sugars that increase the water solubility of flavonoids. Many flavonoids accumulate in the central vacuole, although many of them are appartently synthesized in chloroplasts.

Structural variation is due chiefly to differing oxidation levels of the central ring and to the presence of varying numbers of hydroxyl (and other) substituents attached to the two benzene rings.

Flavonoids are mainly water soluble compounds. They can be extracted with 70% ethanol and remain in the aqueous layer, following partition of this extract with petroleum ether. Since they are phenolic, they change in colour when treated with base or with ammonia; thus they

are easily detected on chromatograms or in solution. Flavonoids contain conjugated aromatic systems and thus show intense absorption bands in the UV and visible regions of spectrum. Finally, flavonoids are generally present in plants bound to sugar as glycosides and any one flavonoid aglycone may occur in a single plant in several glycosidic combinations.

For convenience, flavonoids are divided into classes, depending on the level of oxidation of the central ring. Some classes are very common in nature, i.e. the anthocyanidins, flavones, flavonols and flavan-3,4-diols. Others such as the flavonones, chalcones, aurones and isoflavones, have less frequent occurrence.

Three groups of flavonoids are of particular interest in plant physiology. These are the anthocyanins, the flavonols, and the flavones. The anthocyanins are the most important and widespread group of colouring matters in plants. These intensely coloured water-soluble pigments are responsible for nearly all the pink, scarlet, red, mauve, violet and blue colours in the petals, leaves and fruits of higher plants. They are nearly always present as glycosides, containing most commonly one or two glucose or galactose units attached to the hydroxyl group in the central ring or to that on the 5 position of ring A. The exact colour of the anthocyanins depends firstly, upon the substituent groups present on the B ring. Secondly, carbohydrates-soluble sugars (Nakayama and Powers, 1972) and pectin - are also important. The blue anhydro bases of delphinidin glycosides are stabilized by absorption on or combination with pectin (Bayer *et al.*, 1966) or starch gel (Lowry and Chew, 1974). Finally, the pH of the cell sap influences their colour. Most anthocyanins are reddish in acid solution but become purple and blue as the pH is raised. This probably results from ionization of the OH groups on the B ring, followed by electron shifts in this ring. One of the useful functions of anthocyanins in flowers is apparently the attraction of birds and bees that carry pollen from one plant to another, thus

facilitating pollination (Harborne, 1976).

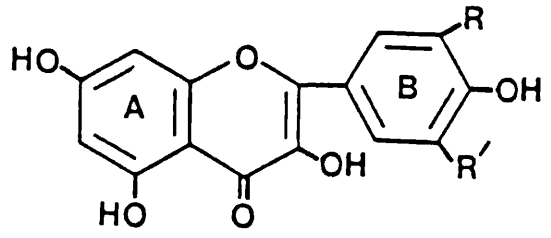
The anthocyanins are all based chemically on a single aromatic structure, that of cyanidin, and all are derived from this pigment by addition or subtraction of hydroxyl groups or by methylation or by glycosylation.

Anthocyanidins are usually named after the particular plant from which they were first obtained. The most common anthocyanidins are: cyanidin, pelargonidin, and delphinidin. These anothocyanidins differ only in the number of hydroxyl groups attached to the B ring of the basic flavonoid structure. Three anthocyanidin methyl ethers are also quite common: peonidin, petunidin and malvidin. Each of these six anthocyanidins occur with various sugars attached as a range of glycosides. The main variation is in the nature of the sugar (often glucose, but may also be galactose, rhamnose, xylose or arabinose), the number of sugar units (mono-, di- or tri-glycosides) and the position of attachment of sugar (usually to the 3-hydroxyl, or to the 3- and 5- hydroxyls).

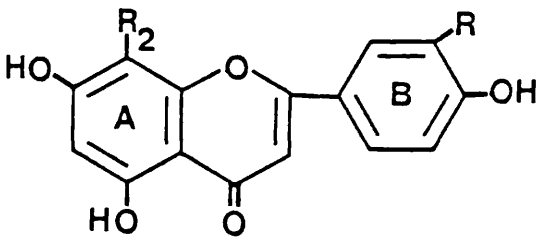
Flavonols are very widely distributed in plants, both as co-pigments to anthocyanins in petals and also in leaves of higher plants. Like the anthocyanins, they occur most frequently in glycosidic combinations. Although there are over a hundred flavonol aglycones known, only three are at all common: kaempferol; quercetin and myricetin (Fig.36). The other known flavonols are mostly simple structural variants on the common flavonols and are of limited natural occurrence.

Flavones only differ from flavonols in lacking a 3-hydroxyl substituent (Fig.36); this affects their UV absorption, chromatographic mobility and colour reactions and simple flavones can be distinguished from flavonols on these bases. There are only two common flavones: apigenin and luteolin, corresponding in hydroxylation pattern to kaempferol and quercetin. Flavones occur also as glycosides, but unlike flavonols, they sometimes occur with sugar bound by a carbon-carbon

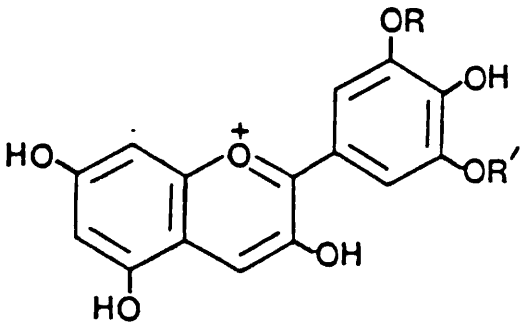
Figure 36 Commonly occurring flavonoids



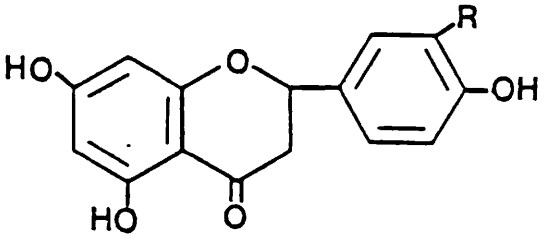
Flavonols: kampferol,R=R'=H
quercetin,R=OH;R'=H
myricetin,R=R'=OH



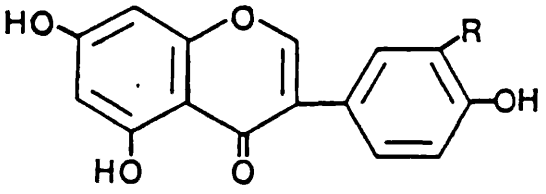
Flavones: apigenin,R=H;R_2=H
luteolin,R=OH;R_2=H
orientin,R=OH;R_2=Glucosyl



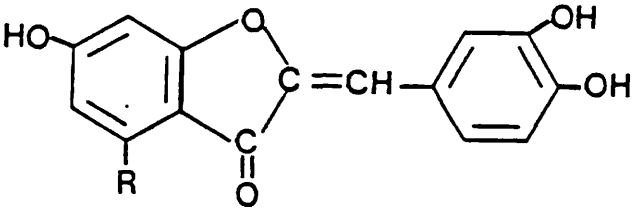
Anthocyanidin: delphinidin,R=R'=H,
petunidin, R=Me;R'=H
malvidin, R=R'=Me



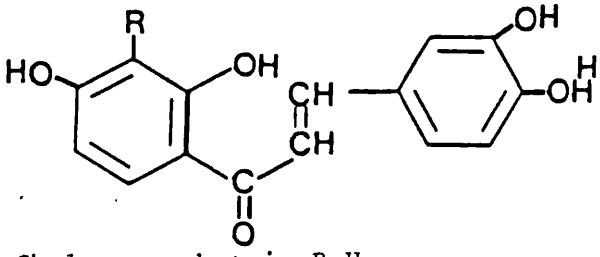
Flavanones: naringenin,R=H
eriodictyol,R=OH



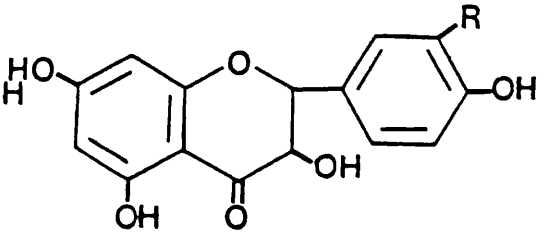
Isoflavones: genistein,R=H
orobol, R=OH



Aurones: sulphuretin,R=H
aureusidin, R=OH



Chalcones: butein,R=H
okanin,R=OH



Flavanonols: dihydrokampferol,R=H
dihydroquercetin,R=OH

bond, one example being orientin, the 8-C-glucoside of luteolin (Fig.36).

Most of the flavones and flavonols are yellowish or ivory-coloured pigments, and like the anthocyanins, they often contribute to the colour of flowers. Even those flavones and flavonols that are not coloured nevertheless absorb ultraviolet wavelength and therefore affect the spectrum of radiation visible to bees or other insects that are attracted to flowers containing them. These molecules are also widely distributed in leaves, especially in the chloroplasts, where they are apparently synthesized (Harborne, 1976). They apparently function there as feeding deterrents and, since they absorb ultraviolet radiation, as a protection against ultraviolet rays.

Other classes of flavonoids such as the chalcones, aurones, flavanones, dihydrochalcones and isoflavones are of limited natural distribution. Occurrence is either sporadic (e.g. the flavanones) or else limited to a very few plant groups (e.g. isoflavones to the Leguminosae and Iridaceae). Chalcones and aurones together comprise the 'anthochlors', yellow pigments which can be detected by the fact that a change to orange or red colour is observed when a yellow petal is fumed with the alkaline vapour of a cigarette or with a vial of ammonia. These compounds occur characteristically in the Compositae, but they have also been recorded in over ten other families. Dihydrochalcones have a different distribution pattern from chalcones, being mainly confined to the Rosaceae and Ericaceae (Williams, 1966). Flavanones are isomeric with chalcones and the two classes are interconvertible *in vitro*. Chalcones are frequently found in nature together with the flavanone analogues, but the converse is not always true. They are colourless substances, which cannot be detected during chromatographic surveys unless chromogenic sprays are employed.

Isoflavones, of which over sixty are known, are isomeric with the flavones but are much rarer in their occurrence. They occur almost

entirely in one sub-family (the Lotoideae) of the Leguminosae. Complex isoflavans such as rotenone are powerful natural insecticides, while the related coumestans such as pisatin are phytoalexins, protective substances formed in plants in response to disease attack (Deverall, 1972).

7.2 Isolation Techniques for Flavonoids

Plant preparation and extraction.

Flavonoids occur in virtually all parts of the plant, the root, heartwood, sapwood, bark, leaf, fruit and flower, and the method of isolation depends to some extent both on the source material and the type of flavonoid being isolated. In cases when flavonoids occur in the surface oils or waxes, they may be obtained simply by scraping or washing the surface with an appropriate solvent (Star and Mabry, 1971) (Wollenweber, 1972). In general, however, the plant material is ground up or macerated before extraction. The possibility of enzyme action occurring during this early period of isolation, leading in particular to hydrolysis of glycosides (Beck, 1964)(Trim, 1955), may be avoided by plunging the plant material into boiling solvent or by rapid drying prior to extraction (Seshadri, 1962). Pre-drying of plant material generally appears to increase the yield of extractives, possibly due to rupture of the cell structure and to the better solvent access provided as a consequence.

Solvents used for extraction are chosen according to the polarity of the flavonoids being studied. The less polar solvents are particularly useful for the extraction of flavonoid aglycones, whilst the more polar solvents are used if flavonoid glycosides or anthocyanins are sought. The less polar aglycones, such as isoflavones, flavanones and dihydroflavonols, or flavones and flavonols which are highly methylated, are usually extracted with solvents such as benzene, chloroform, ether

or ethylacetate (Mukerjee *et al.*, 1969)(Jackson *et al.*, 1967)(Shamma and Stiver, 1969)(Harborne *et al.*, 1963)(Thomas and Mabry, 1968). A pre-extraction with light petroleum or hexane is frequently carried out to rid plant material of sterols, carotenoids, chlorophylls, etc. (Bhutani *et al.*, 1969b)(Clark-Lewis and Porter, 1972). Flavonoid glycosides and the more polar aglycones such as hydroxylated flavones, flavonols, biflavonyls, aurones and chalcones are generally isolated from plant material by extraction with acetone, alcohol, water or a combination of these (Bottomley *et al.*, 1966) (Cambie and James, 1967) (Dreyer and Bertelli, 1967). Boiling water has been found suitable for the extraction of polyglycosides (Okigawa *et al.*, 1971a) and the flavone-polysaccharide compound from *Monoclea forsteri* (Markham, 1972) and also for the isolation of compounds such as flavandiols, catechins and procyanidins.

Preliminary purification

When flavonoids of varying types are to be extracted from a single batch of plant material, a worthwhile method for preliminary separation is sequential solvent extraction with a number of solvents of varying polarity. This can lead to separation of glycosides from aglycones and to the separation of polar from non-polar aglycones (Bhutani *et al.*, 1969b)(Thomas and Habry, 1968).

Treatment of crude plant extracts with charcoal powder is also a useful method for the preliminary purification of flavonoids, particularly glycosides. In one example of this procedure (Mabry *et al.*, 1970), flavonoids from *Baptisia lecontei* were eluted from activated charcoal by washing successively with methanol, boiling water, 7% aqueous phenol and 15% methanolic phenol. The bulk of the flavonoid material appeared in the 7% phenol fraction, which was subsequently ether-extracted to give a phenol-free aqueous solution rich in flavonoid

glycosides. Markham (1972) found dialysis useful in the separation of a flavone-polysaccharide compound (MW about 3000) from an aqueous plant extract of *Monoclea forsteri*. The same technique was also used in the isolation of high molecular weight blue flavonoid 'complexes' from *Centaurea cyanus* (MW 6200) (Bayer *et al.*, 1966) and *Commelina communis* (Takeda *et al.*, 1966) all of which were non-dialysable through a cellulose membrane.

The chromatography of the flavonoid pigments

Paper partition chromatography was first applied to the separation of flavonoids pigments by Bate-Smith in 1948. Since then, it has been used increasingly in the study of these compounds. The flavonoids have just the right range of solubility characteristic for ease of separation and most of them possess characteristic colours on paper in visible or ultraviolet light.

In most survey work, concentrated plant extracts are applied to paper chromatograms, which are developed in one, or more frequently, two dimensions. In addition, or alternatively, acid-hydrolysed extracts are examined in the same way for the presence of aglycones. Surveys of the heartwood extracts of *Pinus* species (Erdtman, 1952) and *Prunus* species (Hasegawa, 1958), of the leaves of *Triticum* species, allied genera (Endo, 1956) and *Camellia* species (Roberts *et al.*, 1958), and of the tubers and flowers of *Solanum* species (Harborne, 1959a) have all been carried out by means of paper chromatography.

On the other hand, large scale separation has been carried out on columns of silica gel, kieselguhr, magnesol, cellulose, alumina, polyamide, Sephadex and ion-exchange resins. The adsorbents of choice have generally been silica gel, cellulose and polyamide. In a typical procedure, a column 6 cm in diameter and 16 cm deep is prepared from a slurry of magnesol in acetone. After washing the column with acetone, the plant extract in the same solvent is applied to it and development

proceeds with water-saturated ethyl acetate (Ice and Wender, 1952).

The method of separating plant flavonoids by paper chromatography consists of banding concentrated plant extracts on sheets of thick Whatman filter paper, developing with a suitable solvent (e.g. butanol-acetic acid-water) and excising, eluting and rechromatographing the individual bands which have separated. Pure, crystalline flavonoids are readily obtained from plant extracts after separation on paper.

Paper chromatography plays an essential role in the identification of flavonoids on a microscale. The combination of this technique with that of ultraviolet spectroscopy has now been used in a large number of investigations for the elucidation of the structure of unknown compounds. The determination of the structure of flavonoid glycosides involves first the identification of the aglycone, and then the determination of the nature, position and number of sugar residues.

7.3 The Chromatographic Analysis of Water Soluble Pigments of *Hybanthus floribundus*

Introduction

During investigation into the metal content of various solvent extracts of *Hybanthus floribundus* the presence of an intense yellow pigment was observed.

Throughout the study, paper chromatography was found to be one of the best methods for the rapid separation of the flavonoid mixtures from the crude extracts of dried plant material. Furthermore, sufficient quantities of the pigments for ultraviolet and other spectral analysis often can be isolated from about thirty chromatograms. The techniques of nuclear magnetic resonance, ultraviolet and mass spectrometry along with elemental analysis were employed as aids to identification.

Materials and reagents

Chromatographic analyses were all carried out on Whatman 3MM chromatographic paper. The solvent systems used for two dimensional chromatography were a 3:1:1 solution of reagent-grade tertiary butanol:reagent-grade glacial acetic acid:water (TBA) and a 15% v/v solution of reagent-grade glacial acetic acid in water (HOAc). A large glass chromatographic tank (57×50×20cm) was used for descending runs whilst ascending runs were carried out in smaller tanks (30×30×30cm) containing a frame support system which allowed five chromatograms to be run at once. Chromatograms were dried under a fume hood either in the frame support or on glass rods suspended from clamps and stands. An ultra-violet lamp was used for viewing the developed chromatograms.

Extraction

Dried leaves of *Hybanthus floribundus* (18g) were finely powdered using a Glen-Creston agate ball mill and shaken at room temperature, overnight, with 150cm^3 of demineralized water. The solid material was filtered off using a millipore filter, and re-extracted with a further 100cm^3 of demineralized water. The two extracts were combined and concentrated using a rotary evaporator at 30^{o}C. The concentrate (60cm^3) was freeze-dried and stored in a sealed container in a refrigerator until required.

Chromatography

About 0.1g of the residue was dissolved in 1cm^3 of methanol (containing a minimum of water to effect solution). This solution was then spotted (using fine drawn capillary tubing) on the lower left-hand corner of a sheet of Whatman 3MM chromatographic paper. A hair drier was used to evaporate off the solvent between repeated applications of the solution to the paper. The final spot, was about 1cm in diameter and 3cm from each edge of the paper.

The chromatogram was developed ascendingly using TBA as solvent. When the solvent front reached to within about 3cm of the upper edge of the paper (∿8hr), the chromatogram was removed and dried. The bands produced were then developed ascendingly in the second direction with HOAc as solvent. This run required about 2hr for completion. The dried two-dimensionally developed chromatogram was viewed in UV light alone and in the presence of ammonia fumes. All the spots detected were circled with a pencil and the colour reactions were noted.

Results and Discussion

The results of the two-dimensional chromatography of the water extract of *Hybanthus floribundus* are set out in Fig.37. At such an early stage in the investigation of these pigments only tentative assignations of the flavonoid types to which these pigments belong can be made on the grounds of both R_f value and colour reaction under UV light. These are set out in Fig.38.

Spot 8 is the yellow pigment of special interest for reasons already given. On the basis of comparison of R_f values with literature results it does not appear to fall into any of the normal flavonoid classes, yet its colour reactions are certainly flavonoid-like.

It is clear that further chromatography will be required to yield the yellow pigment in large enough quantities for specific identication, and that other types of analysis will be necessary for elucidation of its structure.

Preliminary purification of flavonoids in the crude extract

The crude aqueous extract of *Hybanthus floribundus* was dissolved in 125cm^3 of hot methanol. This solution was mixed with 5g of celite and filtered through a Buchner funnel. The celite-residue material was suspended in 50cm^3 of hot methanol and filtered again. The two

Paper Chromatography

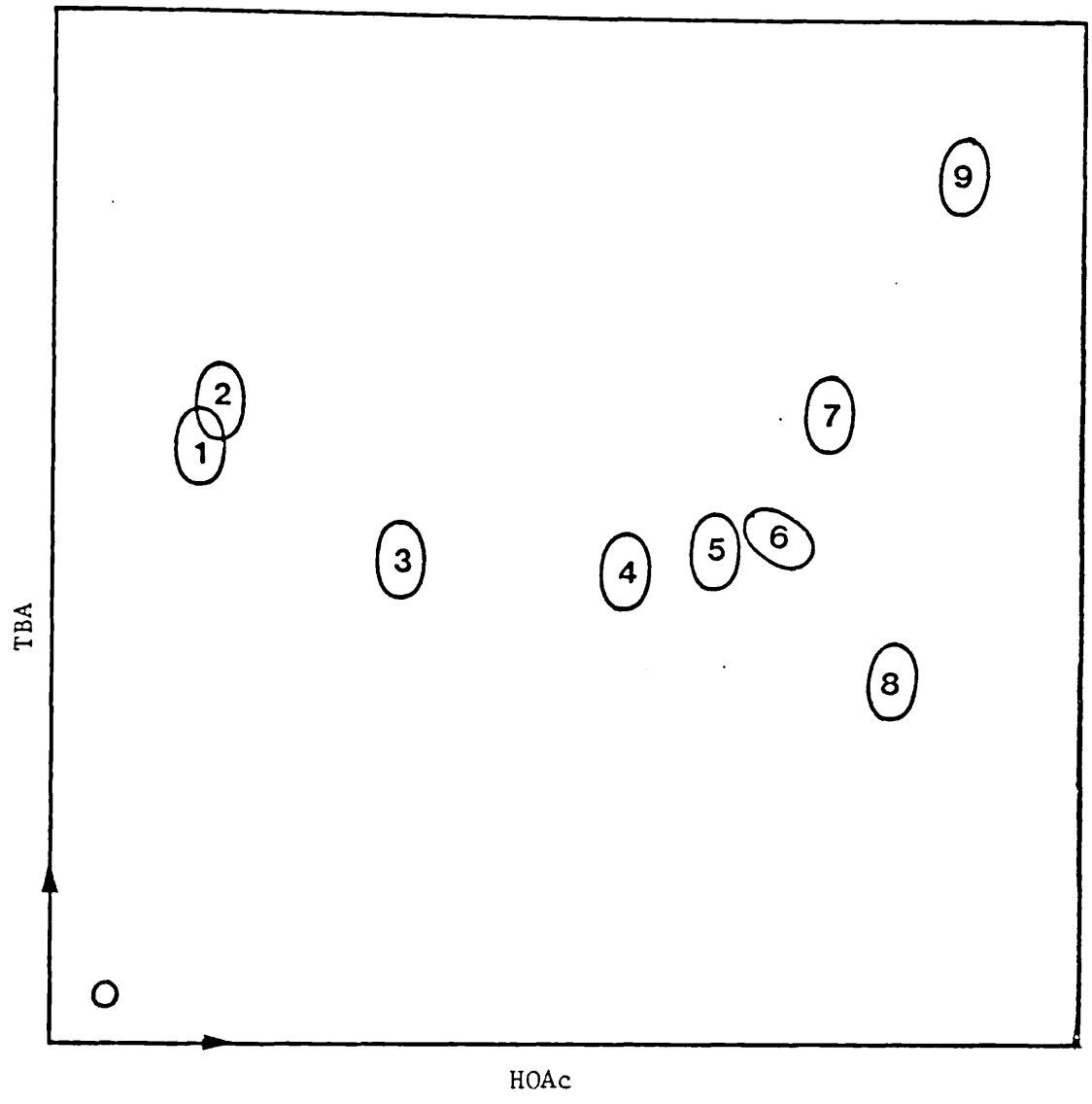


Fig.37. The ascending 2-D-paper chromatography pattern of flavonoids obtained from a water extract of *Hybanthus floribundus*

Spot No.	R_f value		Appearance under UV		Deduction
	TBA	HOAc	without NH_3	With NH_3	
1	0.51	0.05	Yellow	Fluorescent Yellow	Flavone/Flavonol Aglycone
2	0.65	0.07	Yellow	Yellow	Flavone/Flavonol Aglycone
3	0.42	0.20	Blue	Fluorescent Blue	Flavone/Flavonol 7-O-monoglycoside
4	0.41	0.35	Blue	Blue	Flavone/Flavonol 7-O-diglycoside
5	0.45	0.43	Blue	Fluorescent Blue	Flavone/Flavonol 7-O-diglycoside
6	0.41	0.59	Yellow	Yellow	Flavonol 3-O-monoglycoside
7	0.69	0.51	Yellow	Yellow	Flavonol 3-O-monoglycoside
8	0.41	0.69	Yellow	Fluorescent Yellow	?
9	0.72	0.70	-	Fluorescent Blue	Dihydroflavonol 3-O-monoglycoside

Figure 38 Tentative assignations of flavonoid types in *H. floribundus*

filtrates were combined, left standing overnight, and then filtered.

