A Study of the effects of Cadmium on Beans (Phaseolus Vulgaris, cv. Canadian Wonder), its interactions with Zinc and its effect on Chlorophyll.

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H. BENABID

DEDICATION

TO MY PARENTS, TO MARC, NADIR

AND ALL MY FRIENDS

CONTENTS

Chapter 2

Part One

Abreviation for names and units

CEC	Cation Exchange Capacity
µg	Microgram
µg/g	Microgram per gram
D.W	Dry weight
Chl	Chlorophyll
Pheo	Pheophytin
µs	Microsecond
ppm	Part per million
ppb	Part per billion
Rem sol	Remaining solution
t	Tonne
mmole	millimole
Fig	Figure
mg/l	Milligram per litre
TLC	Thin layer chromatography
SD	standard deviation
AA	Atomic absorption
UV	Ultra violet
Phy	Phytol
Bchl	Bacteriochlorophyll
cpm	Counts per minute
KeV	Kilo electron volt
Nutr sol	Nutrient solution
F.W	Fresh weight

µmole	Micromole
NR	Not required
R	Required
LT	Low toxicity
ND	Not determined
µCi	Microcurie

Summary

The thesis concentrates on the environmental implications of Cd. The aspect being dealt with concerns the effect of cadmium on plants and particularly its effect on chlorophyll content. The thesis also deals with the relationship of Cd with pH and other trace metals, particularly zinc.

The first chapter is an introduction to the subject. It briefly reviews the history of studies on trace metals, their relationship and their effects on soils, animals and plants. It also includes information about factors such as pH, organic matter, interactions between elements etc.. affecting the behaviour of trace metals. Chapter I contains a review on cadmium, its behaviour, its phytotoxicity and its presence in biological systems. It also reviews some previous works and suggestions and it ends by suggesting a frame work for this thesis with a brief description of each part.

Chapter 2 is divided into two parts. Part One deals with Cd as it is affected by pH which plays an important role in accounting for Cd behaviour and uptake by plants. Besides an introduction on Cd, this chapter contains a description of the experiments and the techniques used for the analysis. It also contains detailed data and the corresponding graphics.

Investigations carried out in this chapter suggest that pH is undoubtedly an important factor interfering with the transport, translocation and uptake of Cd by plants. Part Two of this chapter is aimed at studying the transport and distribution of Cd in a plant. It is also aimed at demonstrating the preferential localization of Cd by using radiolabelled Cd-109. The analysis was carried out using the solid scintillation technique. Information was obtained suggesting that Cd was preferably taken up by roots than by the other parts of the plant. It also indicates that leaves are less tolerant to high levels of Cd than roots and stems.

Chapter 3 lays emphasis on the interaction of cadmium with other trace metals and most particularly with zinc because of their chemical similarities. The following levels of Cd and Zn are used for the investigation. Cd(μg) : 30 to 180 and Zn(μg): 2000 to 10,000. The interactions of Cd with other trace metals were also reviewed. In this chapter, some conclusions are drawn concerning the mutual effect between Cd and Zn. It also shows that the addition of Zn has a positive result in minimizing the toxic effect of Cd as is expressed in the delay of plant response to Cd compared to the findings in Chapter 2. A chemical approach is suggested to explain the nature of the interaction between Cd and Zn. Two possible types of interaction are more likely to occur: competition for sites at the root level and a direct substitution of Zn by Cd in zinc enzymes.

The chemical approach seems to be valid because of the chemical similarities between the two elements. Symptoms of chlorosis or loss in chlorophyll were shown as a result of Cd effect on beans.

Chapter 4 is devoted to the investigation of this matter aiming at explaining the causes of chlorophyll losses by studying the effect of Cd on chlorophyll formation and content. In Vitro and In Vivo experiments were carried out using atomic absorption and ultra violet spectroscopy. Some conclusions were drawn consequently such as: Cd suppresses chlorophyll content and also decreases Mg content. It is also suggested that Cd may disturb the formation of chlorophyll by interfering with Fe which is important for its synthesis. In this chapter some suggestions are put forward concerning the possible displacement of Mg by Cd.

Chapter 5 carries out an assessment of all the points and suggestions encompassed in this thesis and considers the extent of the practicality of the results. It also assesses the prospects for future research which could be to be carried out in this area.

CHAPTER 1

1. Introduction

1.1 Trace metals in plants and animals.

It has been known for several decades that quantities of certain elements exert a positive or negative influence on plants and animals. These elements were given the name of trace element because they occur in small quantities in organic life. Many of these elements are essential because no organic life can develop or survive without their participation. Trace metals are of great importance to biological function because they are capable of enhancing enzymatic activities by acting as catalysts. They can also be of great danger if they are present in excess or deficient quantities. In addition to the important role played by some trace elements, some analytical techniques such as: polarography, atomic absorption spectroscopy, and neutron activation analysis were developed, thus contributing to an increase in the interest of studying the behaviour of these elements in soils, plants and animals. Through this work, an attempt will be made to answer some questions concerning the behaviour and effects of Cd which is considred as one of the very toxic trace metals.

Below is a list of selected elements considered to be important for soil, plant and animal system. The list also provide information about their role in biological systems. (Aubert, et al 1977; Underwood, 1977; Huber, 1980; and Ike, 1980)

B: Is often found in soil solutions as boric acid [H_3BO_3]. It plays a role in the regulation of carbohydrate metabolism. Boron was also found to stimulate germination in grass and its deficiency affects both leaves and stems.

Cd: Mainly found associated with zinc in zinc ores, it can be discharged from steel industry, coal burning and use of fertilizers. Cadmium has no known function in the three systems mentioned above. Instead it is known to be very toxic and can compete with other divalent metals especially zinc with which it has chemical similarities.