About 250cm^3 of the clear filtrate was mixed with activated charcoal using a mechanical stirrer. Charcoal was added in portions until the supernatant liquid showed no flavonoids as determined by silica gel thin layer chromatography. A total of 30g of charcoal was added, two 10g and two 5g portions.

The charcoal-flavonoid material was filtered onto a small Buchner funnel, and the residue was washed with 500cm^3 of boiling methanol, followed by 500cm^3 of boiling water and finally with 500cm^3 of a boiling solution of phenol:water (7:93). After the phenol-water solution had been concentrated to a small volume on a rotary evaporator, the remaining traces of phenol were removed by ether extraction.

Chromatography

One-dimensional chromatography was carried out on 300×300mm Whatman 3MM chromatography paper in Shandon Glass Tanks. Spots of the above extract were applied to the paper and developed in the ascending mode using n-butanol-acetic acid-water, 4:1:5, v/v/v and n-butanol-acetic acid-water, 12:3:5, v/v/v. The yellow pigment moved as a very distinct spot (R_f value of 0.26 and 0.28 respectively). n-Butanol-acetic acid-water, 3:1:1, v/v/v, caused the pigment to move more slowly (R_f 0.23), but not as cohesively as with the above solvents. Another good solvent for plant phenols is acetic acid-water, 3:17, v/v. This caused the pigment to move with the solvent front (R_f 0.75).

At this stage bands of the concentrated extract were placed along the origin lines of twenty sheets of Whatman 3MM paper, which were developed in butanol-acetic acid-water, 12:3:5, v/v/v, in the descending mode. Thin strips were cut from the sides of the papers and fumed with ammonia vapour to locate the position of the yellow pigment. Compact yellow bands were found at R_f 0.32. The pigment was cut from the papers

and eluted in 150cm^3 of 50% aqueous methanol. The solution was filtered, and concentrated using a rotary evaporator at 30^{o}C. Further chromatographic analysis gave a single spot which appeared yellow under the UV and gave a fluorescent yellow colour in the presence of ammonia fumes.

The paper chromatographic analysis of the sugars obtained from the yellow pigment

0.5mg of the yellow pigment was mixed with 6% aqueous hydrochloric acid (5cm^3) using a minimum of methanol to effect complete solution. The solution was heated on a steam bath for 45 minutes and then cooled and extracted thoroughly by shaking with ether.

Both the ether and the aqueous layer were concentrated separately and were paper chromatographed ascendingly in a solvent of ethylacetate: pyridine:water, 12:5:4, v/v/v, alongside some of the more common sugars such as glucose and rhamnose. The chromatogram was dried and sprayed uniformly with a solution of p-anisidine hydrochloride (1g) and sodium hydrosulphite (0.1g) in methanol (10cm^3) diluted to 100cm^3 with n-butanol. The sprayed chromatogram was then heated at 130^{o}C in an oven for ten minutes during which time the sugar spots turned brown; the R_f values for the coloured spots of the unknown sugars were compared directly with those of the known sugars. The ether extract yielded no sugar while the aqueous extract yielded rhamnose and glucose (Fig.39).

7.4 Spectroscopic Measurements

UV spectra were recorded with a Pye Unicam SP 8-100 ultraviolet spectrophotometer. Mass spectrometry was recorded on a VG MicroMass 12 mass spectrometer. ^{1}H n.m.r. spectrum was measured with a Bruker 400MHz and ^{13}C n.m.r. spectra with Jeol FX90Q and Bruker 400 MHz instruments.

Figure 39

Sugar analysis of yellow pigment in *Hybanthus floribundus*

Spot applied	Colour of spot	R_f value of spot ethyl acetate/pyridine/water	Assignments
L-Rhamnose	Brown	0.56	
L-Arabinose	Red	0.35	
D-Arabinose	Red	0.34	
Glucose	Brown	0.27	
D-Fructose	Brown	0.26	
D-Galactose	Brown	0.20	
D-Xylose	Red	0.33	
L-Sorbose	Brown	0.24	
Lactose	Brown	0.08	
Aqueous layer	Brown	0.56, 0.28	Rhamnose, glucose
Ether layer	-	-	-

7.5 The Structure Analysis of the Yellow Pigment by Ultraviolet Spectroscopy

Preparation of reagent stock solutions and solids

Sodium methoxide (NaOMe). Freshly cut metallic sodium (2.5g) was added cautiously in small portions to dry spectroscopic methanol ($100cm^3$). The solution was stored in a glass container with a tightly fitting plastic stopper.

Aluminum chloride ($AlCl_3$). Five grams of fresh anhydrous reagent grade aluminum chloride were added cautiously to spectroscopic methanol ($100cm^3$).

Hydrochloric acid (HCl). Concentrated reagent grade hydrochloric acid ($50cm^3$) was mixed with distilled water ($100cm^3$); the solution was stored in a glass stoppered bottle.

Sodium acetate (NaOAc). Anhydrous powdered reagent grade sodium acetate was used.

Boric acid (H_3BO_3). Anhydrous powdered reagent grade boric acid was used.

Steps in the ultraviolet spectral analyses

(1) A stock solution of the yellow pigment was prepared by dissolving a small amount of the compound (about 0.1mg) in about $10cm^3$ of spectroscopic methanol.

(2) The methanol spectrum was measured at normal scan speed (about 50nm per min) using $2-3cm^3$ of the stock solution of the yellow pigment.

(3) The methanol spectrum was rerun at slow scan speed (about 10nm per min) in the regions of the peak maxima in order to determine the wavelength (λ) of each maximum more accurately.

(4) The sodium methoxide spectrum was measured immediately after the addition of three drops of the sodium methoxide stock solution to the solution used for steps 2 and 3. After five minutes, the spectrum was rerun to check for yellow pigment decomposition (only the initial spectrum is presented here). The solution was then discarded.

(5) The aluminum chloride spectrum was measured immediately after the addition of six drops of the aluminum chloride stock solution to $2-3cm^3$ of fresh stock solution of the yellow pigment.

(6) The aluminum chloride/hydrochloric acid spectrum was recorded immediately after the addition of three drops of the stock hydrochloric acid solution to the cuvette containing the aluminum chloride (from

step 5). The solution was then discarded.

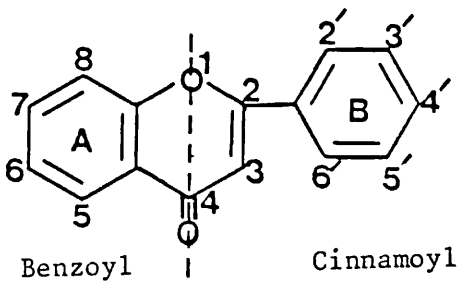
(7) The sodium acetate spectrum of the yellow pigment was determined by the addition of an excess of coarsely powdered, anhydrous reagent grade sodium acetate with shaking to a cuvette containing 2-3cm^3 of fresh stock solution of the yellow pigment. About 2mm layer of sodium acetate remained on the bottom of the cuvette. The sodium acetate spectrum presented here was recorded within two minutes after the addition of the sodium acetate to the solution. A second spectrum was run after five to ten minutes to check for decomposition.

(8) Since no decomposition of the yellow pigment was observed during the recording of the sodium methoxide spectrum, the sodium acetate/ boric acid spectrum was determined by adding sufficient powdered anhydrous reagent grade boric acid to give a saturated solution, with shaking, to the cuvette (from step 7) which contained the sodium acetate. The solution was discarded after the spectrum was recorded.

Throughout the above spectral analyses spectroscopic grade methanol was used as reference. The spectra recorded are presented in Fig.41.

Results and discussion

The methanol spectrum of the yellow pigment exhibits two major absorption peaks in the region 240-400nm. These two peaks are characteristic of methanol spectra of flavones and flavonols and are commonly referred to as Band I (usually 300-380nm) and Band II (usually 240-280nm). Band I is considered to be associated with absorption due to B-ring cinnamoyl system, and Band II with absorption involving the A-ring benzoyl system (Fig.40).



Benzoyl Cinnamoyl

Figure 40

The methanol spectrum shows a pronounced Band I which appears at a longer wavelength and is characteristic of flavones and flavonols oxygenated in the A-ring as well as in the B-ring. The position of Band I (360nm) shows the presence of flavonol with a substituted 3-hydroxyl group (methylated or glycosylated), while Band II appear as two peaks which may be due to the B-ring oxidation pattern.

Sodium methoxide is a strong base and is used mainly to ionize to some extent all hydroxyl groups on the flavonoid nucleus. The addition of sodium methoxide to the yellow pigment resulted in a large bathochromic shift of Band I of about 58nm without a decrease in intensity which is characteristic for the presence of a free 4'-hydroxyl group. Since the absorption peaks did not degenerate in the sodium methoxide spectrum in a few minutes, the absence of free hydroxyl groups at both the 3- and 4'-positions is demonstrated (Fig.41).

Sodium acetate is a weaker base than sodium methoxide and tends to ionize significantly only the more acidic phenolic hydroxyl groups. The addition of sodium acetate to the yellow pigment resulted in a bathochromic shift of Band II of about 14nm, which is characteristic of flavones and flavonols possessing a 7-hydroxyl group. In the presence of sodium acetate, boric acid will chelate with ortho-dihydroxyl groups at all locations on the flavonoid nucleus except at C-5,6. Since no bathochromic shift was observed in Band I relative to methanol spectrum, the presence of ortho-dihydroxyl groups is not possible. This is again confirmed from the aluminum chloride, aluminum chloride/hydrochloric acid spectrum, where no bathochromic shift occurred by Band I with aluminum chloride relative to aluminum chloride/hydrochloric acid spectrum. Thus the presence of an ortho-dihydroxyl group in the B-ring is not possible. However the presence of a 5-hydroxyl group in the yellow pigment is evident by a Band I bathochromic shift of 44nm on the addition of aluminum chloride/hydrochloric acid relative to that in the

CHROMATOGRAPHIC DATA

Spot appearance: (UV) yellow

: (UV/NH_3) fluorescent yellow

R_f Values: 0.42 (TBA), 0.69 (HOAc)

UV SPECTRAL DATA (λ_{max},nm)

MeOH 254,268,308sh,360

NaOMe 266,326,418

$AlCl_3$ 274,310sh,368sh,410

$AlCl_3$/HCl 274,304sh,360sh,404

NaOAc 268,320sh,420

NaOAc/H_3BO_3 254sh,268,304sh,360

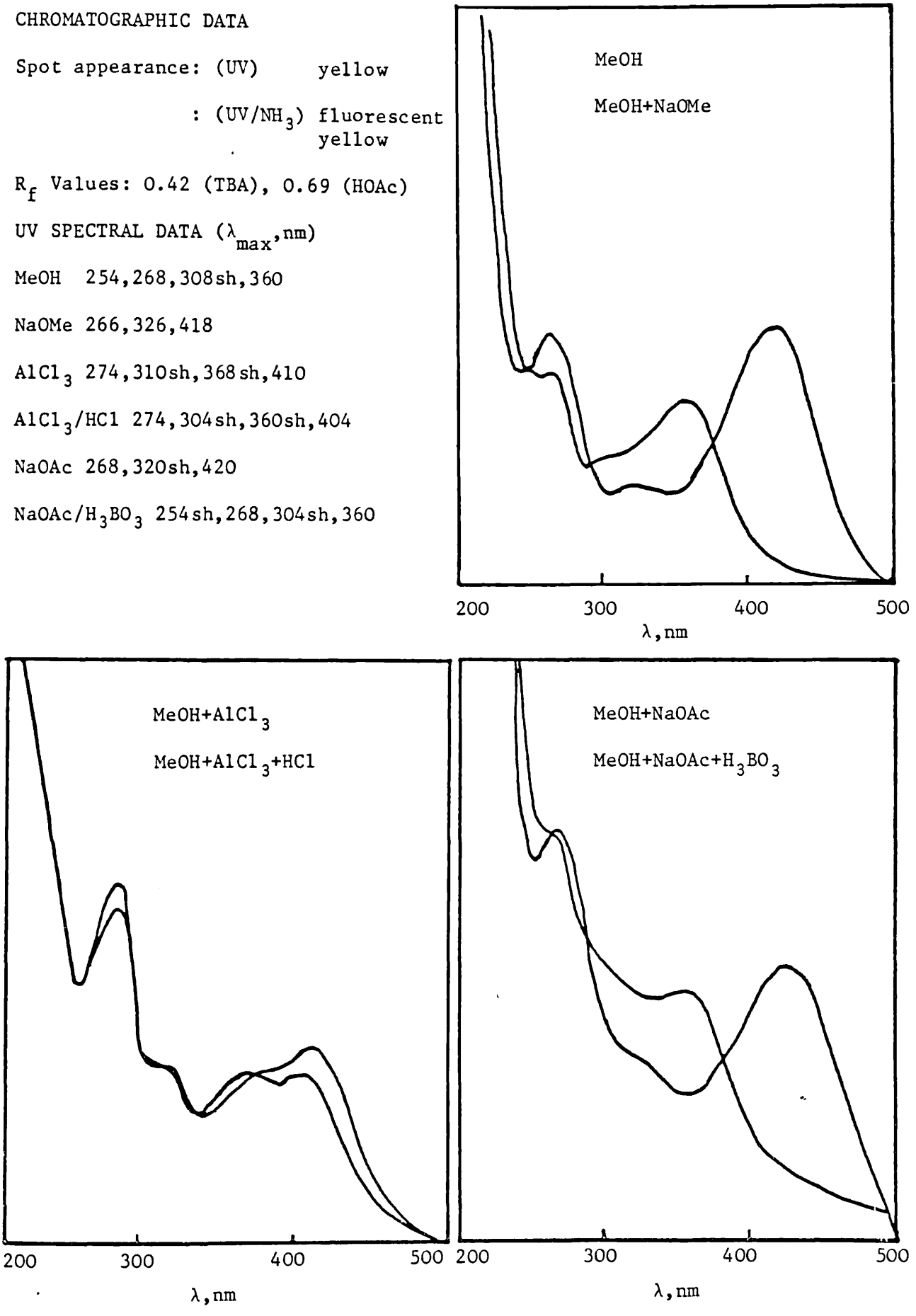
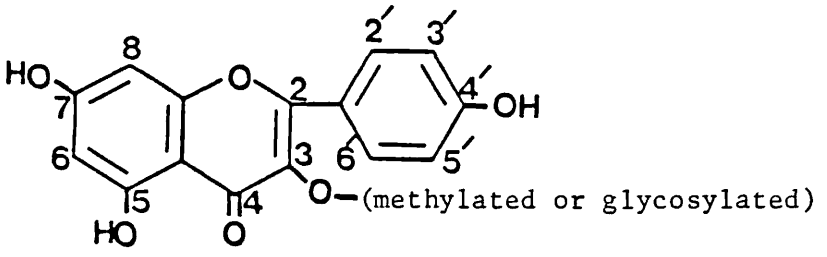


Figure 41. The ultraviolet spectra of the yellow pigment

methanol spectrum.

Thus the probable structure deduced from the spectral measurements for the yellow pigment is:



7.6 Elemental analysis

The elemental analysis of the pigment gave the following results: C,23.5%; H,4%; N,0.0 and 20.7% ash.

The pigment was wet ashed in nitric acid and analysed for metal content using atomic absorption spectrophotometry. No copper, nickel, zinc, iron, magnesium or calcium was detected.

X-ray fluorescence spectrometry, showed the presence of major trace of calcium, and sulphur and minor trace of tin, titanium, lead, zinc, potassium, bromide and chloride.

7.7 Mass Spectrometry of the Yellow Pigment

Perhaps the most useful fragmentations in terms of flavonoid identifications are those which involve the cleavage of intact A- and B-ring fragments. Such ions are designated here as A_1, A_2 and B_1, B_2 respectively (Harbone *et al.*, 1975). Two common fragmentations of flavonoids are those designated below as pathway-I and pathway-II (Fig. 42). The diagnostic fragmentation pattern for flavones and flavonols aglycones are illustrated in Fig.43. The pathway-I process commonly produces two different ions, A_1^+ and B_1^+, the ratio of one to the other

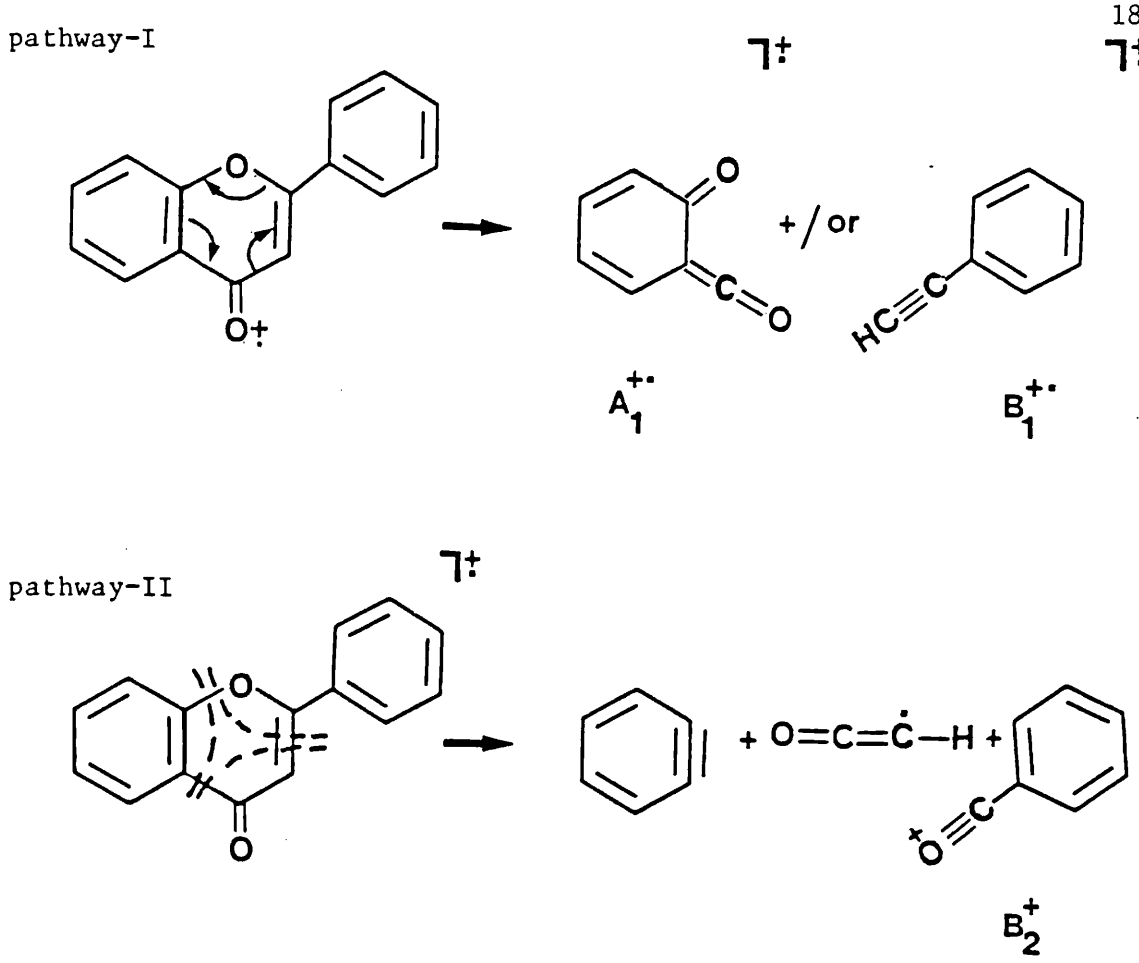


Figure 42 Fragmentation pattern of flavonoids

being indicative of the charge distribution within the parent ion. In contrast pathway-II yields predominantly a single charged species, B_2^+.

Considering the fragmentation pattern for the yellow pigment, the molecular ion peak occurs at m/e 344 (Fig.46). There is strong evidence for the presence of $A_1^{+\cdot}$, whereas the fragment corresponding to $B_1^{+\cdot}$ is not seen (Fig.44).

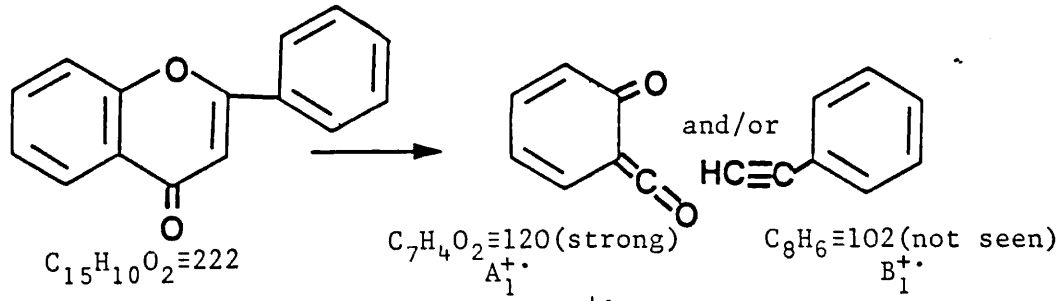


Figure 44 Fragmentation pattern showing $A_1^{+\cdot}$ which is strongly evident.

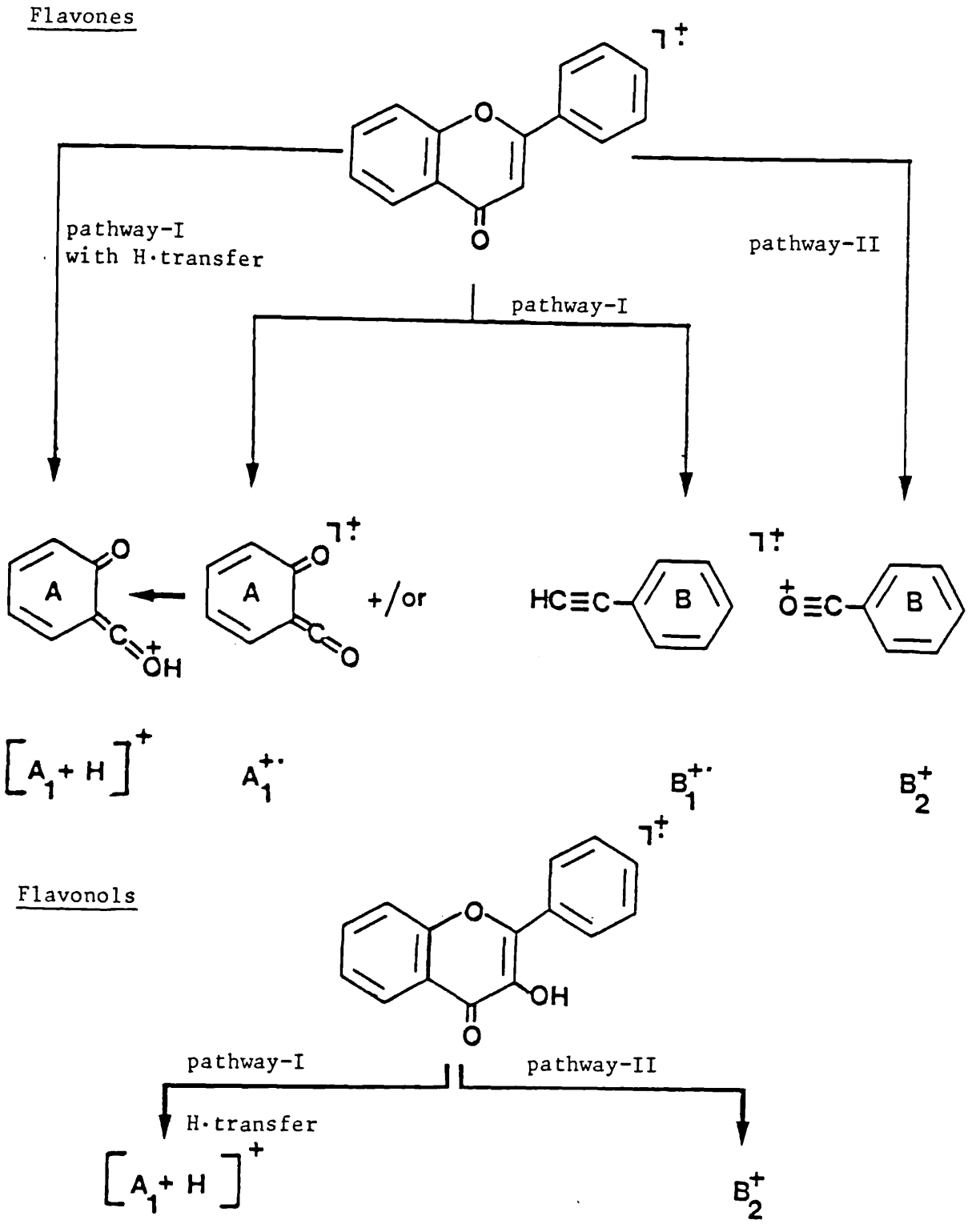


Figure 43 Diagnostic mass spectral fragmentation pathways for flavones and flavonols aglycones

The extra mass units, however (122 m.u.) could only correspond to the presence of three methoxy and two hydroxyl groups or one methoxy, one ethoxy and three hydroxyl groups. Thus the fragmentation should be as follows:-

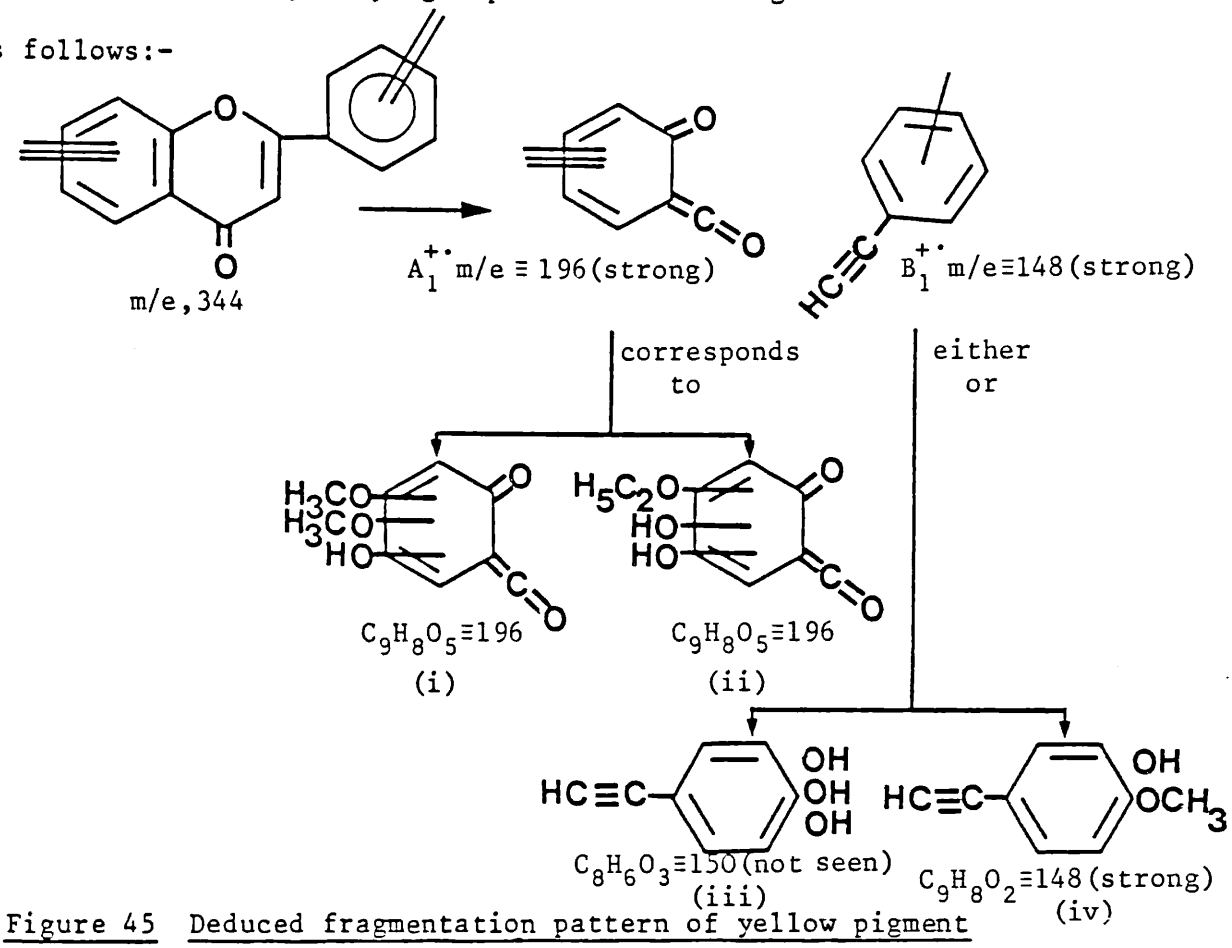
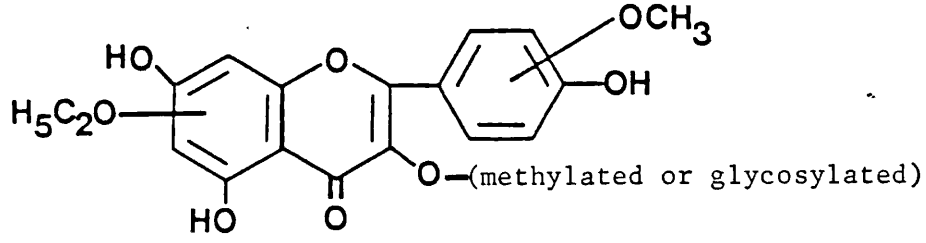


Figure 45 Deduced fragmentation pattern of yellow pigment

Considering the $B_1^{+\cdot}$ fragment, the only possible substitution is (iv) where substitution in $A_1^{+\cdot}$ fragment could either be (i) or (ii). But since evidence from ultraviolet spectroscopy suggests the presence of a 5,7' hydroxyl group, structure (ii) is more favoured.

Thus from the above evidence the possible structure for the yellow pigment is:



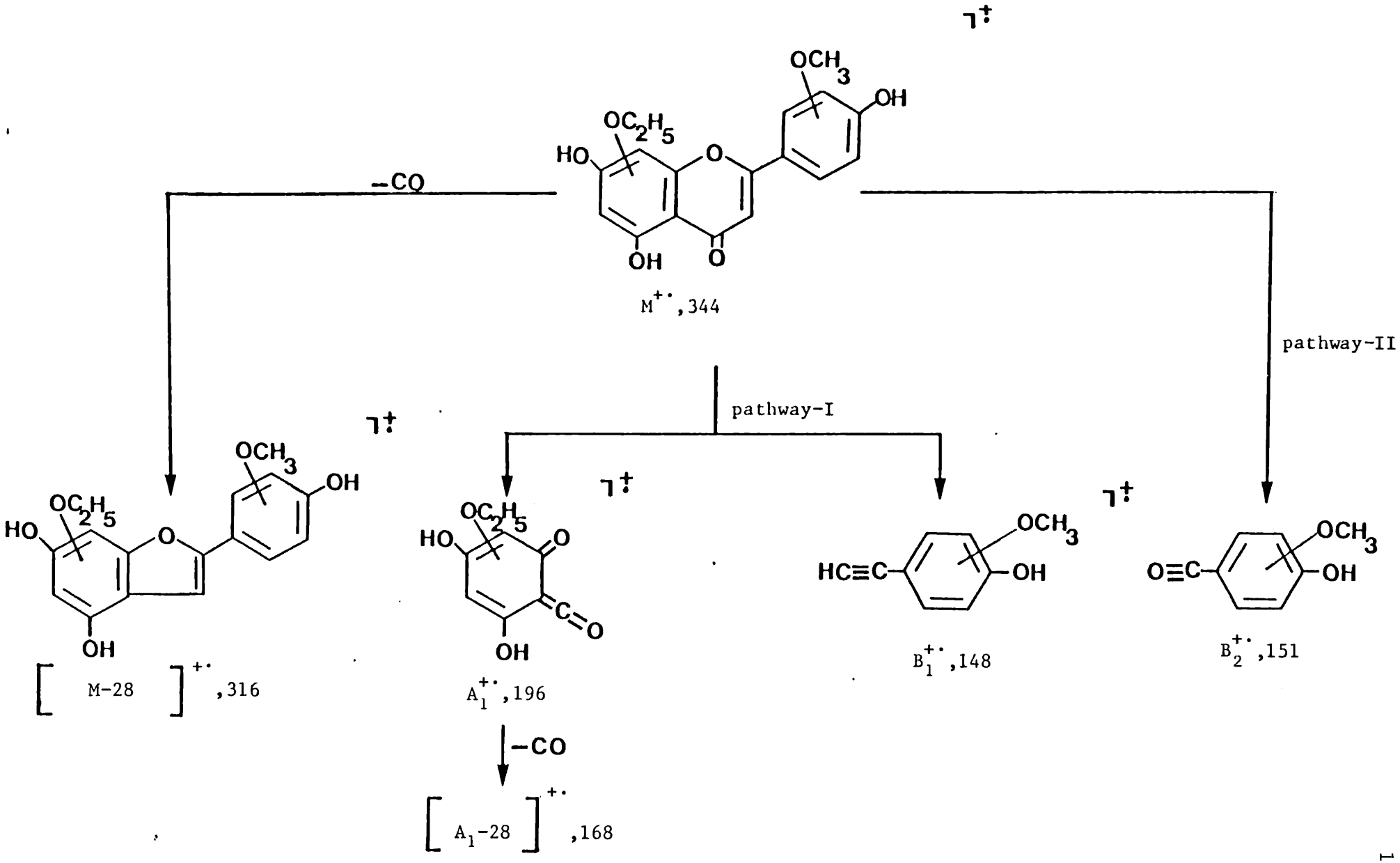
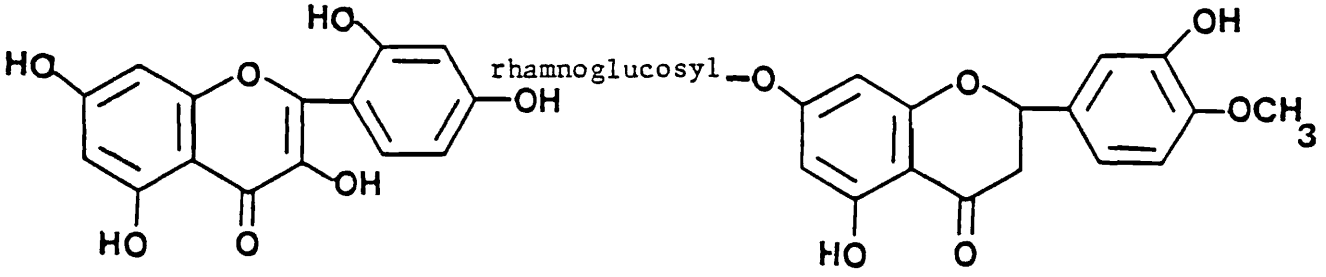


Figure 46 Observed fragmentation in MS of yellow pigment

7.8 Nuclear Magnetic Resonance Analysis of the Pigment

The ^1H n.m.r. spectrum of the yellow pigment measured with a Bruker 400MHz instrument is shown in Fig.49. In D_2O solution, the pigment gives signals in the ^1H n.m.r. spectrum from the glucosyl H-1 group, 5.50 ppm; rhamnosyl H-1 group, 5.00 ppm; OCH_3 group, 3.68 ppm; rhamnoglucosyl group, 3.34 ppm; H-3 trans and H-3 cis, 2.24 ppm and 2.74 ppm; rhamnosyl CH_3 group, 1.29 ppm.

Although proton n.m.r. spectroscopy has been widely used for elucidation of the structures of flavonoids (Mabry *et al.*, 1970), yet there are few published ^{13}C n.m.r. data. In the present study, the ^{13}C n.m.r. spectra of the yellow pigment were recorded with both Bruker 400MHz and Jeol FX 90 MHZ instruments (Fig.50,51) using D_2O as solvent. In order to assist with the spectral interpretation of the pigment, synthetic morin (Fig.47I) and hesperidin (Fig.47II) were studied.



Morin (I) Hesperidin (II)

Figure 47

The spectra were assigned (Table VII) by comparison with ^{13}C n.m.r. data on some flavonoids (Pelter *et al.*, 1976) (Wenkert, 1977). The aromatic carbon shifts of the sugar carbons attached to C-7 in hesperidin were also assigned as shown in Fig.48.

However, the solutions of morin and hesperidin were much more concentrated than that of the unknown pigment and the spectra were clear and much more easily obtained. In hesperidin, the glycoside

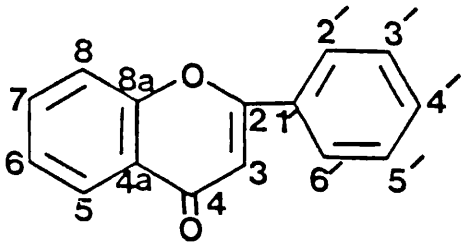


Table VII. ^{13}C N.m.r. spectra of flavonoids. The δ values are in ppm downfield from TMS* or TPS

Carbons	Morin*	Hesperidin*	Yellow pigment	
			400 MHz	90 MHz
2	148.88	78.36		
3	136.17	41.94		
4	176.20	196.81		182.28
4a	103.20	103.33		
5	156.78	162.93		
6	93.54	96.44		
7	163.91	165.01		
8	98.22	95.62		
8a	161.04	162.38		
1'	115.44	130.77		
2'	159.19	113.99	109.35	
3'	103.20	146.28		
4'	160.75	147.88		
5'	109.44	111.95	108.37	
6'	131.68	117.93		
OMe	—	55.63	48.87	51.24

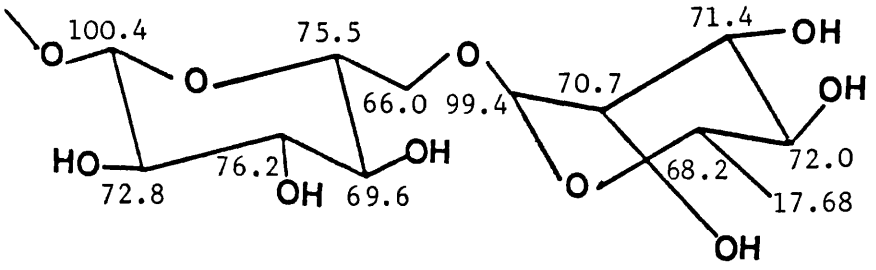


Figure 48

groups are clearly shown between 60 ppm and 100 ppm, whereas in ^{13}C n.m.r. spectra of the pigment, the sugar moeity is not as clear as it is in the ^{1}H n.m.r. spectrum. However, the carboxyl group clearly shows at 182 ppm, as well as the methoxy group. In addition to the assigned signals in table VII, the pigment also shows signals at 22.66 ppm which could be assigned to a methyl group, and at 181.33 ppm, 181.95 ppm and 182.11 ppm. However, both ^{13}C n.m.r. spectra of the pigment were carried out in D_2O as solvent, so possibly the protons in the pigments exchange with deuterium from the solvent.

7.9 Discussion

The flavonoid constitution of *Hybanthus floribundus* is complex. Paper chromatographic examination of water and methanol extracts disclosed the presence of a number of components of flavonoid-like character, as well as a yellow pigment which appeared to be present in large quantities.

The isolation and purification of the pigment was achieved by preparative one-dimensional paper chromatography. Spectrophotometric examination of the pigment was found to be similar to that of isorhamnetin 3-O-rutinoside. However, the appearances of the two spots were different under UV prior to ammonia exposure (Fig.54). Hydrolysis of the yellow pigment showed the presence of glucose and rhamnose.