Co: Is an essential constituent of natural compounds such as B12. It is believed that cobalt plays a role in nitrogen fixation and it is also used in fertilizers to provide soil with certain amount of cobalt to be transfered to animals through plants.

Cu: Is found in primary rocks such as: chalco-pyrite [$CuFeS_2$] and is usually released by weathering. In addition to its essential role in photosynthetic electron transport in plants, copper is involved in many metalloenzymes structures including cytochrome oxidase , plastocyanin, ascorbic acid oxidase and phenolase.

Fe: Is present in substantial quantities in soil as for instance limonite [Fe_2O_3. 3 H_2O]. It is easily absorbed by plants when it is in a ferrous form Fe^{2+} and hardly available as Fe^{3+}. Iron is an important

constituent of the cytochromes and ferredoxins and is involved in chlorophyll formation. Chlorosis is the major symptom of Fe deficiency mostly observed in well-aerated soil where the available Fe^{2+} is transformed by oxidation to Fe^{3+}

Mg: Is present in most types of soil and its absorption as a cation occurs mainly in soil solutions. Magnesium is an essential constituent of chlorophyll and also plays a role of activator of many enzymes related to phosphate transfer.

Mn: Occurs in insoluble oxides in soils and is mostly present as Mn2+ considered to be its most available form. Mn is required for plant growth and it has a role of a cofactor in some enzymes such as: kinase and IAA oxidase.

Mo: Often occurs in soil solutions as molybdates[$HMoO_4^-$ and MoO_4^{2-}] and it is easily taken up as Mo2+. Molybenum is known to be essential for nitrogen fixation and nitrate assimilation. It is also believed that Mo functions as a component of metalloenzymes and is involved in the induction of nitrate reductase.

Zn: Is found in ferro-magnesium minerals such as: magnetite and biotite and is easy decomposed to Zn^{2+}. Zn is important for plant growth and it is required as cofactor in many enzymes. (see full list in chapter III). It is known to affect the activity of the RNA in the cell and also the activity of the cytochrome.

Among a large number of nutrients suggested by Bowen(1979), just some of them are listed in the table below because, apart from Cd which has not been shown to be essential to plants, they are all important for plant growth.

Table 1.1.1 Concentrations of some elements(mg/dm^{-3}) in nutrient solution affecting the growth of seed plants. Bowen(1979)

Element	Deficiency	Normal Growth	Toxic Level	Ref
B	<0.1	0.02_1	1_5	C & H
Cd	_	<0.05	0.2_9	Page et al
Co	0.0006	0.001_0.01	>0.1_3	C & H
Cu	<0.01	0.01_0.1	0.5_8	C & H
Fe	<0.5	0.5_5.0	10_200	C & L
Mg	<5	12_50	_	H
Mn(II)	0.0025_0.02	0.1_1.0	1_100	H
Mo	< 0.01	0.01_0.5	0.5_0.2	C & H
Zn	<0.0006	0.002_0.2	60_400	H

C: Chapman(1966); H: Hewitt(1966); L; Loneragan(1968);

The range of values shown in the table indicates that deficiency or toxicity depends mainly on the plant species and plant tolerance to the element and also depend on pH and the interelations between nutrients. The concentrations of the nutrients are not specified because of insufficient information concerning plant species and instead, optimum concentrations are reported.

More information concerning the elements quoted above were obtained from a study carried out by MacNicol and Beckett (1984) on different plant species. The results shown in table 1.2.2 indicate that the effects of nutrients differs from one plant to another. The effect is also subject to changes in the conditions of the experiment, pH, temperature, and time.

Table 1.1.2 From: MacNicol and Beckett. 1984

Metal	Plant	Cult	Age	pH	Part	Crit	C:L (mg/kgD.W)
Cd	Barley	soln	5 LS	_	Leaf	10	15
Cd	Beans	soln	5 W	5.0	Leaf	10	5
Cd	Cabbage	soln	5 W	5.0	Leaf	10	200
Co	Barley	Soln	5LS	_	Leaf	10	6
Co	Beans	Soln	_	_	Leaf	25	4-40
Co	Cabbage	Soln	8W	_	Leaf	10	10
Cu	beans	soln	_	_	Leaf	10	30
Cu	lettuce	soln	6 W	_	Top	10	5-1
Cu	Cabbage	Soln	8W	_	Leaf	10	25
Mn	beans	soln	Mat	5.4	Leaf	10	100
Mn	barley	soln	Mat	5.5	Top	10	120-300
Mn	Cabbage	Soln	8W	_	Leaf	10	200
Zn	beans	soln	_	_	Leaf	10	130
Zn	barley	soln	4 LS	_	Leaf	10	290
Zn	Cabbage	Soln	8W	_	Leaf	10	10

B, Fe, Mg and Mo were not determined.

Cult = Culture; Crit = Criterion of toxicity(% depression of yield);

C.L = Upper critical level; LS = Leaf stage; Mat = Maturity; W = Week;

Table 1.1.3: Trace Metal requirement for plants and domestic animals: IU(1981).

Metal	Plant (ppm)	Domestic animals (ppm)
Co	0.1-0.67	0.25
Cu	5-20	60
Fe	25-500	80
Mn	20-500	20
Zn	25-150	50

1.2. Trace Metals In Biological Systems

Great progress was made during the last decade in the studying of interactions between trace metals, amino-acids, proteins, enzymes and other molecules in the biological system. The capacity of trace metals in combining with organic molecules was confirmed by Passow et al (1961). They concluded that trace metals react with ligands present in all proteins and exercise an inhibitory effect on enzymes. Most trace metals are capable of forming complexes with ligands containing sulphur, nitrogen or oxygen which play a role of electron donors. The following ligands can be expected to be present in any living cell: -OH, -COOH, $-PO_3H_2$, -SH, $-NH_2$.