Fig.49. ^{1}H n.m.r. spectrum of the yellow pigment. The δ values are in ppm downfield from TPS (400MHz)

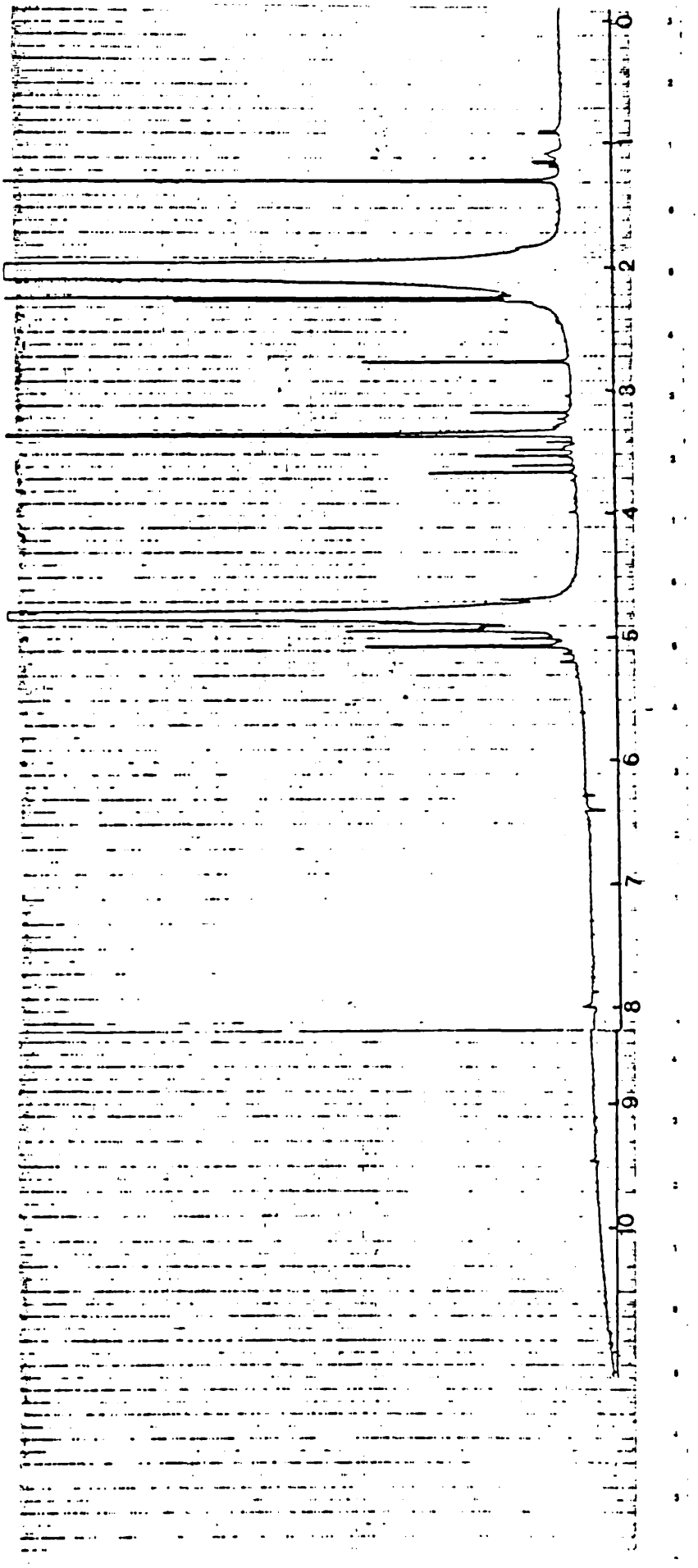
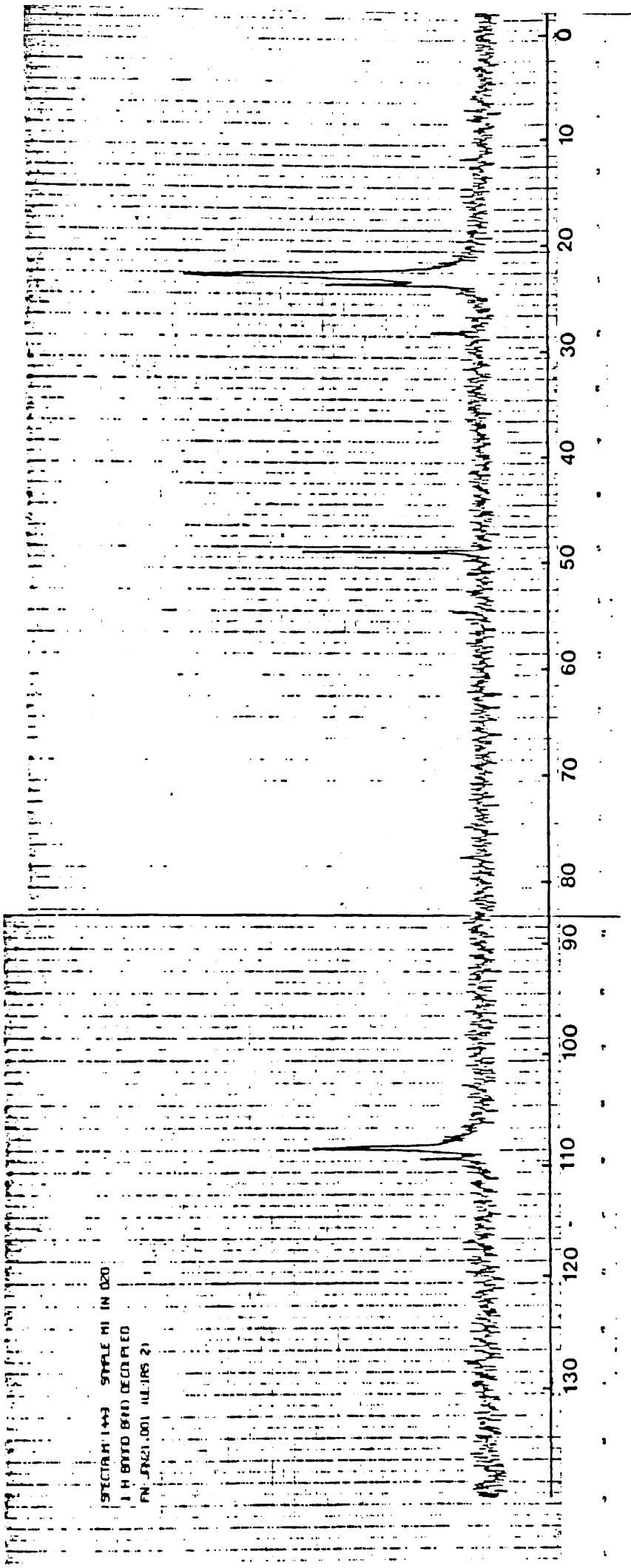


Fig.50. ^{13}C n.m.r. spectrum of the yellow pigment. The δ values are in ppm downfield from TPS (400MHz)



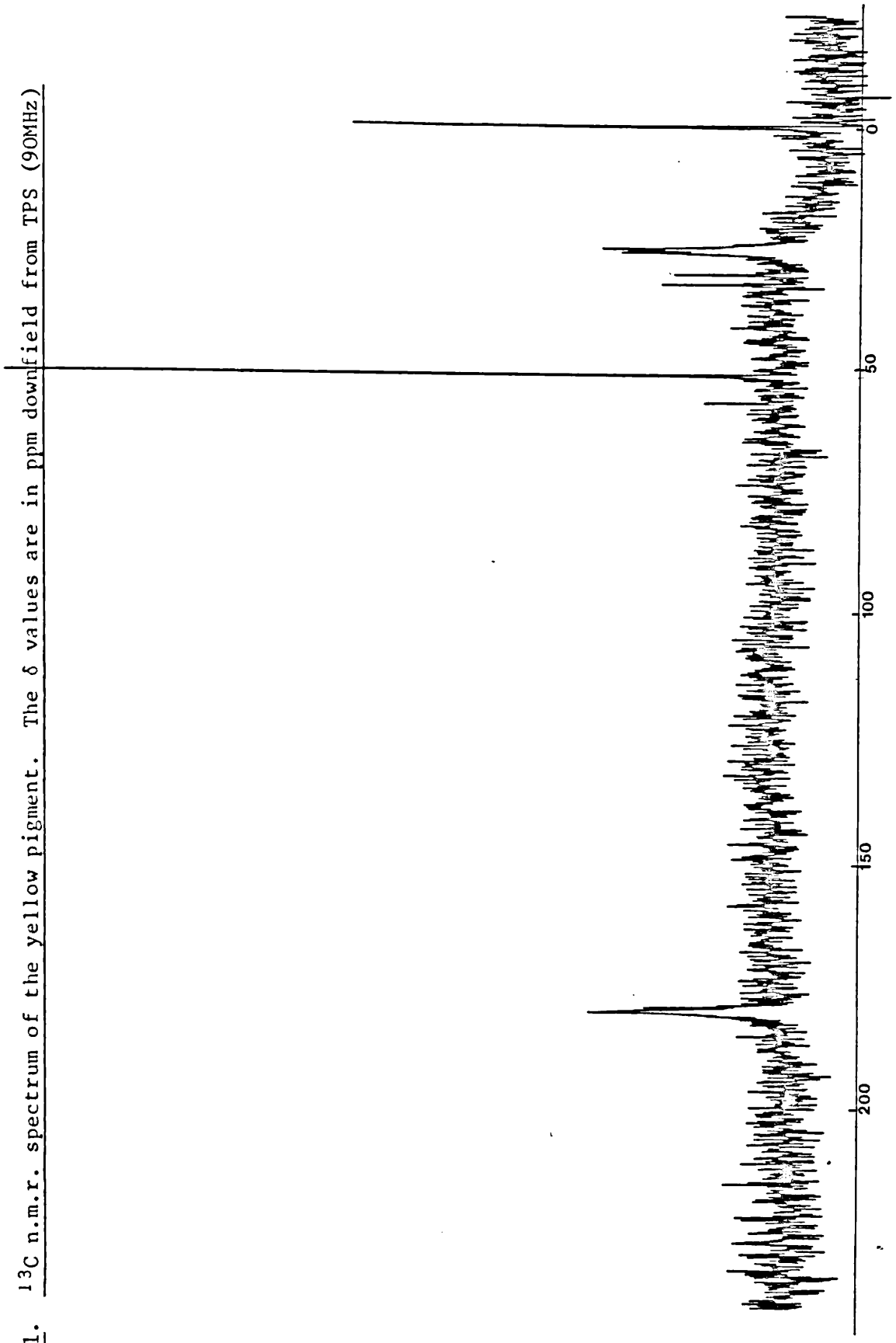


Fig.51. ^{13}C n.m.r. spectrum of the yellow pigment. The δ values are in ppm downfield from TPS (90MHz)

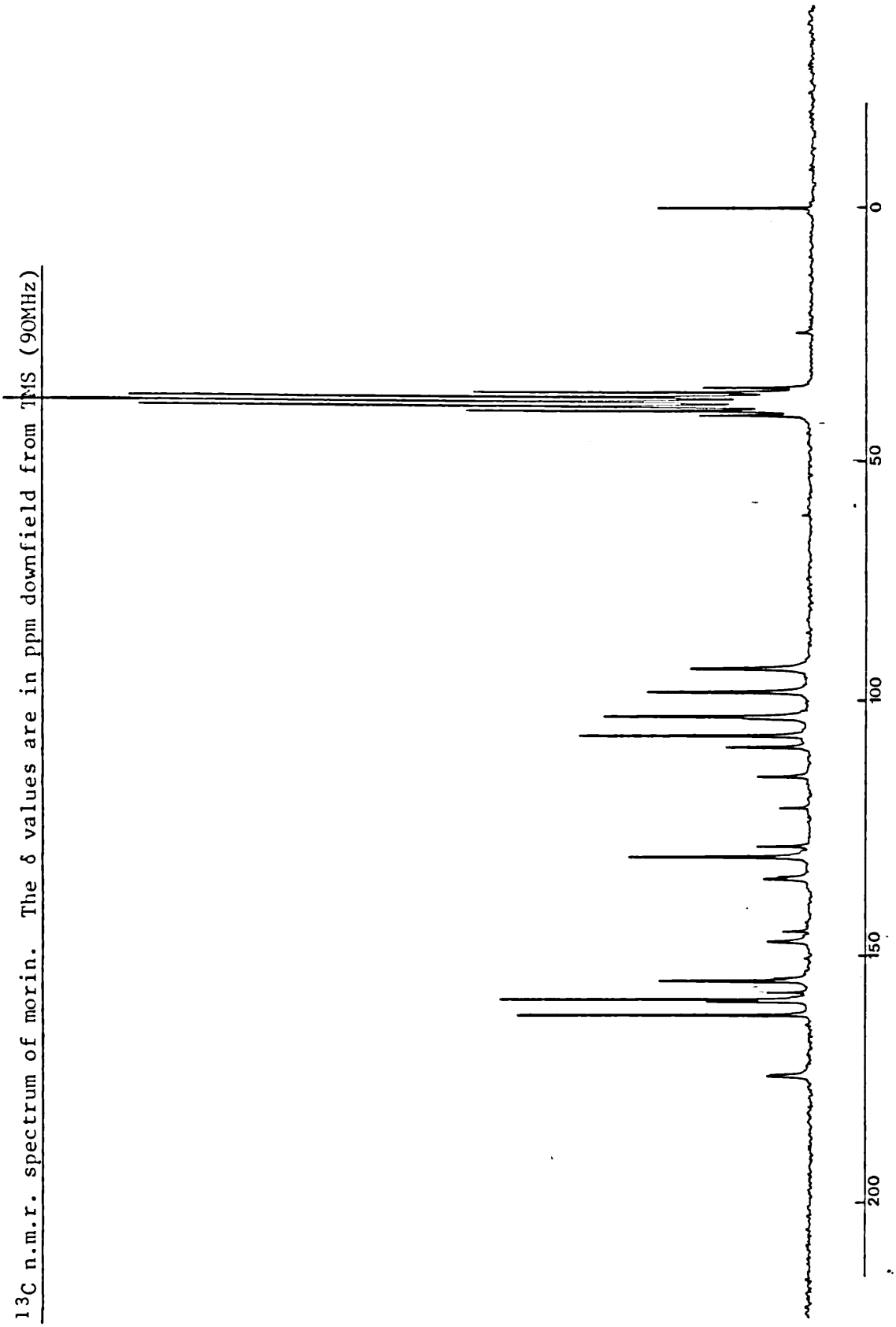


Fig.52. ^{13}C n.m.r. spectrum of morin. The δ values are in ppm downfield from TMS (90MHz)

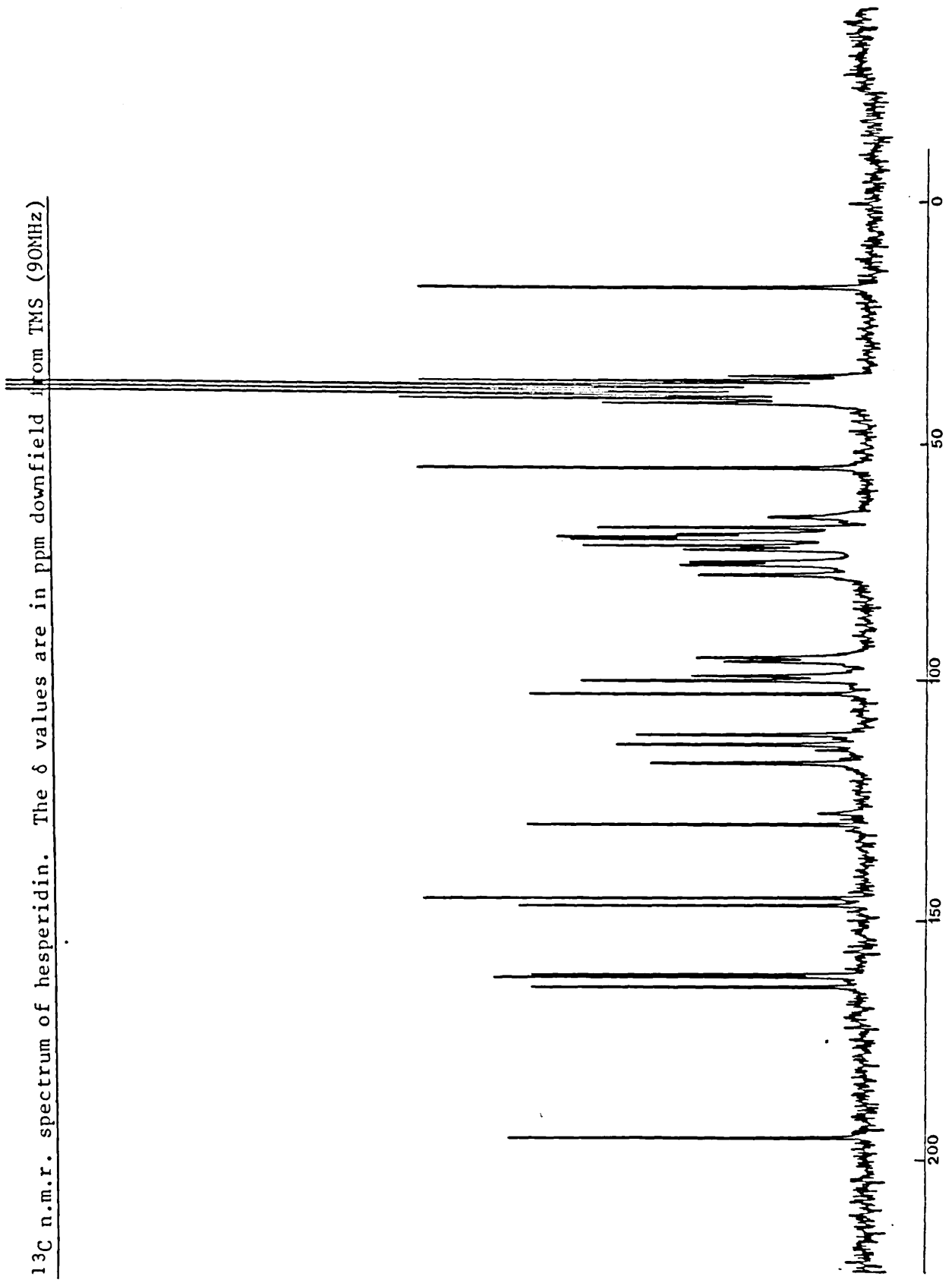


Fig.53. ^{13}C n.m.r. spectrum of hesperidin. The δ values are in ppm downfield from TMS (90MHz)

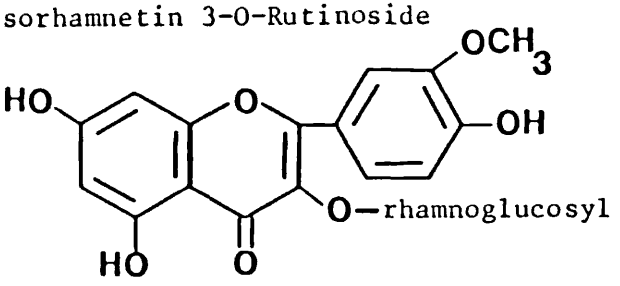
Yellow Pigment	Isorhamnetin 3-O-Rutinoside 
Spot Appearance: (UV) yellow : (UV/NH_3) fluorescent yellow R_f Values: 0.42 (butanol-acetic acid-water) : 0.69 (glacial acetic acid-water) UV SPECTRAL DATA (λ_{max},nm) MeOH 254,268,308sh,360 NaOMe 266,326,418 $AlCl_3$ 274,310sh,368sh,410 $AlCl_3$/HCl 274,304sh,360sh,404 NaOAc 268,320sh,420 NaOAc/H_3BO_3 254sh,268,304sh,360	Spot Appearance: (UV) deep purple : (UV/NH_3) yellow R_f Values: 0.45 (butanol-acetic acid-water) : 0.61 (glacial acetic acid-water) UV SPECTRAL DATA (λ_{max},nm) MeOH 254,265sh,305sh,356 NaOMe 271,328,414 $AlCl_3$ 268,278sh,300sh,369sh,402 $AlCl_3$/HCl 267,275sh,300sh,359sh,399 NaOAc 271,320,396 NaOAc/H_3BO_3 254,267sh,304sh,360

Figure 54. Comparison of data for yellow pigment and isorhamnetin 3-O-rutinoside

The suggestion that the yellow pigment could be a carotenoid and in particular crocin was considered. Although crocin could be mistaken for a yellow flavonoid, yet the spectrum is typically carotenoid ($\lambda_{max.}$ 414,437 and 458 nm in ethanol). However, no absorption in this region was observed for the yellow pigment.

Mass spectrometry of the yellow pigment clearly demonstrated fragmentation pattern characteristic of flavonoids.

Although the pigment was obtained in a crystalline form and purity was tested with paper chromatography, yet elemental analysis showed the C% to be lower than expected. Ashing the pigment with nitric acid, followed by atomic absorption analysis for metal content showed the pigment to be devoid of copper, nickel, zinc, iron, magnesium and calcium. These analyses were similar to those reported by W.A. Mullen (personal communication) which confirms our results. Thus, the yellow pigment is not likely to be a mixture, since different preparations have same analysis. X-ray fluorescence spectrometry, showed the presence of major trace of calcium and sulphur and minor trace of tin, titanium, lead, zinc, potassium, bromide and chloride.

However both ^{1}H n.m.r. and ^{13}C n.m.r. spectra look unusual. This may be because the solution is very dilute. The flavonoid would be very volatile and would have high absorbance in the UV, and thus would show in mass spectrometry and UV spectroscopy, but not enough is present for good n.m.r. studies. Another possibility is that the protons in the pigment exchange with deuterium from the solvent (D_2O). However, the glycoside can be seen from the ^{1}H n.m.r. spectrum; the methoxy and aromatic carbon atoms are seen in the ^{13}C n.m.r. spectra. Thus although not giving definitive information, the n.m.r. spectra are not inconsistent with the other results.

It seems possible from elemental analysis (ash 20.7%), that the yellow pigment may be present together with some inorganic material

e.g. as a calcium salt. X-ray crystalographic analysis would characterise the compound completely.

Finally, further investigation of other pigments shown in Fig.37 were not completed and additional work should be carried out to elucidate their structure.

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CHAPTER VIII

A SPECTROSCOPIC STUDY OF TRANSITION-METAL ION COORDINATION BY PECTIN

8.1 Introduction

The pectins or pectic substances are found universally in the primary cell walls and intercellular layers in land plants. They are most abundant in soft tissues such as sugar beet pulp (25%), and apples (15%), but are present in only small proportions in woody tissues. The term pectic substances is used generally to refer to the group of complex plant polysaccharides in which D-galacturonic acid is the principal constituent, and the term pectin is used in relation to the gel-forming, water-soluble polysaccharides. Polysaccharides in which a proportion of the D-galacturonic acid residues are present as methyl esters are designated pectinic acid, and those devoid of ester groups as pectic acids. Although D-galacturonic acid is the main sugar constituent of the pectic substances, various proportions of other sugars including D-galactose, L-arabinose, D-xylose, L-rhamnose, L-fucose, and traces of 2-O-methyl-D-xylose and 2-O-methyl-L-fucose are usually also present as constituents (Fig.2) (Section 1.2).

Metal components of pectates are well known and the retention of metal ions by acid polysaccharides is of considerable interest in view of its biological role in the availability of nutrients to plants (Veis, 1970)(Muzzarelli, 1973). It has been demonstrated that ill-defined gels on the root surfaces of theplants remove nutrient cations directly from the solid phase of the soil by a contact ion-exchange process. In this mechanism metal complexes with pectin and/or acid polysaccharides are formed (Ramamoorthy, 1977)(Leppart, 1971)(Jenny, 1966). Earlier investigations of complexation of galacturonic and glucuronic acids in solution often contradict each other with respect to

the binding of the ions. Some have suggested a chelate structure involving carboxyl and hydroxyl groups (Anthonsen *et al.* 1972, 1973) (Grasdalen *et al.* 1975). The formation of such chelates probably represents a common reaction of polyuronates or, generally, of polysaccharides containing uronic acid residues. Schweiger (1966) suggested that pectates form chelates with a number of divalent metal ions involving both carboxyl and hydroxyl groups. With most metals, the hydroxyl groups have to be present in vicinal pairs, i.e. belong to the same uronic acid residue. However, no exhaustive spectroscopic data have been reported for transition-metal ions binding to pectin. Therefore, we have examined the coordination of some selected metal ions, in order to clarify on the basis of spectroscopic results, the environment of specific sites for ion uptake by pectic substances.

8.2 Preparation of the Samples

Metal pectates and pectinates were prepared according to literature methods (Schweiger, 1963)(Wunsch, 1952). The preparations were carried out by adding weighed amounts (1-2g) of pectin in 65% aqueous isopropyl alcohol (15cm^3) to sodium hydroxide ($\sim$0.15g). The suspension was then stirred for 15 minutes until the hydroxide pellets had completely dissolved. Varying concentration of metal salts in a small volume of 65% isopropyl alcohol was added to the suspension and the mixture was allowed to equilibrate at room temperature under magnetic stirring. The solid samples were filtered and washed several times with 65% isopropyl alcohol to remove soluble salts. After the alcohol rinse, the complex was air dried in the funnel, and then kept over phosphorus pentoxide (under vacuum). When dry, the product was weighed, ground using a pestle and morter and left over phosphorus pentoxide. For metal pectates, pectic acid was used instead of pectin and the method of preparation proceeded as described for the preparation of metal pectinates.

Copper galacturonate was prepared by adding weighed amounts (0.5-1g) of the acid to 0.1M solution ($\sim$100cm^3) of copper sulphate solution and allowing the suspension to equilibrate at room temperature under magnetic stirring. The complex formed was then filtered and washed several times with water.

Elemental analysis

Elemental analysis of the complexes prepared gave the following results:

Table VIII. Elemental analysis of pectin-type compounds

Sample (air dried and stored over P_2O_5)	Calculated (for formulae see table IX)		Found	
	C%	H%	C%	H%
Pectin	33.9	5.6	34.1	5.5
Galacturonic acid	30.2	5.8	30.3	5.6
Aluminum pectinate	30.8	5.1	30.8	5.3
Cobalt pectate	29.9	4.5	29.4	4.9
Cobalt pectinate	32.3	4.0	32.8	4.8
Copper pectate	29.6	4.5	29.2	4.6
Copper pectinate	29.6	4.5	29.0	4.4
Copper galacturonate	26.7	4.8	26.9	4.9
Nickel pectate	28.8	4.8	28.8	4.9
Nickel pectinate	28.8	4.8	28.2	5.1
Zinc pectate	29.5	4.5	29.5	4.8
Zinc pectinate	33.2	3.6	33.2	3.9

From the results in Table VIII, the formulae deduced for the complexes are:

Table IX. Formulae of pectin-type compounds deduced from analytical results

Sample	Formulae
Pectin*	$C_{12}H_{16}O_{12}.4H_2O$
Galacturonic acid	$C_{12}H_{16}O_{13}.6H_2O$
Aluminum pectinate*	$C_{12}H_{14}O_{12}Al.5H_2O$
Cobalt pectate	$C_{12}H_{14}O_{12}Co.4H_2O$
Cobalt pectinate*	$C_{12}H_{14}O_{12}Co.2H_2O$
Copper pectate	$C_{12}H_{14}O_{12}Cu.4H_2O$
Copper pectinate*	$C_{12}H_{14}O_{12}Cu.4H_2O$
Copper galacturonate	$C_{12}H_{14}O_{13}Cu.6H_2O$
Nickel pectate	$C_{12}H_{14}O_{12}Ni.5H_2O$
Nickel pectinate*	$C_{12}H_{14}O_{12}Ni.5H_2O$
Zinc pectate	$C_{12}H_{14}O_{12}Zn.4H_2O$
Zinc pectinate*	$C_{12}H_{14}O_{12}Zn.H_2O$

*for pectin and pectinates 7% methoxy was included in the calculations e.g. pectin is $C_{12.07}H_{16.14}O_{12}.4H_2O$

8.3 Spectroscopic Measurements

Infra red spectra were recorded with a Perkin Elmer 197 spectrophotometer in KBr phase. Electronic spectra were recorded with a Unicam SP700 spectrophotometer. Electron spin resonance spectra were obtained on a Decca X-3 spectrometer at about 9270.301 MHz, fitted with a proton resonance probe for field determination and using 100 KHz modulation. The ^{13}C solid nuclear magnetic resonance spectra were recorded with an FX-60 QS spectrometer with magic angle spinning.

Results

IR and Electronic Spectra

Infra red spectra of metal pectates and pectinates exhibit two strong bands in the range 1600-1635 cm^{-1} and 1400-1410cm^{-1}, assigned respectively to the $\nu_{as}(CO_2)$ and $\nu_{sym}(CO_2)$ modes of carboxylate groups,

Table X. Carboxylate stretching frequencies (cm^{-1}) in metal pectates and pectinates

Metal complex	State of the sample	$\nu_{as}(CO_2)$	$\nu_{sym}(CO_2)$	Δ
Pectin	air dried	1610,1730	1410	
Zinc pectate	air dried	1610s	1410s	200
Zinc pectinate	air dried	1610s	1410s	200
Cobalt pectate	air dried	1610s	1410s	200
Cobalt pectinate	air dried	1610s	1410s	200
Nickel pectate	air dried	1610s	1415s	195
Nickel pectinate	air dried	1610s	1415s	195
Copper pectate	air dried	1620s	1405s	215
Copper pectinate	air dried	1620s	1405s	215

as listed in Table X. In addition to this the ligand absorbs at 1730cm^{-1} due to presence of free carboxyl groups. The mode of coordination of carboxylate groups has often been deduced from the magnitude of Δ, the difference between $\nu_{as}(CO_2)$ and $\nu_{sym}(CO_2)$. Monodentate coordination will shift ν_{as} to higher and ν_{sym} to lower frequencies (Nakamoto, 1963). From the results listed in table X, it is reasonable to assign a bidentate structure to carboxylate groups in Zn^{2+}, Ni^{2+} and Co^{2+} complexes. Their frequency values agree with those reported for other M^{2+} carboxylates whose bidentate coordination mode is noted from crystal structures (Nakamoto, 1963, 1968). Instead, the higher Δ values observed for Cu^{2+} complexes suggest carboxylate groups less symmetrically bound to the ion or monodentate coordination through an oxygen atom only.

The electronic spectra of Co(II)-complexes (Fig.55,56) are typical

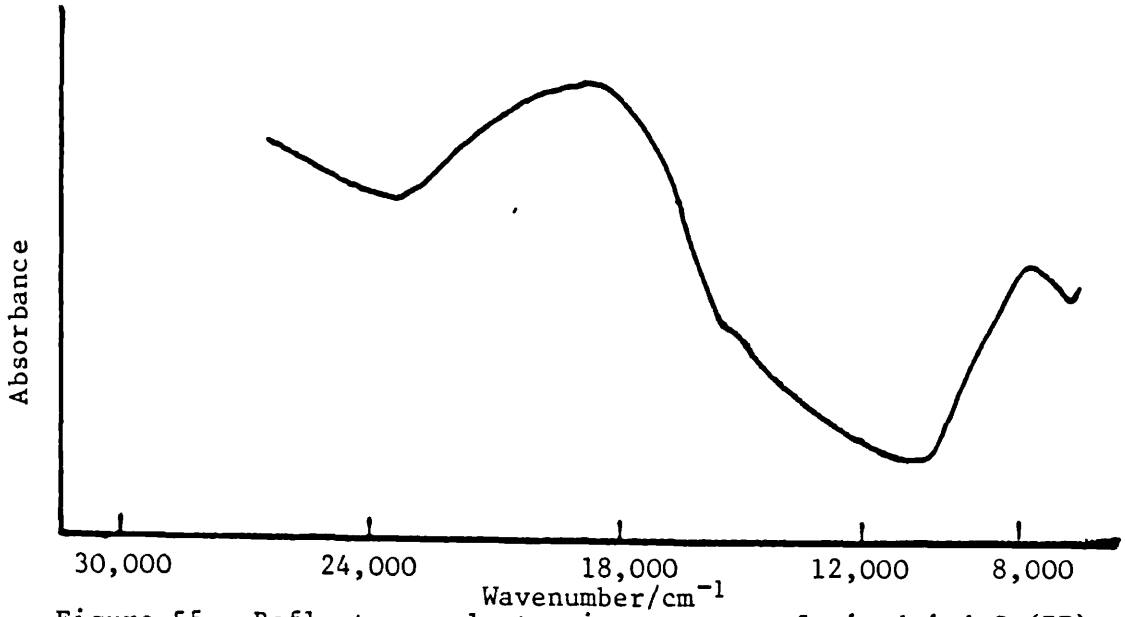


Figure 55. Reflectance electronic spectrum of air-dried Co(II) pectate

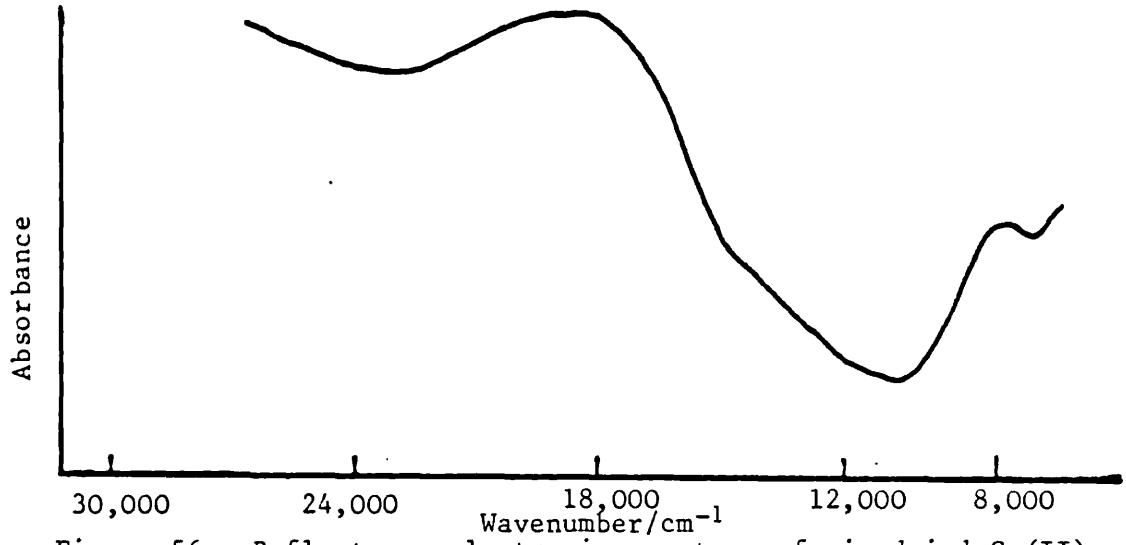


Figure 56. Reflectance electronic spectrum of air-dried Co(II) pectinate

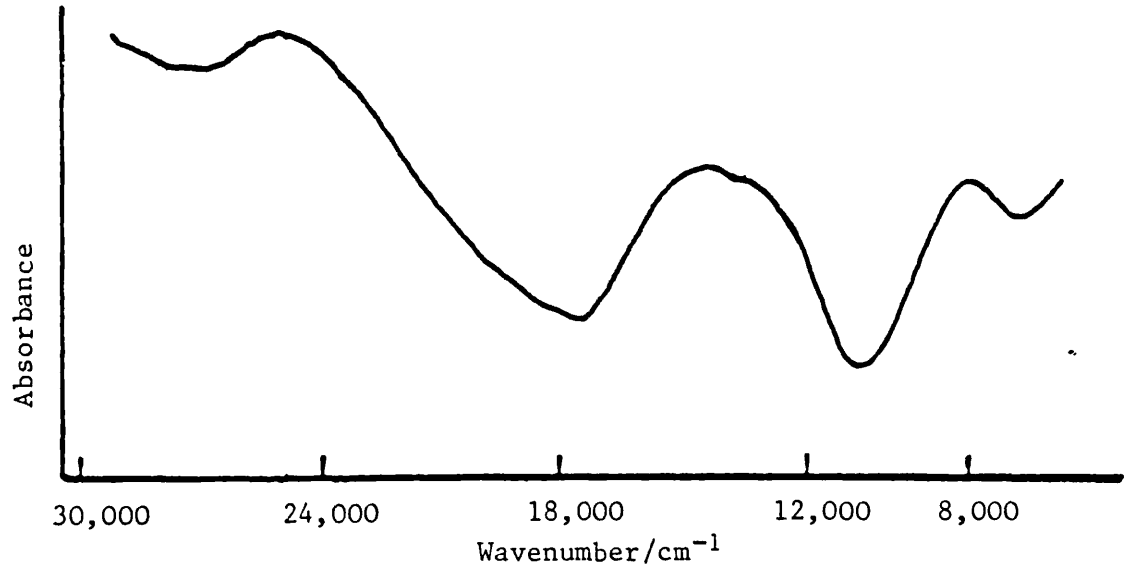


Figure 57. Reflectance electronic spectrum of air-dried Ni(II) pectate

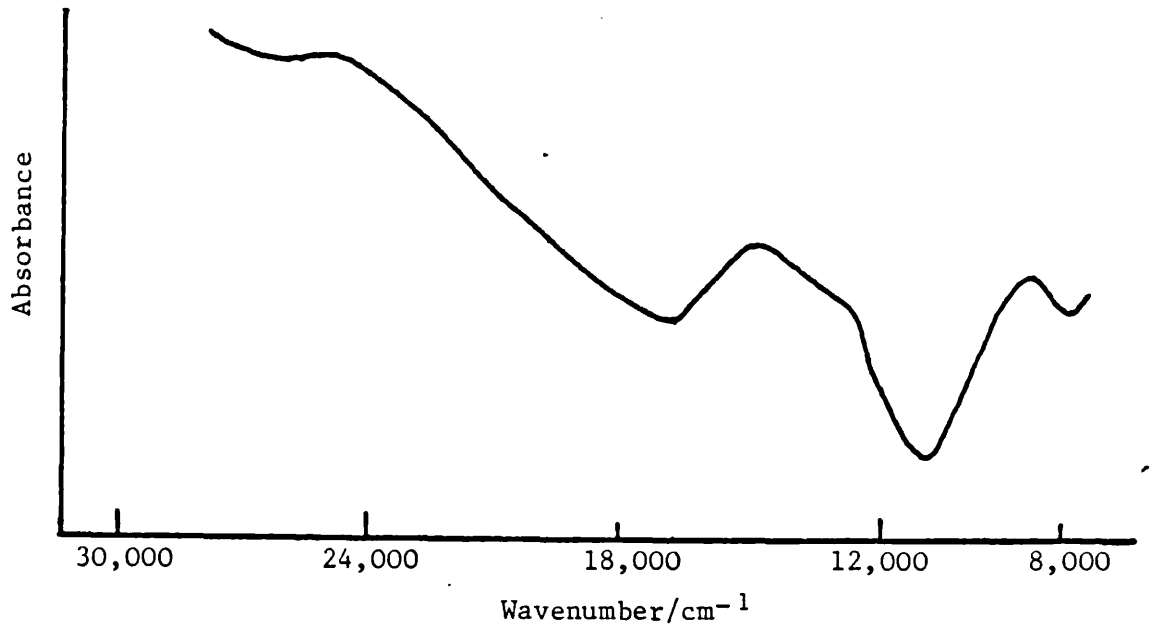


Figure 58. Reflectance electronic spectrum of air-dried Ni(II) pectinate

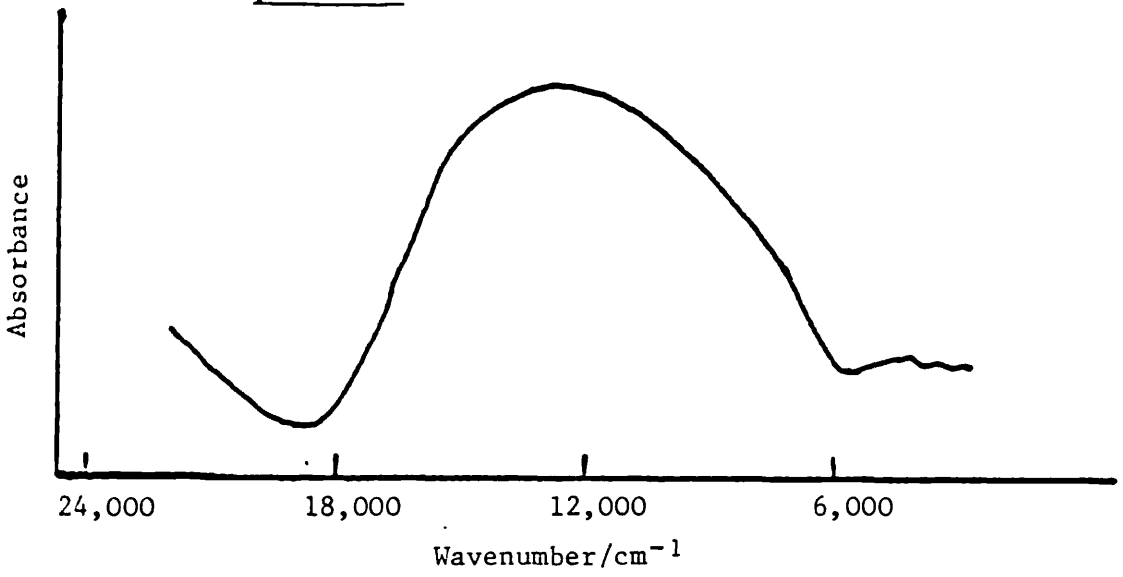


Figure 59. Reflectance electronic spectrum of air-dried Cu(II) pectate

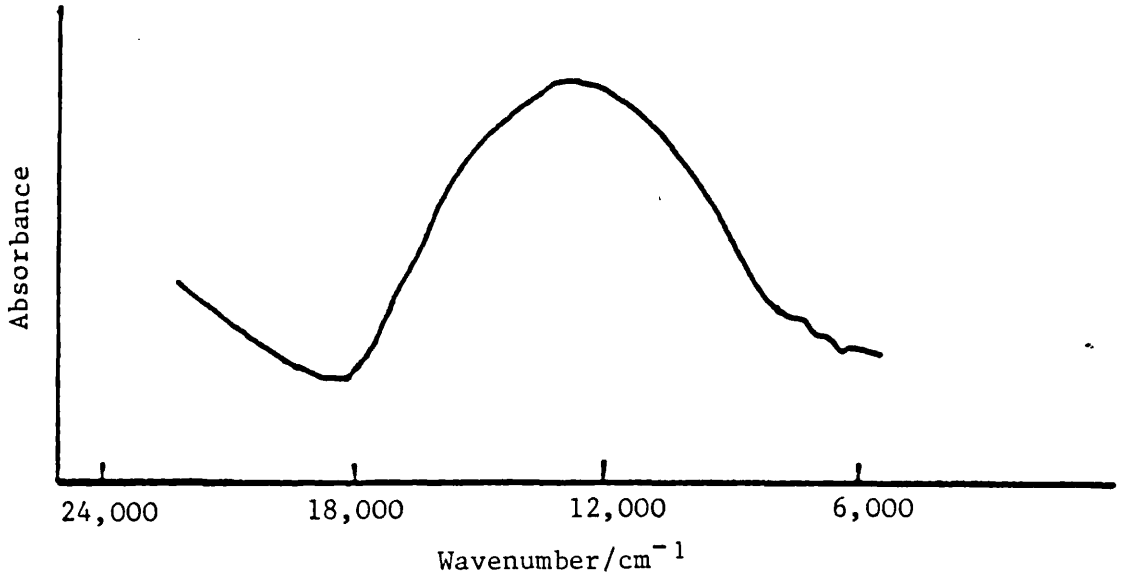


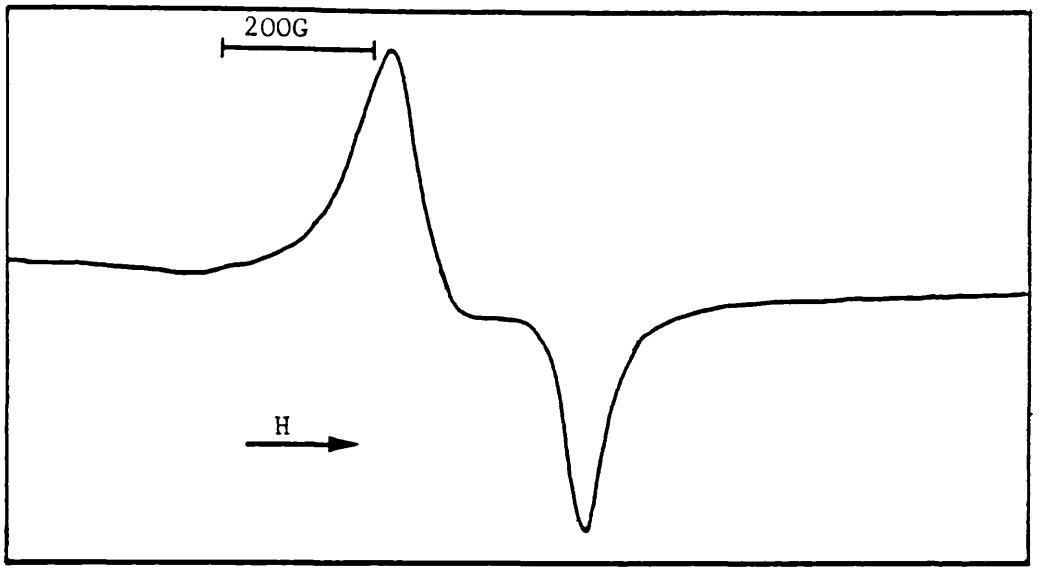
Figure 60. Reflectance electronic spectrum of air-dried Cu(II) pectinate

of a pseudo-octahedral configuration. Both the pectate and pectinate complexes show bands occurring at 8,000, 14,500 sh, and 18,500 cm^{-1} which may be assigned to the $^4T_{1g} \rightarrow {}^4T_{2g}$, $^4T_{1g} \rightarrow {}^4A_{2g}$ and $^4T_{1g} \rightarrow {}^4T_{1g}(P)$ transitions respectively. Similarly, Ni(II) complexes (Fig.57,58) exhibit absorption bands which indicate octahedral coordination, occurring at 8,000 cm^{-1} in pectate and 8,500 cm^{-1} in pectinate (ν_1: $^3A_{2g} \rightarrow {}^3T_{2g}$), a shoulder at 13,500 cm^{-1} in both pectate and pectinate ($^3A_{2g} \rightarrow {}^1E_g$), a band at 14,500 cm^{-1} in pectate and 15,000 cm^{-1} in pectinate (ν_2: $^3A_{2g} \rightarrow {}^3T_{1g}(F)$) and 25,000 cm^{-1} in both pectate and pectinate (ν_3: $^3A_{2g} \rightarrow {}^3T_{1g}(P)$). The electronic spectra of copper compounds (Fig. 59,60) are characterized by a broad structureless band showing a maximum around 12,500 cm^{-1}. The position and shape of the absorption suggest tetragonal coordination.