Klotz and Klotz(1959) listed some trace metals and classified them according to their solubility product for various inorganic sulfides: $Hg^{2+} > Ag^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Ca^{2+} > Mg^{2+}$. One of the major roles played by most trace metals in their interference with the action of enzymes is their inhibitory effect (Klotz 1954). The mechanism of metal interference with enzymes was well reported by Passow et al(1961).

Table 1.2.1: Some enzymes and biological functions of the elements quoted above: Compiled from Underwood 1975 and Mengel & Kirby 1978,

Metal	Enzyme	Function
Mn	Arginase	Urea formation
	Haemoglobin	O_2 transport
Fe	Ferredoxin	Photosynthesis
	Cytochromes	Electron transfer
Co	Cobamide coenzyme	N_2 fixation and N-metabolism
Cu	Plastocyamin	Photosynthesis
Zn	Carbonic anhydrase	CO_2 formation and regulation of acidity
	Carboxypeptidase	Protein metabolism

1.3 Trace metals in soil.

1.3.1 Introduction

The source of trace metals in soils are the rocks, which by undergoing some treatment such as: biological, chemical,and physical are transformed into parent material then to soil with water, air, organic matter and inorganic materials as the main constituents. Many plants and animals rely on soil as their major source of trace metals. The presence of trace metals in soils may be defined as that of those metals present in very small amounts and are measured in parts per million (ppm). The concentration of trace metals in soils is related to rocks from which they are derived, as reported by Hodgson(1963) in table 1.3.1

Table 1.3.1 Micronutrients in soils and rocks (in ppm). (Hodgson, 1963).

Element	Earth Crust	Basic Rocks	Acid Rocks	Sedimentary Rocks	Soils
B	10	10	15	12	10
Cu	70	140	30	57	20
Co	40	45	5	23	8
Fe	50,000	86,000	27,000	33,000	38,000
Mn	1,000	2,000	600	670	850
Mo	2.3	1.4	1.9	2.0	2.0
Zn	80	130	60	80	58

1.3.2 Distribution and forms of trace metals in soils.

The distribution of trace metals in soils has been shown to be closely related to the composition of parent material. Soil clay has been a subject of interest to relate trace metals content of soil to its clay content (Hodgson, 1963). Hodgson investigated this matter and reported that trace metals can be bound in soil by being associated with soil surfaces, with organic or inorganic material, by precipitating with other components to form new phases and also by incoporation in biological systems and their residues, and also by occupying sites in soil minerals. In that respect, Hodgson suggested two major forms in which trace metals can be found:

1. Surface adsorption of trace metals

Hodgson reported that the soil chemistry of the remaining micronutrient cations appears to be regulated by reactions with minerals and organic surfaces. Colombic forces are involved in attracting metals, with the exceptions of copper, cobalt and zinc which need additional forces of attraction for bringing about their interactions with organic and clay mineral surfaces.

2. Reaction with organic matter

The role of organic matter in the reaction of micronutrients has been examined and studied by many workers. The high capacity of organic soils to fix micronutrients confirmed the significance of the

important role that organic matter can play in the soil.

Hodgson (1963) suggested four ways which can be used to assess the contribution of organic matter to the chemistry of micronutrients in soils.

a. The association of organic matter with the distribution and availability of micronutrients.

b. The effect of organic matter removal on the activity of soils and the behaviour of micronutrients.

c. Characterization of organic matter and its reaction with sites.

d. Direct attempts to determine the amount of an element present in the organic form.

Stevenson and Ardakani (1972) and Schnitzer and Khan (1972) also reported that the reactions of trace metals with soil organic matter involve ion-exchange chelation and complex formation. Their suggestion is supported by the functional groups of complexes and chelates, such as: - COOH, phenolic-OH, -NH2 and C=0. According to Loneragan(1975), the order of bonding of divalent metal ions varies with organic matter in different soils. Copper (Cu^{2+}) is generally the most strongly bound as was reported by Leeper (1972) (see section on organic matter). The binding of Zn^{2+} and Mn^{2+} is usually weaker. Here as well, pH plays a significant role in affecting the solubilities of the formed metal-organic complexes.

With increasing pH, the stabilities of those metal-organic complexes increases to the breaking point of the complexes. As an example, Schnitzer and Skinner (1963) found that equimolar complexes of fulvic acid with Al^{3+} ions broke up at pH8 and at pH9 with Fe^{3+}. They also reported on the ability of soil organic matter to compete strongly with oxides for several metals. Rossel and Babcock (1968), found that the complexation of a mixture of humic acid and fulvic acid with manganese from Mn^{3+} and Mn^{4+} oxides occluded Mn^{2+} hydroxides. Such reactions may be very important in keeping these metals in soluble forms in alkaline soils.

1.3.3 . Trace metals in soil solution.

Soil solution, as defined by Somerville (1980), is the aqueous liquid phase of soil particles and other soluble materials. It can be considered the most important source of trace metals and other nutrients for plants and animals. Soil solution plays a role of medium transporting trace metals or other vital nutrients to plants. Loneragan (1975) compiled a list showing the concentrations in part per billion (ppb) of some important metals in soil solutions as shown in table 1.3.2.

Table 1.3.2 (Loneragan, 1975)

Element	Concentration (ppb)
Mn	1.1-3,740
Fe	2.2-280
Cu	0.6-38
Co	0.4-16
Zn	1.9-196

Some other workers Hodgson et al (1965) and Hodgson(1966), Geering and Hodgson(1969) also reported the concentration and degree of complexing of the elements quoted above (except iron) as shown in table 1.3.3.

Table 1.3.3 : Hodgson, (1965 and 1966) and Geering and Hodgson(1969).

Element	Total element in soil sol in mmoles	Degree of complexing (%)
Co	<0.007-0.2	8-50
Cu	<0.01-0.6	89-99.8
Mn	<0.02-68	84-99
Zn	<0.03-3	28-99

The data shown above have been obtained as a result of an experiment carried out on two soils in two different areas, NewYork and Colorado. The elements listed above were displaced from the surface horizons of these soils.