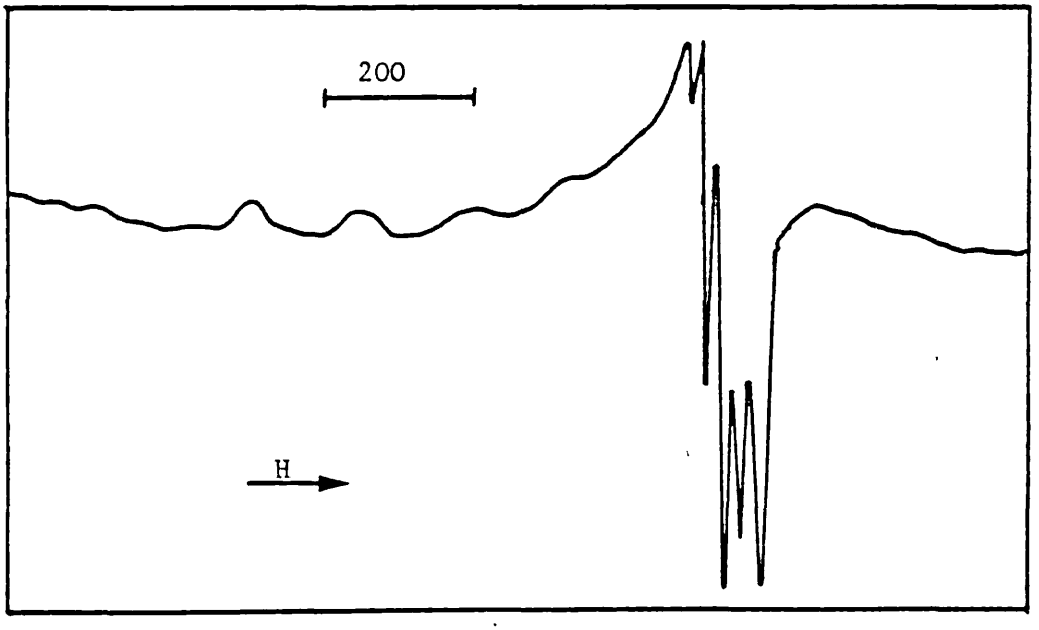
Electron Spin Resonance Measurements

The room temperature spectrum of copper galacturonate loaded with copper and washed free of excess ion is shown in Fig.61. In the air-dried samples, two absorptions at g_{11} = 2.263 and $g_{\perp}$ = 2.08 were observed. A frozen solution (10^{-3}M) in acetate buffer (pH=5) gave anisotropic axial spectra with spectral parameters (g_{11} = 2.354, $g_{\perp}$ = 2.063, A_{11} = 140G and $A_{\perp}$ = 20G) which are characteristic of a copper environment with a relatively strong axial distortion (Hathaway, 1970). On increasing the ion concentration a symmetrical line with $g_{av.}$ = 2.18 and δH = 180G appeared. These values are close to those reported for copper-polygalacturonate (Deiana *et al.* 1980) and was attributed to a weak exchange among closely spaced copper ions.

The ESR spectra of polycrystalline copper pectinate and copper pectate in different hydration states are shown in Fig.62,63. As for copper galacturonate, anisotropic spectra with coincident spectral parameters are observed in air-dried and frozen solution (in sodium hydroxide (0.1M)) complexes. In the frozen solution, however, the spectra

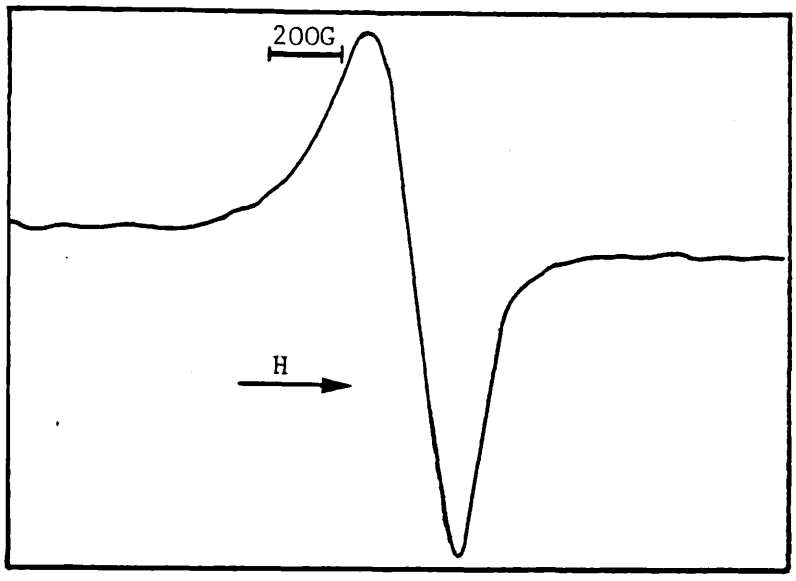


Room temperature ESR spectrum of copper galacturonate

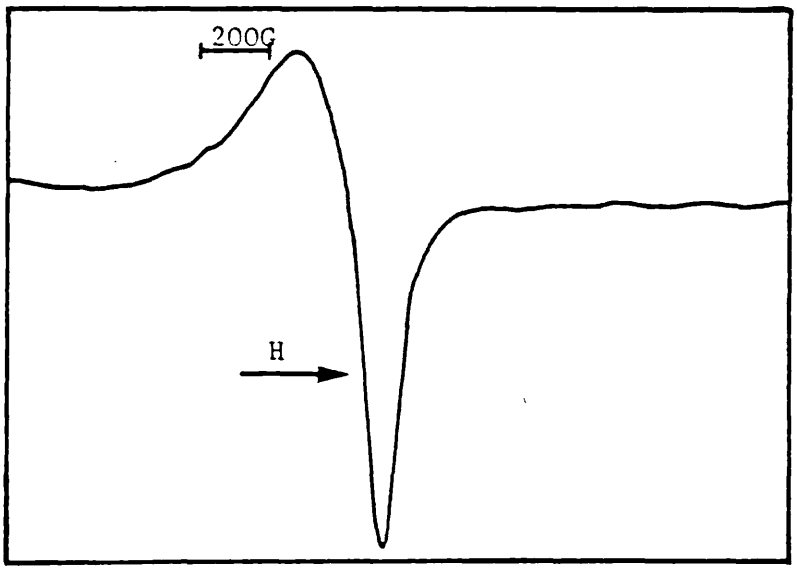


ESR spectrum of frozen solution of copper galacturonate

Figure 61

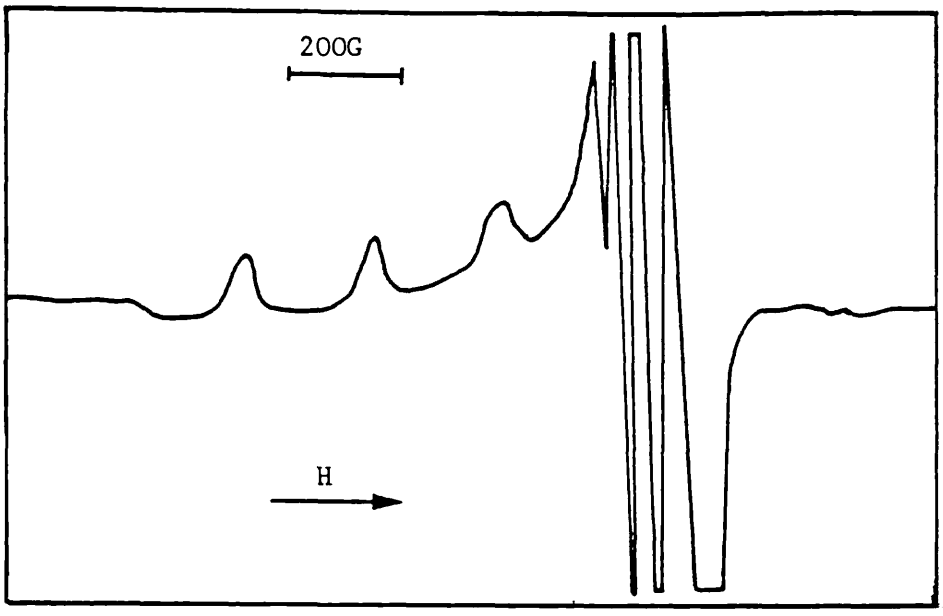


Room temperature ESR spectrum of copper pectate

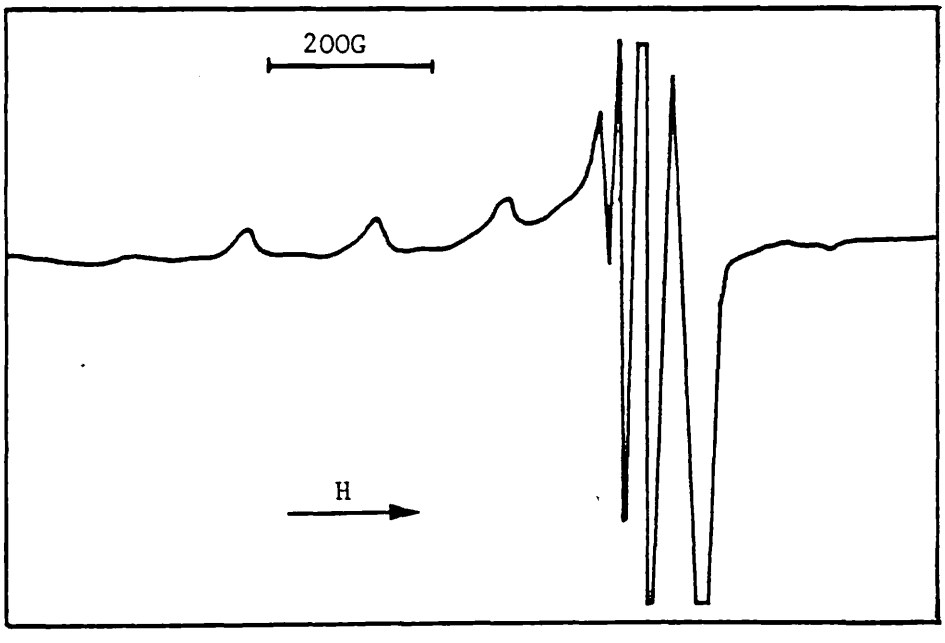


Room temperature ESR spectrum of copper pectinate

Figure 62



ESR spectrum of frozen solution of copper pectate



ESR spectrum of frozen solution of copper pectinate

Figure 63

Table XI E.S.R. parameters of copper complexes

Complex	State	g_{11} (±0.005)	$g_{\perp}$ (±0.005)	A_{11} (± 5 Gauss)	$A_{\perp}$ (± 5 Gauss)	Reference
Copper galacturonate	Solution	2.354	2.063	140	20	
Copper pectate	Air-dried	2.380	2.062	120		
Copper pectate	Solution in NaOH	2.252	2.062	185	30	
Copper pectinate	Air-dried	2.375	2.060	130		
Copper pectinate	Solution in NaOH	2.251	2.062	185	35	
Copper polygalacturonate	Air-dried	2.389	2.070	120		(Deiana *et al.* 1980)
Copper polygalacturonate	Hydrated	2.390	2.070	122	20	(Deiana *et al.* 1980)

are well resolved and the spectral parameters (table XI) are characteristic of a copper environment with a relatively strong axial distortion (Hathaway, 1970). The values were also close to those of copper complexes with water molecules coordinated in the metal plane and carboxylate groups in the axial position (Nyberg, 1971)(Dejehet *et al*, 1978).

ESR of dried powdered material of leaves of *Hybanthus floribundus* was attempted. The signals recorded were not well resolved. The spectra showed weak broad signal which may be due to nickel and which was overlapped by the strong signals due to manganese. Further work should be carried out at liquid helium temperature in order to resolve the nickel signals.

^{13}C Nuclear Magnetic Resonance Measurements

Various investigations (Angyal, 1969)(Jochims *et al*. 1967) have envinced the utility of proton magnetic resonance spectroscopy for establishing the configuration and conformation of carbohydrates in solution. Although the proton resonances of the sugars are often heavily overlapped, it has been possible to identify many of them with the aid of double-resonance techniques.

The binding of metal ions to active sites in uronic biopolymers play an important role in determining their physical and biological properties. The exploration of possible metal-ion binding-sites in sugar molecules have recently been intensified (Anthonsen *et al*. 1972, 1973)(Angyal, 1972, 1973) with the main aim of obtaining a better understanding of the rather unique ion-binding properties of polyuronides (Haug, 1970). Information on binding sites and complex-formation constants is thus of great importance, and we have applied ^{13}C nuclear magnetic resonance spectrometry to obtain such information. The study, however, was limited to the solid state, because of the insolubility

of the sugars and their derivatives. The assignments of resonances shown in table XII are tentative and are derived largely from correlation with ^{13}C nuclear magnetic resonance studies of carbohydrates (Douglas, 1970) (Perlin, 1969).

It is evident from Table XII that the resonances of C-6 remain relatively constant throughout the series of carbohydrates and complexes studied, and are always the highest field peaks in the spectra. Comparison of the position and intensity of C-6 resonances in pectin relative to the complexes, suggests the carboxy group being involved in the binding of metal ions in hexapyranoses. There is one peak at approximately 101 ppm throughout the series which is common to both ligands and complexes and seems most reasonably assigned to carbon 4 on the basis that this centre is relatively remote from the anomeric centre. The signals of methyl groups in pectin and derivatives absorb at substantially lower fields (53 ppm) and the identification of these resonances in each spectrum is therefore straightforward.

However, further assignment of resonances to the remaining peaks at this stage is not possible, as they all seem to absorb in the range 69 ppm to 84 ppm, and further studies should be attempted to identify C-1, C-2, C-3 and C-5 resonances.

8.4 Discussion

The reported data evidence that sorption of the examined ions by pectates and pectinates is accompanied by the formation of stable carboxylate complexes. Their insolubility in water makes reasonable the hypothesis that ions act as cross-linking agents in the polymer network (Jellinek, 1972). It has be suggested (Deiana et al. 1980) that in the air-dried state, Co(II) and Ni(II) ions satisfy the geometrical requirements for hexacoordination, being surrounded by bidentate carboxylate groups and two axial water molecules. On the contrary, in Cu(II) complexes two

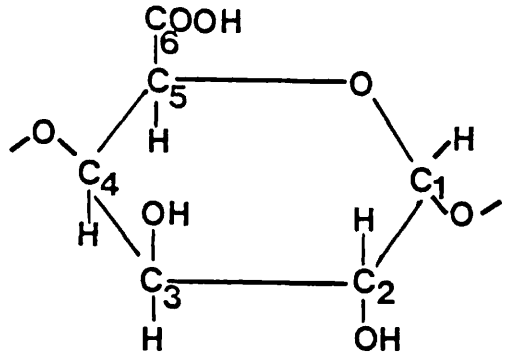


Table XII Tentative assignment of ^{13}C n.m.r. signals of pectin and derivatives, polygalacturonic acid and D-galacturonic acid (δ in ppm)

	Polygalac-turonic	D-Galac-turonic	Pectin	Nickel pectinate	Zinc pectinate	Aluminum pectinate
CO_2H	171.90	173.4	171.78	175.5	174.70	174.38
C_4	101.90	93.50	101.12	101.2	102.2	101.38
CH_3	-	-	54.37	53.9	53.60	54.11