The following table is a list of a selected number of elements suggested by Allaway(1968) indicating levels of these elements in soil, plants and animals. It also indicates the required , the deficient and the toxic level to the plant and animal diet.

Table 1.3.4 Concentrations and amounts of selected trace metals in various phases of the environment(Allaway, 1968).

Element	Conc in soil (ppm)	Conc in plant (ppm)	Critical level in animal diet (ppm)
B	20-100	5-30	NR
Cd	0.01-7	0.2-0.8(NR)	NR
Cu	2-100	R:2-4	R: 1-10
		T:>20	LT
Mg	ND	ND	ND
Mn	100-4000	R:15-100	R:10-40
Mo	0.2-5	1-100	R: < 0.1
Co	1-40	0.05-0.5	R: 0.07
			by ruminant
Fe	ND	ND	ND
Zn	10-300	R: 8-15	R: 10-40

1.4 Factors affecting the availability of trace metals in soil to plants.

Many factors were shown to be affecting the availability of trace metals and their distribution and transport to plants. Many of the investigators came up with different conclusions and their approach on the degree and the way some factors such as pH and organic matter affect the availability of trace metals in soils. Those differences depend on the type of crop, type of soil and also on the type of trace metals and their forms in the soil.

1.4.1 pH

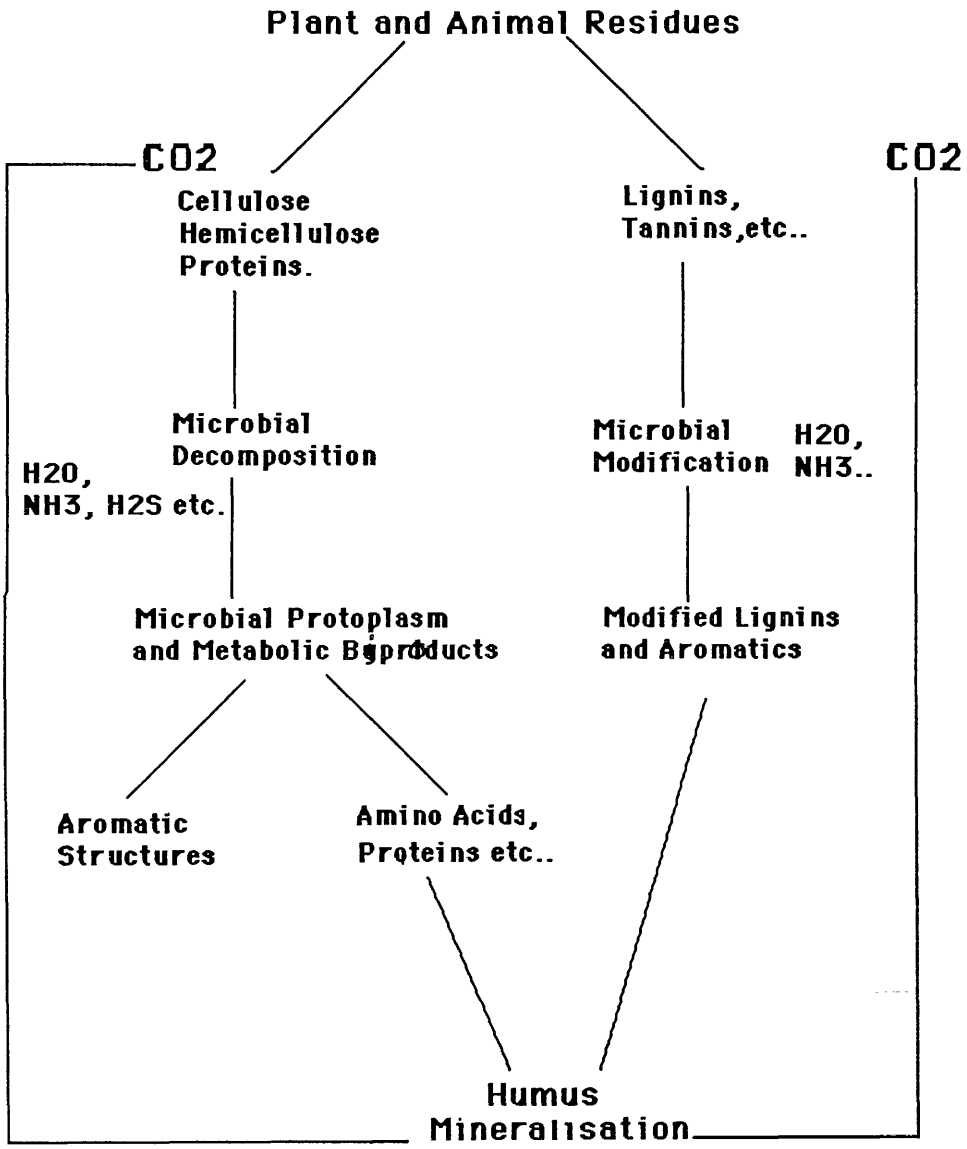
The importance of pH to the availability of trace metals was illustrated by many investigators. McHargue(1923) noticed that manganese added without lime was toxic to plant whereas when added after liming, the same amount of manganese increased yields. Delas , (1960) reported that increasing pH reduced copper toxicity. It was also found that zinc uptake from applied fertilizer was affected by changes in pH (Wear, 1956). Lindsay (1979) indicated that the solubilities of zinc and copper depend strongly on pH and decrease two fold for each unit of increase in pH. Contrary to Delas(1960), Leyden and Toth (1960) observed that a rise in pH increased the uptake of native soil zinc by plants while the absorption of zinc from fertilizers decreased. Manganese, iron and cobalt were also found to decrease in solubilities with increase in pH (Shuman,1975 and Loganathan et al 1977).