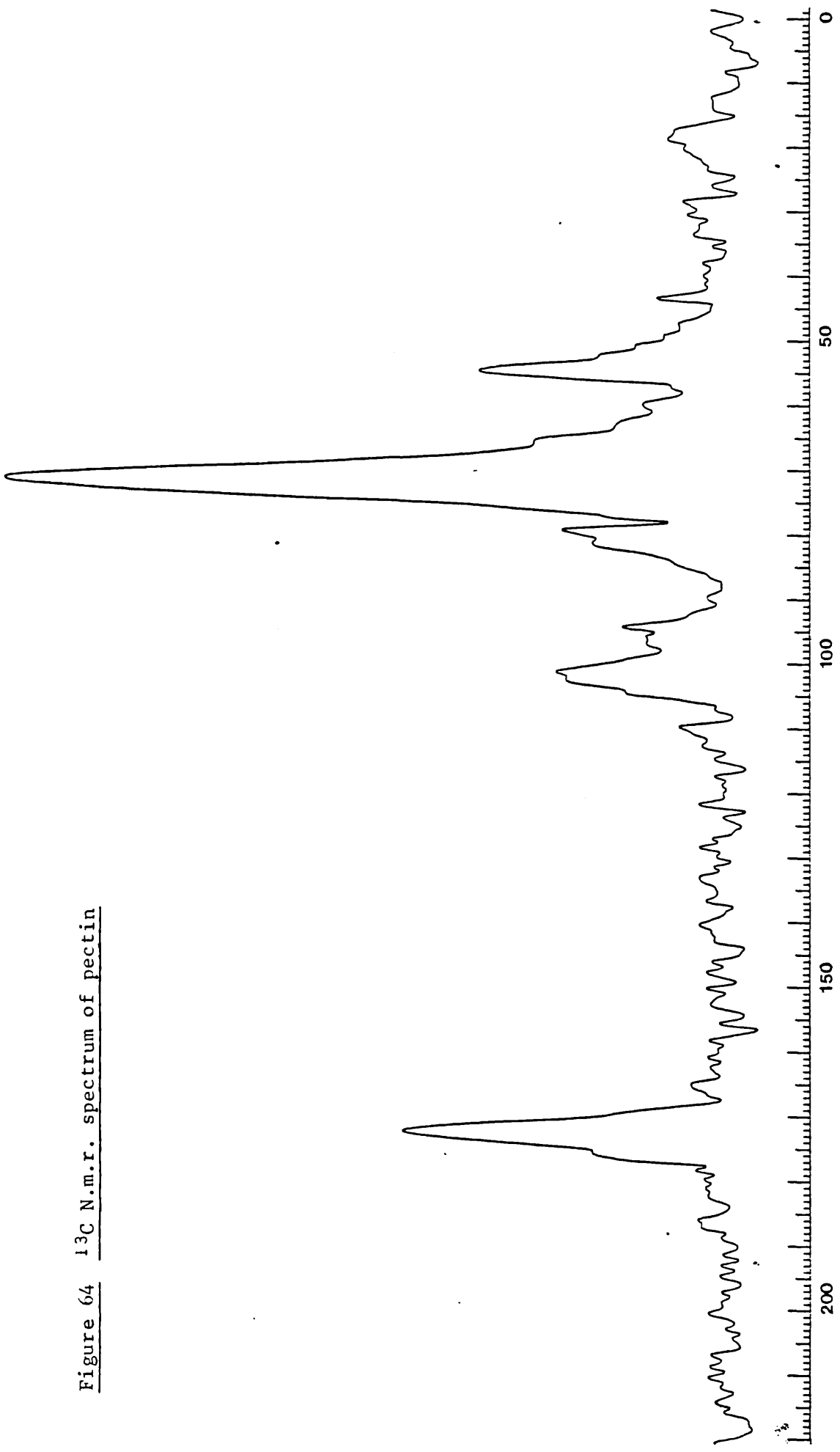


Figure 64 ^{13}C N.m.r. spectrum of pectin

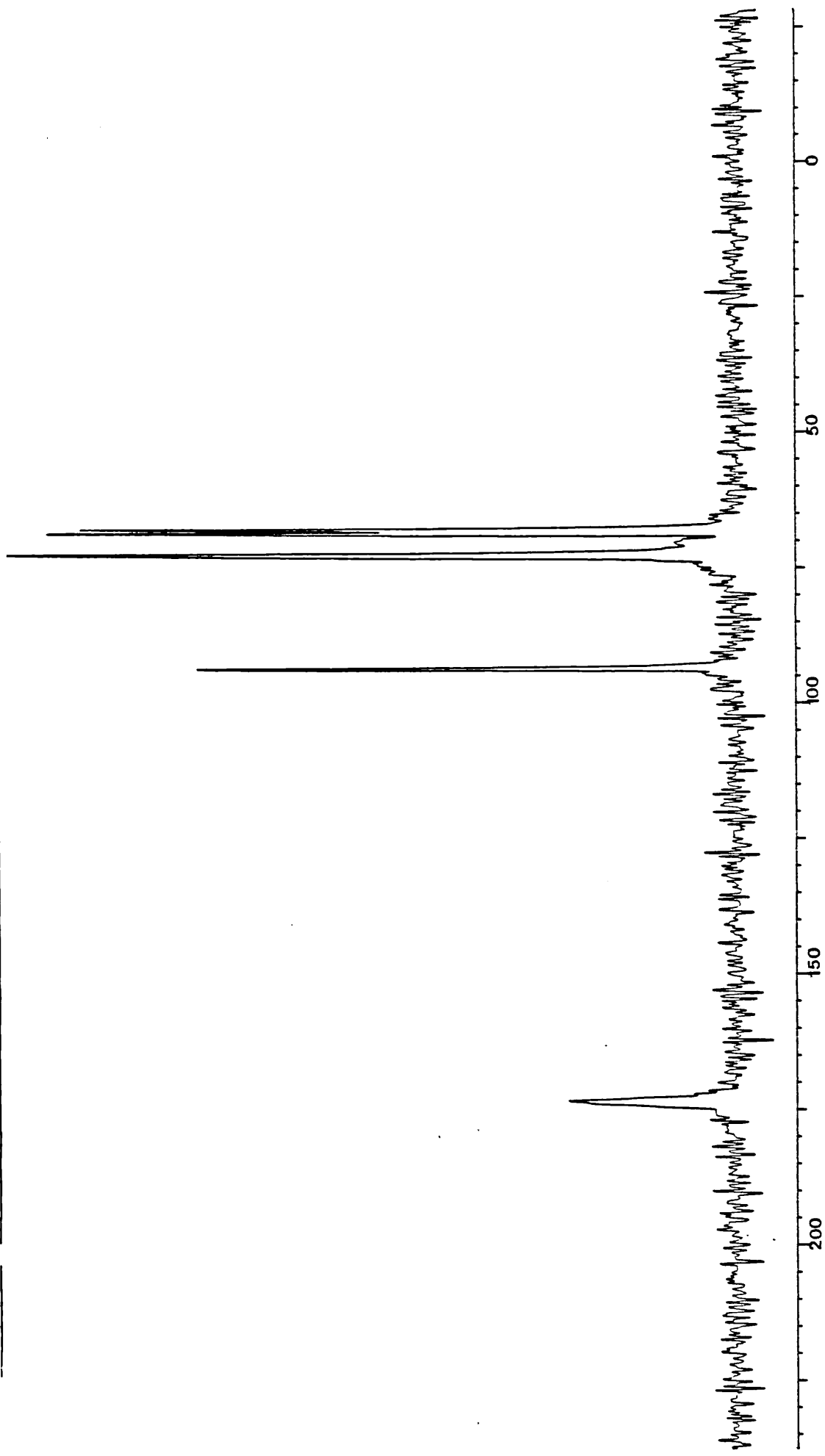


Figure 65 ^{13}C N.m.r. spectrum of D-galacturonic acid

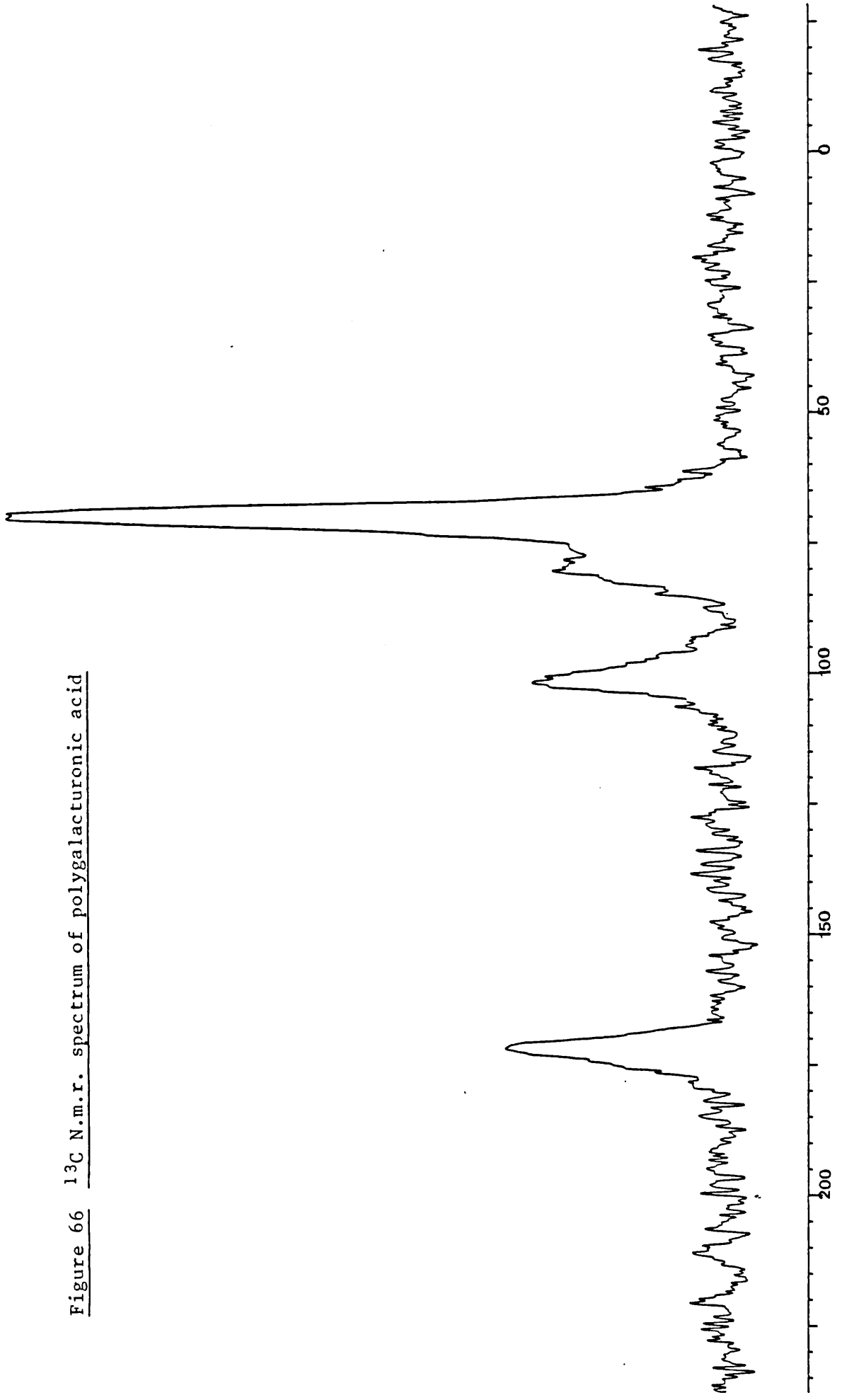


Figure 66 ^{13}C N.m.r. spectrum of polygalacturonic acid

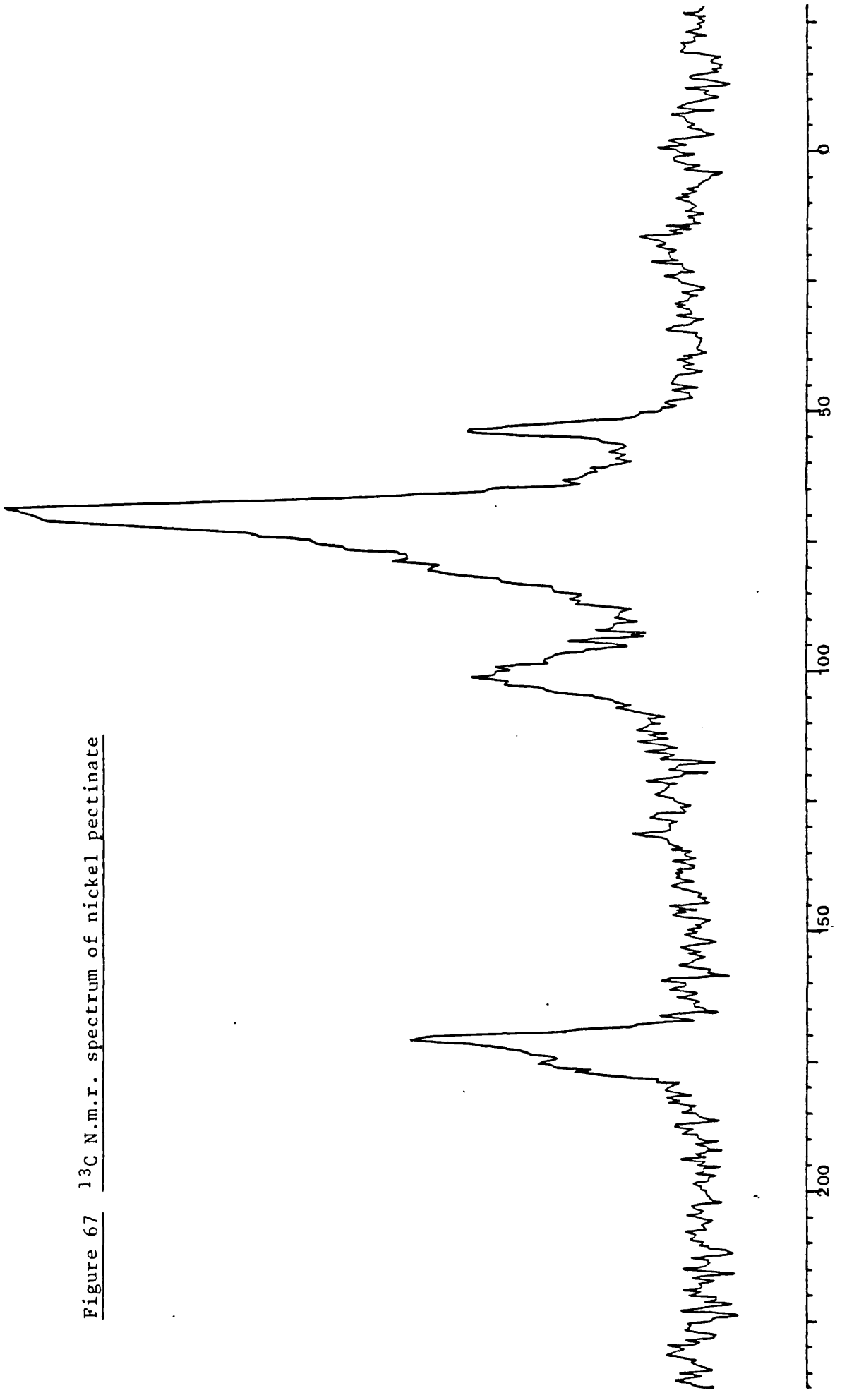


Figure 67 ^{13}C N.m.r. spectrum of nickel pectinate

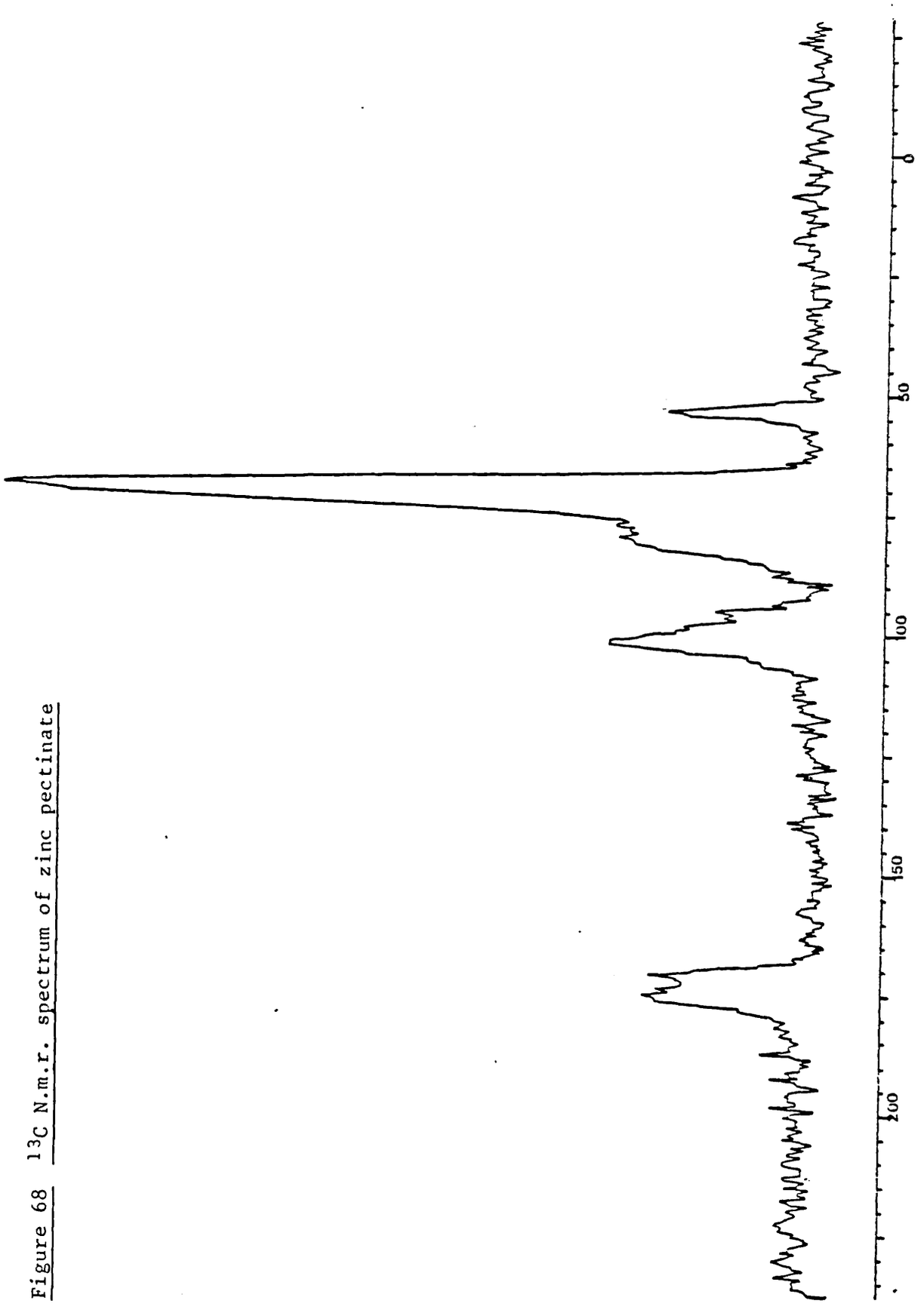


Figure 68 ^{13}C N.m.r. spectrum of zinc pectinate

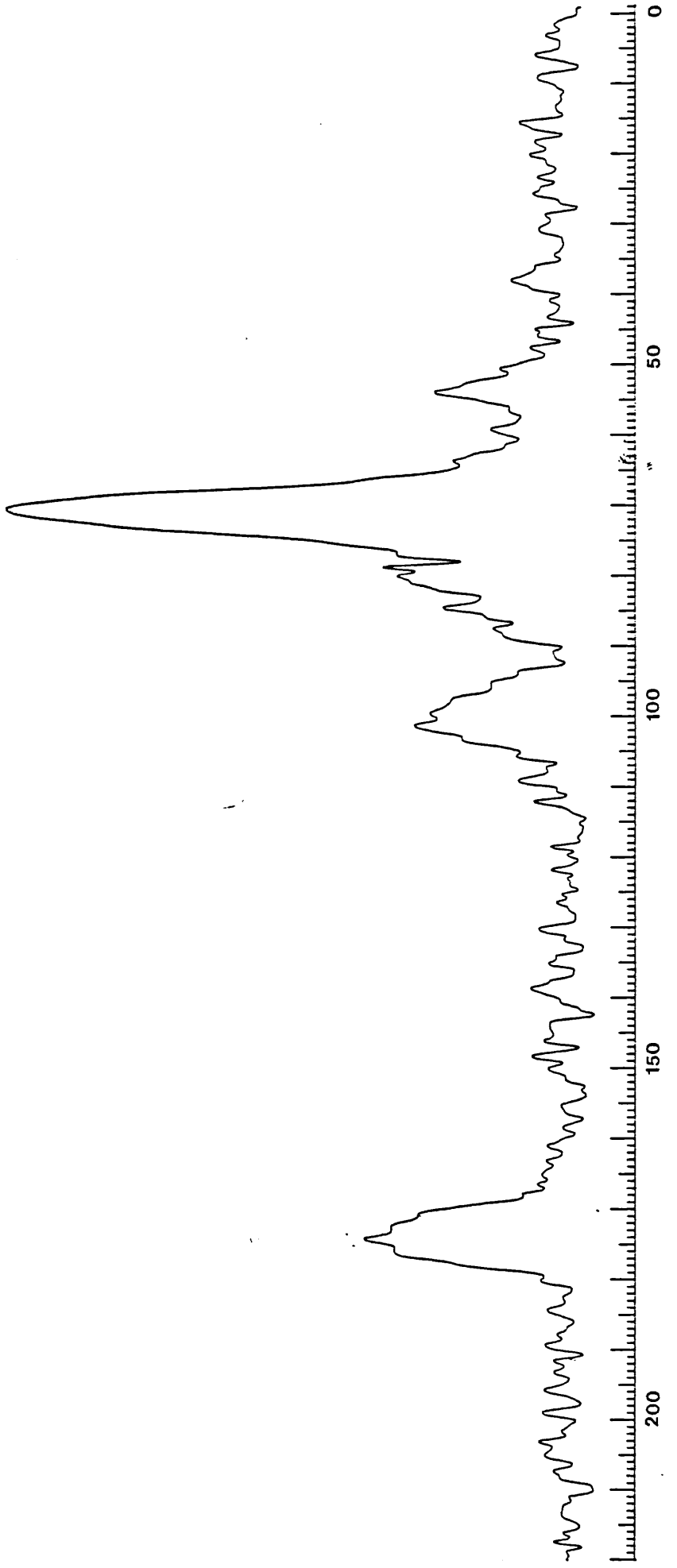
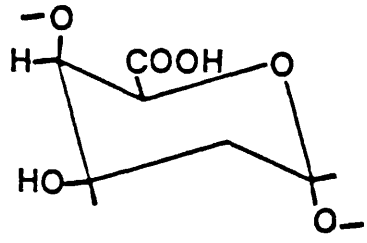


Figure 69 ^{13}C N.m.r. spectrum of aluminum pectinate

carboxylate groups act as unidentate ligands. The octahedral environment is completed by water molecules. Excluding direct intermolecular interactions, in the latter ion, which is not evidenced by spectral results and unlikely because of steric hindrances, a possible assumption is that water molecules are substituted by weaker interacting groups. A reasonable hypothesis is the binding of sugar ring - or glycosidic-oxygens. However, for Cu(II) complex ESR data might be in accord with tetragonally distorted octahedral geometry, which could be explained only by taking into account four sugar oxygens in the ion coordination sphere.

As noted in the introduction (section 8.1) the basic unit of which pectates are comprised is the D-galacturonic acid molecule (though rhamnose and galactose are also present). These units combine in β 1-4 linkage to give polygalacturonic acid chains.



D-galacturonic acid unit

The $-COO^-$ and -OH groups can donate electron pairs to metal cation and in most cases a sparingly soluble compound results if most metal sites are occupied (Jellinek and Sangal, 1972). The affinity of ions in solution for the polymer decrease in the order Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+} (Jellinek and Sangal, 1972). However this is merely a tendency and not a specificity. Thus pectates can form chelates with a number of divalent metal ions involving both carboxyl and hydroxyl groups.

Schweiger (1964) suggested that gelation of alginate with certain metal ions occurs through chelation involving both carboxyl and hydroxyl groups. He further suggested that in calcium alginates, calcium

with its primary valences connects to carboxy groups, probably on neighbouring units. Coordinate bondings are extending to two vicinal hydroxyl groups of a third unit (Fig. 70). This third unit may be in the same chain and thus keep the macromolecule in a coiled shape or it may be in another chain forming a huge molecule with a net structure. He concluded that the fact that gels are formed when complex-forming metal ions are added is evidence for the latter case and that it is very likely that both intra- and intermolecular complexes exist.

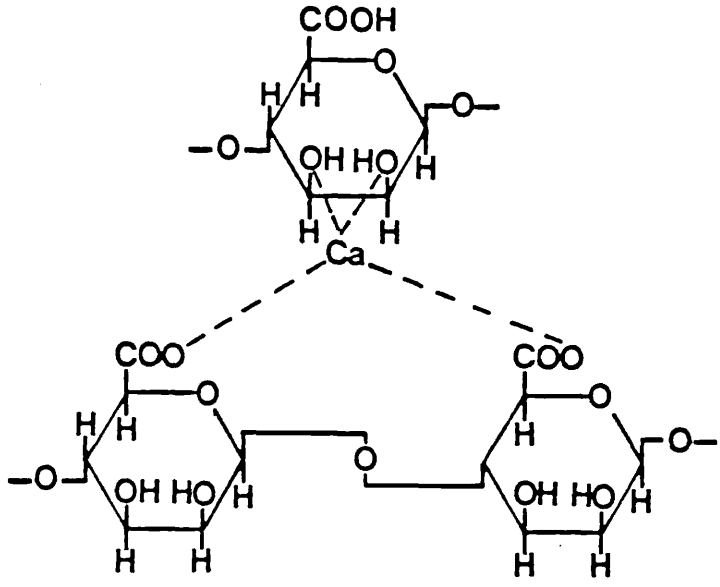


Figure 70

Schweiger (1966) suggested that the formation of such chelates is not limited to alginate only but also applies to pectate and suggested that such chelation may probably represent a common reaction of polyuronates or, generally, of polysaccharides containing uronic acid residues. Thus in pectates, chelation may involve two carboxy groups and one or two pairs of hydroxyl groups per metal ion. These chelates may be intermolecular (Fig. 71a) i.e. carboxyl and hydroxyl groups belong to different chains, or intramolecular (Fig. 71b), in which case the groups involved in chelation are present in a single residue, or, generally, in residues of the same chain.