These are some metals known to be susceptible of forming soluble complexes at different pH values: pH>7: Mo^{6+}, Mo^{5+}, Se^{6+}, Cr^{6+}, and at wide range of pH: B, I, Li and Rb, and Co, Fe^{2+} Mn^{2+}, Cu^{2+}, Zn^{2+} and Co^{2+} soluble at pH<7 , (Vingradov, 1959). Thus, pH is considered as a major factor affecting the solubilities and availability of trace metals in soil to plants and is involved in many changes in soil properties caused by changes in the redox potential and in pH itself.

1.4.2. Organic Matter

Organic matter in a soil consists partly of living organisms and dead plants and animal residues (Diagram 1.3.2). It is considered to be the most chemically active part of the soil, (Hodgson,1963). Organic matter is a source of cation exchange activity (CEC), serves as a soil pH buffer and plays the role of a carbon reservoir. Organic matter contributes to plant growth through its effects on the chemical, biological and physical properties of soils. It also affects the availability of many micronutrient cations. For instance, inorganic precipitation in soils of high pH greatly reduces the solubility and availability of many micronutrients. This can be improved by adding organic substances such as: manure and sewage sludge to correct the deficiency of some trace metals and in particular Fe and Zn. Organic matter can also reduce the availability of toxic metals such as Cd and Hg by combining with them as well as with other micronutrients present at high concentrations. The importance of soil organic matter was recognized through its important role played in keeping trace metals in a soluble form in soil solution.

Diagram: 1.3.2
Organic matter decomposition and formation of humic acid substances.(From: F.E. Bear ed. Chem of the soil, 1964)



Therefore organic matter has been a subject of investigation undertaken by many workers. Shuman (1975) reported that in sandy soils low in organic matter, added Zn may be expected to be readily available to plants due to the inability of the soil to absorb it in large amounts, whereas in soils high in clay (usually high in organic matter), large amounts of added zinc can be absorbed by soils without being toxic to plants. This is due to the great absorptive capacity and bonding energy of the soil. Hodgson (1963) greatly contributed in examining and studying trace metals and reported that the presence of organic matter may promote the availability of certain elements presumably by supplying soluble complexing agents that interfere with their fixation. According to Hodgson (1963), the role and effect of organic matter in soil can be resumed into the production of complexing agents, the decrease in the oxidation potential and the stimulation in microbial activity that results in incorporation of manganese in biological tissue. Adding to that, the observations made by Leeper(1972) who reported that certain trace metals have a tendancy to combine with certain chelating group and become fixed. Below are some metals classified in decreasing order of their chelating tendency :

$$Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > \ldots\ldots > Mn^{2+} > Zn^{2+}$$

1.4.3. Redox Potential

The redox potential can be measured in soil, marine, geochemical and biological systems because electrons are essential reactants to organic, inorganic and biochemical reactions. The redox potential affects the oxidation state of many elements amongst other, H, C, Mn, S, Fe, ...etc, Bohn(1971).

The redox potential equation is given by:

$$Eh = E_0 + \frac{RT}{nF} \ln \frac{a(OX)}{a(RED)}$$

Where:

Eo = The standard oxidation - reduction potential.

R = The gas constant.

T = The absolute temperature.

n = The number of electrons involved.

F = The Faraday (96,500 Coulombs).

a = Activities of oxidized and reduced species.

This equation can be used to measure the tendency of the solution to gain or lose electrons, to reduce or oxidize a substance added to it. It can also evaluate the great importance of the state of oxidation or reduction

in soil which is involved in all the chemical transformations and binding of micronutrients. In that respect, Mitchell(1955) observed that the uptake of a number of trace metals was increased when plants were grown in poorly drained soils, (i.e. swamps soils or marsh soils). The reason is that increasing water content of a soil decreases its oxygen content which leads to reduction of the metals previously present in an oxidized form. Under these reducing conditions, soluble organic matter formed from decomposition of plant material becomes more effective in solubilizing trace metals and therefore making them more available to plants, (Kee and Bloomfield, 1961 and 1962). As a result of these chemical changes, the Eh of the soil decreases and the pH approaches neutrality (Bohn, 1968 and 1971) and (Patrick and Turner, 1968). With the decrease of Eh, the amount of some available trace metals increased(Mn, Fe, Co), while that of Zn and Cu decreased with the increase of Eh. In the case of acid soils, the increase of the Eh leads to a decrease in pH to neutrality. In the case of well drained soils, (Arids and Semi Arids: Middle East and North Africa) which are well aerated and in which most of the oxygen is taken from the air; oxidation forms are dominant. Under these conditions, most of the metals are oxidized, thus forming insoluble complexes of minerals, and trace metals tend to bind to salts of complexing agents. (See: Patrick and Turner, 1968 ; Gotoh and Patrick, 1974 and Khalid et al 1977).

1.4.4 Microbial activity.

Microbiological activity plays a great role in recycling trace metals from organic matter by decomposing it and releasing the metals held (IU, 1981).

Many elements undergo microbiologically induced transformations following the type of reactions listed below (Alexander, 1977).

a. The release of inorganic ions during the decomposition of organic materials as in ammonification and phosphorus mineralization.

b. The reduction of an oxidized state of the elements under conditions where oxygen is limited. Nitrates, sulphates, carbonates and ferric ions can play the role of election acceptors in the absence of oxygen.

As mentioned earlier, microbiological activity has an effect on the availability of micronutrients. An important study on this matter has been carried out by Hodgson(1963) involving the oxidation and reduction of the iron ion Fe^{3+}_ Fe^{2+} and manganese Mn^{4+}_ Mn^{2+}. He also reported that the microbiological decomposition of organic matter stabilizes reduced forms of Fe and Mn and provides indirect means of promoting oxidation of these elements. Such decomposition can also serve to transform other elements to a less available form. The importance of the microbial activity was also shown in the case when drainage becomes impeded and oxides of Fe^{3+} and Mn^{3+} are readily reduced.

Their reduction is an energy requiring conversion. Therefore, the addition of easily decomposible organic matter is required to demonstrate the importance of microbiological activity in these transformations, (Hochster and Quastel ,1952 ; Rao, 1956)

1.4.5 Trace metal interactions

The concentrations of other micronutrient ions present in solution can strongly influence trace metal absorption. The effect varies greatly with the concentration of the absorbing and interfering ions. Bowen (1969) has shown that zinc and copper have mutually competitive activities on absorption by sugarcane leaf-tissue. Loneragan (1975) also concluded that interactions among trace metals do exist and that they are important. He found that the absorption of zinc as Zn^{2+} was strongly inhibited by copper weakly by cobalt and not at all by manganese or iron. This conclusion was shared by other investigators, among them Hawf & Schmidt(1967), Bowen(1969) and Chaudry & Loneragan (1972). In addition to the above, Tiffin (1967) reported that both zinc and manganese ions inhibited iron absorption from Fe^{2+} by tomato roots and by tobacco leaf cells (Kannan1969).

Since Cd is the principal element for the investigation, below is a review of its background, its relationship with other elements and its effects on soils, plants and animals.

1.5. Cadmium background

Cd like other heavy metals is a serious toxic element. It is acknowledged as one of the most hazardous environmental pollutants; not only to soil and plants, but also to animals. The use of heavy metals in agriculture, mercury as fungicides, lead as "Anti-Knock" compound has consequently increased concerns about pollution caused by these heavy metals. Cd is also a major source of pollution since it is commonly used as pigment, or as additive in rubber, plastics and batteries. Thus, metal pollution became no longer an isolated phenomenon, but a very worrying and dangerous problem. Cd occurs naturally in small quantities and is closely associated with zinc. It is found in Zn ores to a proportion of 1:350 (Fergusson ,1982). Besides Zn ore sources, many other sources exist. Some of them are listed below:

a. steel industry.
b. use of phosphate fertilizers.
c. application of sewage sludge.
d. coal combustion.
e. volcanic action.

1.6 The use of Cd and its repercussions on the environment.

This increasing emission of Cd from a variety of sources created a great interest about the possible movement of Cd and other heavy metals into the atmosphere, water and food chain as a result of plant uptake. This led to the great concern shown about the eventual effects of Cd on soil, plants and animals. As a consequence, the EEC reacted to this major problem by unifying their assessment of Cd consumption, predictions and means of tackling Cd pollution and minimizing its effects. The following results provide information about Cd consumption and predictions of Cd discharges in the EEC for 1990 and 2000, (From, MARC report number 26, 1982). MARC: Monitoring& Assessment Research Centre.

Table 1.6.1

Estimated discharges (in tonnes) of Cd from the European countries iron and steel industry for 1979 and predictions for 1990-2000.

	Discharge medium		
Country	Air	Solid waste	Slags
France	6.3	46	21.7
United Kingdom	5.9	40	16.4
West Germany	10.7	59.5	40.9
Predictions, 1990/2000			
France	8.5/10.4	66.8/81.7	27.5/33.6
United Kingdom	6.3/7.7	55.5/67.1	20.9/25.5
West Germany	10.0/12.1	94.6/115.8	52.0/63.3

The data shown in the table above indicate the discharge medium of Cd produced from iron and steel industries. The investigation was mostly carried out by the United Kingdom which provided most of the data. Estimation of the quantities of Cd released from the other EEC countries was added for the completion of the assessment.

Table 1.6.2

Estimated discharge of Cd from Coal consumption in the European countries, 1978.

Country	Coal consumption 1978(x 10^6)	Cadmium in discharge medium from coal burning (Tonnes) Atmospheric emissions	Ashes
France	44.2	0.9	43
United Kingdom	120.6	2.4	118
West Germany	81.9	1.6	80

From this table, one can notice that coal consumption is high in the UK and West Germany because they are the two largest coal consumers in the EEC. This creates high discharges of Cd from coal consumption followed by quantities of Cd found in ashes. Coal is mainly used in power stations and some industries such as, steel manufacturing. Coal is also used for domestic heating.

Table 1.6.3

Estimated discharge of Cd from the manufacture and from agricultural applications of fertilizers in the EEC in 1979.

Country	Landfill Cd(t/year)	Water Cd(t/year)	Cd from Phosphate fertilizers	applications
France	2.6	27	144	
UK	20	7.5	45	
W. Germany	0.02	0.05	44	

The discharge of Cd from phosphate fertilizers is caused by the process of phosphoric acid production. The process requires sulphuric acid to form phosphoric acid and calcium sulphate or gypsum. The two formed products contain cadmium originally present in phosphate rocks and removed by sulphuric acid action. France seemed to be the country producing most of the Cd discharged from phosphate fertilizers applications because it is the largest phosphoric acid producer in the EEC. West Germany took a step forward to minimizing Cd pollution by recovering most of the gypsum and using it as construction material.

Table 1.6.4

Assumed Cd content of phosphate fertilizers according to country of origin.

Country	Cd content g Cd/t of P_2O_5
Senegal	255
Togo	16
Morocco	60
Tunisia/Algeria	60
USA	35
USSR	0.8

Countries quoted above are the major exporters of rock phosphate to the EEC.

Table 1.6.5. Cadmium input to the European community environment (tonne/year).

	Compartment		
Source	Air	Land	Water
Volcanic action	20	ND	ND
iron and steel prod.	34	349	ND
Fuel combustion			
- coal and lignite	8	390	ND
- oil and gas	0.5	14.5	ND
Sewagesludge	2	130	33
Phosphate fertilizers	-	346	62

This table has been illustrated as an attempt to estimate Cd input into the environment. Clear indications appeared to confirm that iron and steel productions, coal consumption and phosphate fertilizers applications are the three major sources of Cd discharges.