Presumably the formation follows an equilibrium with an equilibrium constant characteristic for each metal. The ratios of

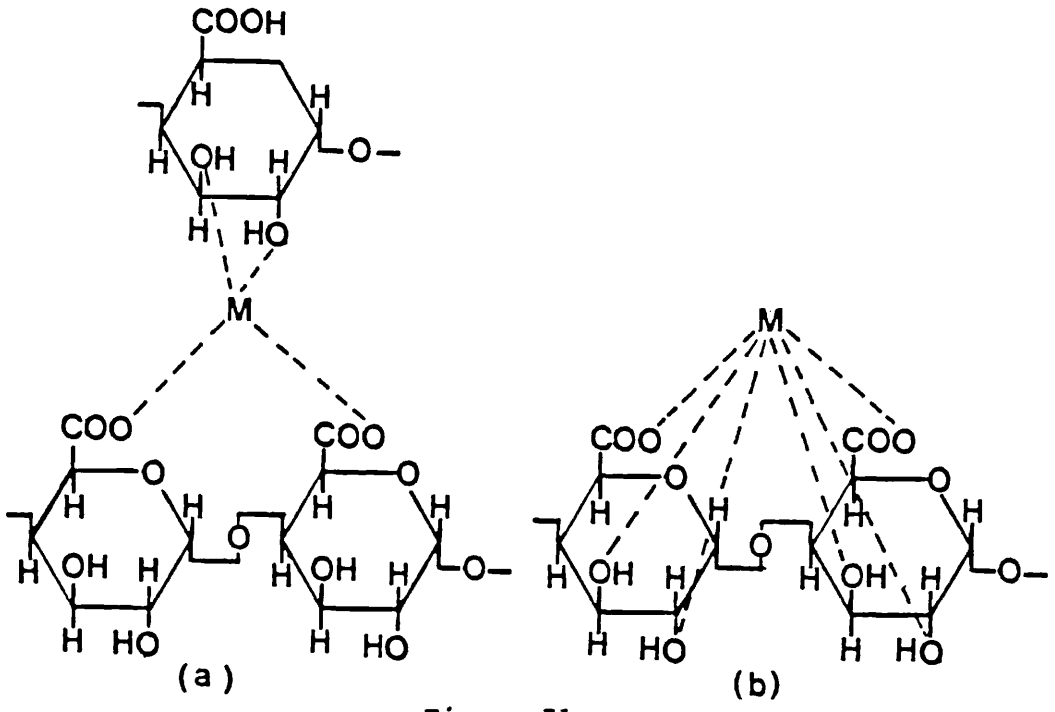


Figure 71

inter- to intramolecular forms apparently are in the following order: Cu^{2+}, Ca^{2+} > Mg^{2+} > Ni^{2+}, Sr^{2+} > Mn^{2+}, Co^{2+}, Ba^{2+} > Cd^{2+} > Zn^{2+}. For Zn-pectate the equilibrium apparently is highly in favour of intramolecular form. It is most probable that zinc is chelated in the manner with the pectin layer of cell walls in *Triodia pungens*. Work on two Australian species has shown that, although zinc is associated with cell wall pectic materials, these show a greater affinity for Cu^{2+} than for Zn^{2+} (Farago, 1977). They thus display the 'normal' affinity of polygalacturonic acids for metal ions, i.e. Cu^{2+} > Ca^{2+} Zn^{2+} > Ni^{2+} (Jellinek and Sangal, 1972). It appears (Farago, 1977) that although the zinc ions are stored in ion exchange sites in the cell wall carbohydrates, this may not constitute the specific tolerance mechanism. Zinc and nickel are closely related in terms of ionic size (zinc 0.69Å; nickel 0.68Å) and it was believed that specificity could be achieved through a control mechanism which was restricted to ions of the same size as zinc. However magnesium (0.65Å), lithium (0.68Å) and Cobalt (0.70Å) have ions of similar dimensions to those of zinc and nickel, and indeed cobalt is also like nickel in its chemical and physical properties, but none of these ions could substitute for zinc or nickel in the tolerance mechanism (Gregory, 1964).

However, for Cu(II) complexes our results suggest that in

hydrated system the exchange-sites of galacturonic acid, pectinates and pectates are probably accounted for only by carboxyl groups, which is in agreement with the conclusion drawn by Deiana *et al.* (1980). Further, our conclusions support those drawn by Khon (1968), who on the basis of theoretical consideration, suggested that in pectates calcium ions are bound to carboxyl groups by electrostatic forces, excluding the chelate intramolecular binding proposed by Schweiger (1964). Lakatos *et al.* (1977) also considered pectates as outer-sphere complexes. However this behaviour cannot be assumed as a general rule. According to the ion properties, the electrostatic attraction may be able to overcome the metal-H_2O bonds, affording the insertion of carboxylate groups in the inner hydration sphere of the cations. On this point of view, the strong affinity of Cu(II) for these systems (Ramamoorthy, 1977), in comparison with alkaline and alkaline-earth cations, can be attributed to the covalence in the metal bond with carboxylate groups.

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CHAPTER IX

General Discussion

The nutritional problems of plants growing in mining regions and in acid and saline soils have been well reviewed (Bollard and Butler, 1966) (Baumeister, 1967). In spite of the high concentrations of various metals in these soils many plant species are tolerant and survive even though others are poisoned and therefore excluded from these habitats. The mechanisms whereby heavy metal tolerance is achieved are largely unknown and in fact at the present moment there is little information available concerning the physiology of metal tolerant plants. In general, storage mechanisms appear to involve the cell wall carbohydrates, but the mechanisms of uptake transport and in particular, specificity are not yet understood. The studies presented in this thesis developed from a number of analyses performed on plants collected from zinc and nickel rich soils. *Hybanthus floribundus*, *Triodia pungens* and *Eriachne mucronata* proved to be obvious candidates for more detailed chemical analysis.

Triodia pungens and *Eriachne mucronata*, found growing on the same Australian zinc site, were found (Chapter III) to possess high concentrations of the metal in their aerial tissues, levels which would be considered toxic in most other plants. Studies have been made of the metal levels in the aerial parts of both *Triodia pungens* and *Eriachne mucronata* sampled over a range of soils (Nicolls *et al.*, 1965). In both species the relationship between zinc uptake and the zinc content of the soil is linear, even at very high soil levels. There thus appeared to be no restriction of zinc uptake. Chromatographic experiments show that the zinc in *Triodia pungens* is partially present as ionic zinc and partially coordinated to another ligand, which is not ninhydrin positive. Extraction of this latter substance followed

by hydrolysis, shows the presence of galacturonic acid. We conclude therefore that zinc is coordinated to pectin. For the aerial parts of *Eriachne mucronata*, the soluble zinc proved to be the Zn^{2+}(aq.) ion. Analyses of the nickel accumulating plant *Hybanthus floribundus* from a metalliferous area in Western Australia show very high nickel levels in the plant tissue with values tending to be highest in the leaves and higher in the stems than the roots. Levels of over 13,000 p.p.m. were found in the leaves of samples collected at Widgiemooltha. The highest concentration in the surface soil in this area is 8,800 p.p.m. More than half of the nickel in the green parts of the plant is water soluble, the rest being released by acid. In the twig material, considerably less nickel was water soluble, more than 80% of the nickel appearing in the acid fraction.

For soluble nickel compounds in *Hybanthus floribundus* chromatographic and electrophoretic examinations revealed that the nickel in the aqueous and alcoholic extracts existed almost entirely in the form of double-charged ions or nickel complexes because the spots coincided very nearly with a nickel standard. A whole range of chromatographic systems were attempted to find one capable of separating the complexes of nickel and it has been shown that soluble nickel in *Hybanthus floribundus* leaves is associated with pectic carbohydrates. The amino acid content of this species is reported and was found to be not identical with that from less mineralised area in Queensland (Kelly *et al.*, 1975). The difficulty of detecting nickel-amino acid complexes in biological materials has been demonstrated since unlike those of copper, the nickel complexes dissociate during paper chromatography and behave similarly during T.L.C. and electrophoresis. In contrast to *Hybanthus floribundus*, chromatographic studies show that both *Triodia pungens* and *Eriachne mucronata* contain high levels of proline in the tops: 27.7% and 7.9% respectively of the ninhydrin positive

material. The high proline content is probably not connected with zinc accumulation, but rather with copper-tolerance as in *Armeria maritima* (Farago, 1979,1981). The response of both *Eriachne mucronata* and *Triodia pungens* to copper are somewhat different. The response of *E. mucronata* to increasing copper in the soil is typically that of an excluder, whereas that for *Triodia pungens* could be interpreted as being similar to that with zinc i.e. the response of an indicator, but with a much lower gradient than that with zinc. Proline is one of the amino acids contained in all proteins, and plant tissues under various types of environmental stress have been shown to accumulate proline. These environmental conditions include water stress (Stewart, 1972) (Huang, 1979) (Tulley *et al.*, 1979), high salt conditions (Stewart, 1974), and temperature stress (Chu *et al.*, 1978). One further possibility is that high proline are a result of the collection and air-drying of the samples in the field. This seems unlikely since proline accumulation starts when 30% of the total tissue water is lost (Singh *et al.*, 1973). However, both *Hybanthus floribundus* and *Eriachne mucronata* were subject to the same stress and did not show very high proline levels. A question which needs to be resolved is whether or not heavy metal tolerance is also correlated with the capacity to accumulate proline.

The single most important tool for 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, which include a proteolytic enzymic digestion. It was devised to obtain maximum information about the chemical environment of the metal, whilst limiting the possiblity of more than one distinct class of compounds being extracted or precipitated by the same treatment. Extraction scheme A for stems and leaves material of *Hybanthus floribundus* revealed that 57% of the stems and

87% of the leaves nickel is associated with pectic, protopectic and similar polysaccharidic material. 23.66% of the metal in the stems was solubilized by the proteolytic enzyme pronase compared with 3.34% in the leaves. 24.9% in the stems and 5.24% of the metal in the leaves is removed with soluble and insoluble proteins or polypeptides. The twigs also contains nickel involved with pectic substances, but has substantially more nickel removed in the low molecular weight extractable fraction. Extraction scheme A for aerial parts materials of *Triodia pungens* and *Eriachne mucronata* reveals that 45.5% in *Triodia pungens* and 18.56% in *Eriachne mucronata* of the zinc is water soluble pectates. As much as 76.37% in *Triodia pungens* and 37.22% in *Eriachne mucronata* of the zinc extracted is associated with materials based on uronic acids.

Sectioning of the plant parts found most nickel present in *Hybanthus floribundus* in epidermal cell walls and the phloem. Since the pectic substances are found almost entirely in the cell wall, this provides supportive evidence that nickel is linked largely to cell wall pectates. Several other studies have implicated the cell wall pectates as the major deposition site for toxic metals (Turner and Gregory, 1967) (Peterson, 1969) (Turner and Marshall, 1971), and a mechanism of tolerance was suggested that higher plants generally keep metal ions away from active metabolic sites by chelation at the cell wall. The nature of the chelation process has been little investigated but it would need to be highly metal-specific to explain the specificity of tolerance. In *Crotalaria novae hollandiae* (a zinc accumulator) (Farago, 1977) zinc accumulates at the cell wall and at the phloem in association with carbohydrates. In the copper containing *Becium homblei* the metal has been found in association with amino acids (Reilly *et al.*, 1970). Copper is similarly associated with amino acids in *Armeria maritima* (Farago, 1979). Thus there is evidence that the mechanisms of

accumulation of copper and of zinc and nickel are different. For both *Crotalaria* and *Hybanthus floribundus*, the metal has been detected in the phloem in addition to the cell wall. It seems unlikely that nickel is travelling in the phloem in high concentrations. But it is more likely that the phloem is the repository for a significant fraction of the total nickel present. It seems likely that the phloem membrane restricts entry to nickel ions as it does to calcium ions. Thus large concentrations of the metal ions become immobile at the membrane; this mechanism perhaps becomes operational only when very large amounts of metal are accumulated, as in *Hybanthus floribundus*.

Phenolic compounds present in *Hybanthus floribundus* were examined and findings reported include the accumulation of a yellow pigment in large quantities by the plant. Analyses of the pigment by paper chromatography, mass spectrometry and UV disclosed the presence of a component of flavonoid-like character. The pigment was obtained in crystalline form and purity was tested with paper chromatography. ^{1}H n.m.r. and ^{13}C n.m.r. studies did not completely confirm the structure proposed, although they were not inconsistent with it. In conclusion, the information gleaned so far would seem to indicate that the yellow pigment is not a simple flavonoid. It is possible that it may be present together with some inorganic material i.e. as a salt, thus giving very dilute solutions which are not suitable for n.m.r. studies.

Finally, the interaction between pectin with Cu(II), Ni(II), Zn(II) and Co(II) has been studied with the aid of e.s.r., n.m.r., electronic and infrared spectroscopy. The visible spectra (diffuse reflectance, dry material) of the nickel and cobalt compounds showed band at 25,000, 15,000, (13,500 sh), 8,500 cm^{-1} and 18,500, (14,500 sh), 8,000 cm^{-1} respectively, indicating octahedral coordination. Copper pectate and pectinate showed a broad structureless band showing a maximum around 12,500 cm^{-1}. The position and shape of the absorption

suggest tetragonal coordination. It can be suggested that in the air-dried state, Co(II) and Ni(II) ions satisfy the geometrical requirements for hexacoordination, being surrounded by bidentate carboxylate groups and two axial water molecules. On the contrary, in Cu(II) complexes two carboxylate groups act as unidentate ligands. The changes observed in spectral parameter in different states suggest the substitution of water molecules by sugar oxygen atoms in order to preserve the stereogeometry of the ion.

Information on binding sites was further extended to ^{13}C n.m.r. spectrometry. The study, however, was limited to the solid state, because of the insolubility of the sugars and their derivatives. The assignments of resonances presented are tentative and are derived largely from correlation with ^{13}C n.m.r. studies of carbohydrates in the literature.

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APPENDIX 1

Experimental conditions for Atomic Absorption Analysis

Element	Lamp Current (mA)	Slit Width (nm)	Air/Acetylene Flow Rates $dm^3/min.$	Wavelength (nm)
Calcium	10	0.2	5.0/1.0	422.7
Cobalt	15	0.2	5.0/1.0	240.7
Copper	10	0.2	5.0/1.0	324.8
Iron	10	0.2	5.0/1.0	248.3
Magnesium	10	0.2	5.0/1.0	285.2
Nickel	15	0.2	5.0/1.0	232.0
Zinc	8	0.2	5.0/1.0	213.9

APPENDIX 2

Locating reagents employed in paper and thin-layer chromatography

Nickel

Dimethylglyoxime 1gm in 100cm^3 ethanol

Rubeanic acid 0.1% (w/v) in ethanol/water (60:40 by volume)

Zinc

Dithizone in chloroform 0.005% (m/v)

Amino acids

Ninhydrin 8% solution in butanol

Phenols

Ferric chloride-ferricyanide 0.05M solution of each

Carbohydrates

Aminobiphenyl-orthophosphoric acid solution, 0.3g

O-aminobiphenyl + 5 cm^3 H_3PO_3 in 95 cm^3 ethanol

p-anisidine-phthalic acid, 1.23g p-anisidine + 1.66g phthalic acid in 100 cm^3 methanol

p-anisidine hydrochloride (1g) + sodium hydrosulphite (0.1g) in 10 cm^3 methanol diluted to 100 cm^3 with n-butanol

Carboxylic acids

Bromocresol green 0.1% in ethanol. 1.0M NaOH solution added to chromatogram.

PUBLICATION

A study of the amino acid content of *Hybanthus floribundus*, a nickel accumulating plant and the difficulty of detecting nickel amino acid complexes by chromatographic methods. Farago, M.E., Mahmoud, I., and Clark, A.J. (1980), Inorg. Nucl. Chem. Letters, 16, 481.

INORG. NUCL. CHEM. LETTERS Vol.16, pp.481-484.
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THE AMINO ACID CONTENT OF *HYBANTHUS FLORIBUNDUS*, A NICKEL ACCUMULATING PLANT AND THE DIFFICULTY OF DETECTING NICKEL AMINO ACID COMPLEXES BY CHROMATOGRAPHIC METHODS

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ABSTRACT

The amino acid content of *Hybanthus floribundus* from a metalliferous area in Western Australia is reported. The amino acid profile is not identical with that from a less mineralised area in Queensland (1). The difficulty of detecting amino acid complexes in biological materials has been demonstrated since unlike those of copper, the nickel complexes dissociate under normal chromatographic conditions.

INTRODUCTION

The Australian shrub, *Hybanthus floribundus* (Lindl.) F. Muell., has been discussed in recent publications (1-6). *H. floribundus* was first found in the Eastern Goldfields area of Western Australia (7,8) and was later reported growing over a wider area of Western Australia where there are nickel toxic soils (3) and in Queensland (1). Brooks and co-workers have investigated the amino acid profiles of nickel accumulating *Hybanthus* species from New Caledonia and that of *H. floribundus* from Queensland (1).

EXPERIMENTAL

Aqueous extract. 2g dried milled plant material was refluxed with methanol (100 cm^3) for 30 min. The residue was filtered off, washed with warm methanol, dried in a stream of air, and finally extracted with 100 cm^3 water at 75-80° (water bath) for 1 h. The residue was filtered off, and the extract was freeze dried.

Paper chromatography. Ascending paper chromatography was carried out using a Shendon frame tank (polythene frame) and Whatman No.1 paper. The solvent volume was 200 cm^3 with prior equilibration.

The following solvents were used: S1, butanol-acetic acid-water (120:30:50 by volume); S2, phenol-water (500g: 125 cm^3); S3, acetone-water (30:20 by volume); S4, ethylacetate-pyridine-water (55:25:20 by volume); S5, *n*-propanol-pyridine-water (4:16:220 by volume); S6, *n*-butanol-pyridine-water (1:1:1 by volume); S7, *n*-butanol-ethanol-water (2:2:1 by volume); S8, phenol-ethanol-ammonia (0.88d) (75:10:5 by volume).

Chromatography of aqueous extracts of *Hybanthus floribundus*. Samples of the freeze dried extracts were dissolved in a minimum of water. Two dimensional chromatography was applied using solvent S1 followed by solvent S2 for 6 and 6.5 h respectively. Marker amino acids were run at the same time for comparison. Fig.1 shows the amino acids present (in addition a yellow 'pigment' was always found, this is at present under investigation). Preliminary assignments of the amino acids

were as follows: 1, leucine; 2, isoleucine; 3, valine; 4, proline; (5, pigment); 6, alanine; 7, glutamic; 8, hydroxyproline?; 9, glycine; 10, serine; 11, aspartic; 12, cysteine. Treatment of the paper with dithiooxamide or dimethylglyoxime resulted in a streak not far from the origin and another spot which corresponded to aspartic acid.

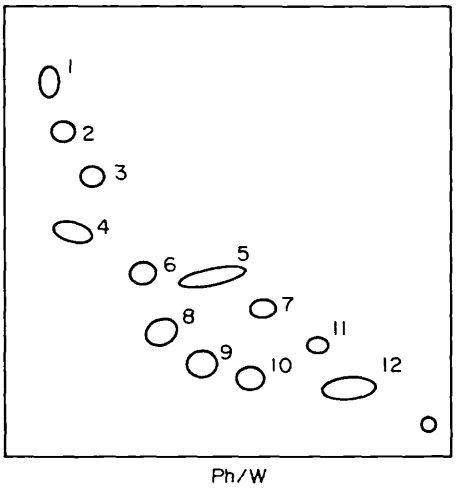

Fig.
Two dimensional paper chromatography of aqueous extract of *H. floribundus*

Chromatography of nickel amino acid complexes. Equimolar (0.01M) solutions of 'Analar' $NiCl_2.6H_2O$ and amino acid each in 10% aqueous *iso*propanol were mixed (1:2 by volume). The amino acids were: aspartic, glutamic, serine, glycine, threonine, alanine, valine, leucine, isoleucine, histidine, argenine, phenylalanine, tryosine, tryptophan, proline, hydroxyproline methinionine. Solid amino acid complexes were prepared and analysed by standard methods. In all cases results obtained using solid complexes were identical to those from the mixed solutions. In solvent S1, all complexes, except that of histidine which was partially dissociated, dissociated showing amino acid spots corresponding to the free amino acids. Similarly the R_f value for nickel was that for ionic Ni^{2+}. In S2 only complexes of aspartic acid and histidine (R_f values 24 and 98) did not dissociate. Adjustment of the pH of S2 to 7 with ammonia did not improve the situation. The results for S4 were similar, again only the complexes of aspartic acid and histidine did not dissociate. The argenine complex showed partial dissociation. S4 showed partial dissociation for complexes of aspartic acid, valine and histidine.

Solvent S5, which has been used in the chromatography of iron complexes (9), and solvents S6 S7 and S8 gave similar effects. Amino acids tend to run fast, with little differentiation of R_f values. Most complexes dissociated, showing long streaks when treated with the nickel reagent

Amino acid analysis of *Hybanthus floribundus* extract. The samples for amino acid analysis were prepared from the water extract by Sevag's method (10), and were run on an LKB 4101 Amino Acid Analyser with a single column system. The amino acids present are shown in Table 1. No cysteine or hydroxyproline were found to be present.

TABLE 1

Amino acid analysis of *Hybanthus floribundus* expressed as percentages of total ninhydrin material, excluding ammonia

Aspartic, 9.13; Serine + amide, 33.28; Glutamic, 6.90; Proline, 1.54; Glycine, 7.14; Alanine, 6.24; Valine, 5.59; Methionine, 0.27; Isoleucine, 6.92; Leucine, 4.74; Tyrosine, 1.95; Phenylalanine, 1.56; γ-amino-*n*-butyric, 6.59; Histidine, 3.10; Lysine, 3.27; Argenine, 1.76.

DISCUSSION

Although preliminary paper chromatographic results indicated the presence of cysteine and hydroxyproline, the amino acid analysis showed conclusively that these acids were absent from the extract. This result is in agreement with that of Brooks *et al.* (1) who found that they were absent from *H. floribundus* from Queensland. In other respects our results differ from those of Brooks *et al.* Our sample contains serine, (which probably corresponds to their first unknown), proline and isoleucine. The last two amino acids were found in other *Hybanthus* species but not in *H. floribundus* from Queensland. We have shown that large amounts of proline are present in the roots of copper tolerant *Armeria maritima* (11), but not in the roots of non-tolerant plants (12). There appears to be no such correlation with *Hybanthus* (Table 2).

TABLE 2

Presence of proline and isoleucine compared with nickel content of *Hybanthus*[a]

	isoleucine	proline	% Ni
H. caledonicus A	+	+	0.88
H. caledonicus B	+	-	0.17
H. austrocaledonius via *linearfolia*	+	+	1.03
H. austrocaledonicus	+	-	1.46
H. floribundus Queensland	-	-	0.006
H. floribundus W.A.	+	+	1.2

[a] Results from ref.(1) except for last entry, this work.

Amino acids have been postulated to play a role in the transport of copper through membranes in human serum (13). In plants high levels of organic solutes, including proline, have been found in the tissues of sodium chloride-tolerant plants (14-16). It has been suggested that the accumulation of these compounds provides a mechanism for the equalisation of the water potentials of the cytoplasm and the vacuole, where the sodium chloride is preferentially accumulated (16,17). The function of high concentrations of amino acids in metallophytes is not yet fully understood.

It seems unlikely that there is a nickel complex of aspartic acid present, since this complex dissociates in solvent S1. Work on the chromatographic identification of amino acid complexes of copper (18) and nickel (19) has been described. There are great difficulties involved with those of nickel, since these mostly dissociate during paper chromatography and behave similarly during TLC and electrophoresis. The nickel complexes are under further investigation.

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