1.7. Factors affecting the uptake and distribution of Cd in plants.

From all the experiments carried out on Cd, it was noticed that its uptake and distribution were subjected to many factors.

1.7.1 pH

Many investigations on different ranges of crops showed that at low pH, Cd solubility increased so did its availability and uptake(Lagerwerff and Biersdorf, 1971 and John, 1972a). The same conclusion was drawn on the behaviour of other metals. John (1972b) reported that for metals present in solution culture, their solubility is pH dependent. A decrease in pH affects the solubility and availability of trace metals by increasing their concentrations.

1.7.2 Presence of other metals

Many metals have shown the capacity of increasing, minimizing or even decreasing the effects of some other metals. The combined use of different metals creates a kind of action such as, additive, synergistic or antagonistic. The behaviour of Cd in the presence of other elements was investigated by many workers amongst them, Lagerwerff and Biersdorf(1971); Turner(1973) and Haghiri (1974). The interaction between Cd and Zn was the obvious choice to receive most of the attention because of the chemical similarities between them.

1.7.3 Time

Time is a factor as important as the ones mentioned previously. Cd-uptake has been shown to be directly proportional to time as reflected in the following table(Cutler and Rains, 1974).

Table 1.7.1. Data of an experiment carried out on(plant) studying the uptake of Cd as affected by time. (From Cutler and Rains, 1974).

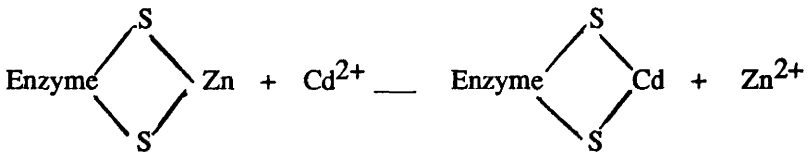
Time(day) (After Cd addition)	Tissue Cd content (µg/g D.W.)	
Days	Roots	Shoots
10	155.5	24.9
14	191.2	36.1
21	225.3	48.7
28	249.4	55.9

1.7.4 Temperature.

Schmidt et al (1965) and Rains(1969); also found that the transport of metal ions was strongly temperature dependent, and net transport was halted at temperatures approaching $0^{o}C$.

1.8 Cadmium in biological systems

Zn, Cd and Hg are in the same group of the periodic table. Zn is classified as relatively non-toxic compared with Cd and Hg which are very toxic. Being the middle member of the periodic sub-group consisting of Zn, Cd and Hg, Cd reveals intermediate properties. All three have the capacity of combining with the group -SH which is considered to be the active centre of the enzyme. The stability of such complexes increases in the order Zn< Cd < Hg. It was mentioned previously that Zn was an important element involved in a number of enzymes. Therefore, as a result, Cd competes and displaces Zn in a number of Zn-containing metalloenzymes by irreversibly binding to active sites, thereby destroying their normal metabolism. This can be illustrated as follows (Fergusson,1982):



The displacement of Zn occurs by bonding to the -SH group of the amino-acid cysteine and the metallothioneine. Cd enzymes are inactive as a result of the interference between Cd and Zn causing the blockage of the active site of the enzyme by Cd. According to Lewis(1923), Cd is considered to be a soft acid, relatively large and polarizable compared to Zn. However, since sulphur is considered a soft base and also polarizable, the Cd-S bond is stronger than that of the Zn-S.

Cd, Zn and many other elements are known to compete with each other in plant as well as in animal. Liver and kidney are considered to be appropriate medium for such competition (Webb, 1972, and Bremner, 1974) because they both contain metallothioneine with cysteine containing an -SH group known to favor binding to trace metals especially Cd. This topic will be reviewed with more details in Chapter 3. Below are some enzymes inhibited by toxic metal (Fergusson, 1982)

Cd^{2+}: Alcohol dehydrogenase; Carboxypeptidase; Carbonic anhydrase; Adenosine triphosphatase;...

Hg^{2+}: Alkaline phosphatase; Lactic dehydrogenase; ...

Pb^{2+}: Alkaline phosphatase; Cytochrome oxidase; Acetylcholinesterase; Adenosine triphosphatase;...

1.9. Effects of Cadmium

Cd has no known function in plants or animals but it is known to be toxic since it is readily taken up by plants.

1.9.1. Effect of cadmium on soil and plants.

Since the use of fertilizers and sewage sludge has increased, a great dispersion of Cd in the environment has been observed and the potential effect of Cd became greater.

Some major effects are shown below:

a. Decrease in yield especially in soils with high phosphate fertilizers applications (Bingham et al,1975).

b. Plant injury symptoms due to soil Cd contamination. Plant injury depends upon the plant species and its tolerance to cadmium.

1.9.2. Effect of Cadmium on Human Beings.

Cd is easily extracted from soils and is accumulated by plants. Consumption of plants grown in a Cd-contaminated soil and the excessive Cd-intake from polluted air and water cause health problems. Thus Cd is believed to be responsible for a number of diseases (Lagerwerff and Biersdorf, 1971) such as:-

a. Testicular damage. (Parizeck, 1957; Gunn et al, 1961; Lagerwerff and Biersdorf, 1971).

b. Cardiovascular diseases (Shroeder and Buckman , 1967).

c. Pulmonary diseases. (Shroeder and Buckman,1967)

c. The endemic "OUCH-OUCH" or "ITAI-ITAI" (Tsuchiya, 1969)

Measures were taken to tackle the adversities caused by Cd, among them the study of the mobility and availability of Cd in soil and the control of its pathological activity in plants.

One of the objectives of this thesis is aimed to find out about the behaviour of Cd and its effects on plants. French beans (Phaseolus vulgaris) is the model chosen for this purpose because it is easy to grow and also because it responds quicker to trace metal effects than other plant species.

1.10. Phytotoxicity of Cd and Plant Tolerance

The phytotoxicity of Cd was recorded in a number of plant species grown in high Cd concentration solutions (Page et al, 1972; Turner,1973). The plant response to Cd was markedly different because of their difference in tolerance. Spinach and soybean were sensitive to Cd whereas tomato and cabbage were resistant (John et al, 1976). As far as Cd symptoms were concerned, investigators came up with suggestions concerning the obvious responses of plants to Cd toxic effects such as : chlorosis, Stunted growth and decrease in yield. Some other workers also reported a decrease in chlorophyll content of the leaves (Root et al, 1975; Petterson and Alloway,1980) and inhibition of photosynthesis (Bazzaz et al 1974b). This matter will be investigated in Chapter 4.

1.11. Previous works.

The growing interest in Cd mobilized many research teams and individuals to look seriously into problems caused by Cd. Various studies were undertaken to determine the behaviour of Cd and its effects on many edible crops. To cite just some studien, Lagerwerff and Biersdorf (1971) used corn and radish in solution and soil; Cunningham et al (1975) and Wallace and Romney(1977) grew respectively soybeans and beans in hydroponic solutions ; and there are many others to be cited in the following chapters. Some used the same species, others used different species and most of them carried out their experiments under different experimental conditions viz, nutrient solution composition, age of the plant and pH. It was noticed by many workers among those quoted above, that Cd was easily taken up by most of the species studied and the amount of Cd tolerated varied from one crop to another. It was also observed that Cd uptake and transport were affected by many factors: eg. pH, metal-interactions etc.. Cd can be translocated throughout the plant subsequent to uptake by roots. Its distribution between roots, stems and leaves varies with plant species and time of treatment (Cutler and Rains, 1974). The distribution of Cd can be an exchange absorption of Cd uptake in short term experiments especially for large Cd amounts, it can also be a diffusion of the element and its competition for sites within the cell walls or it can follow a symplastic movement. These suggestions were shared by other investigators among them: Lagerwerff and Biersdorf, (1971); Page et al (1972); and Cunningham et al (1975).

They concluded that Cd like many other elements can be taken up, transported, distributed and accumulated within plants. Differences in plant responses to Cd effects exist but the symptoms of the effects remain the same in most of the cases.

1.12. Conclusions

From this review, one can get a clearer idea about the importance of these elements and the danger they create and they are still causing great concern. However, while investigators answered many questions, many others questions are still emerging as a result of the increasing use of these metals. Among the questions answered were those of Cd-toxicity symptoms,(Page et al,1972) and Cd-interaction with other heavy metals (Turner, 1973 and Haghiri,1974). Cadmium interaction with zinc is the best known example reported and confirmed by many investigators. They only disagree on the nature of the interaction. Some reported two different interactions between Cd and Zn (Lagerwerff and Biersdorf, 1971). Viz: Synergistic: Zn uptake increased in the leaves when Cd concentrations in the solutions were increased. Competitive: This type of interaction was noticed when concentrations of Zn were increased and the uptake of Cd from culture solutions decreased . These suggestions were shared by many others (Haghiri, 1974; Root et al 1975; Jarvis et al,1976) who confirmed the existence of a competition and interaction between Cd and Zn and between Cd and other elements such as, Se, Ni, and Cu.

1.13. Aim of the thesis.

The main objective of this thesis was to find out about the behaviour, transport , and distribution of Cd within bean plants and its effects on them and on chlorophyll content. The interaction between Cd and Zn was also investigated. The framework of the thesis is summarised as shown.

Chapter 1.

Chapter 1 is an introduction on trace metals in soil, plants and animals with a review on Cd and its relationship with other trace metals.

Chapter 2.

Part 1. This part was aimed at studying the relationship between Cd and pH and the effect of pH on Cd behaviour, Cd availability and Cd uptake by plants. The investigation was carried out in a hydroponic medium using a range of Cd levels of 20 to 140 μg at pH values ranging from 2.9 to 7.8.

Part 2. This part was aimed at studying the transport and the distribution of Cd in bean plants. The investigation was carried out using radioactive cadmium(Cd-109) to determine its distribution and its localization within the plant. A solid scintillation counter was used for the counting of Cd retained by each part of the plant(root, leaves, and stem).

Chapter 3.

This chapter was aimed at investigating the interactions between Cd and Zn. It was also aimed at studying their relationship and its implications on on their uptake by bean plants. A range of combined levels of the two elements was used and added to the culture solution as $Cd(NO_3)_2$ and $ZnSO_4$.

Chapter 4.

This chapter was aimed at studying the effects of Cd on chlorophyll content. The purpose of the investigation was to find out about the effects of Cd on chlorophyll biosynthesis and its content in plants. It also dealt with the relationship between Cd and Mg and also Cd and Fe which are considered to be important for the chlorophyll formation. Atomic absorption and UV spectroscopy were used for the analysis of chlorophyll and equations were used for its estimation.

Chapter 5.

This chapter takes in an assessment of the work and conclusions drawn from previous investigations. It also an assesses the practicality of the findings and the prospects for future research.

CHAPTER 2

Part One.

The effect of pH on Cd behaviour, its availability and its uptake by plants.

2.1. Introduction

2.1.1. Effect of pH on trace metals in soil.

As already mentioned, pH plays a major role in the transport, distribution and behaviour of trace metals in soils. It was shown by many investigators to have great effect on solubility, availability and uptake of trace metals from soil by plants(Bowen, 1969; Miller et al 1976; and John et al 1976). In this Introduction, some of the results and suggestions achieved by many workers who studied this matter and reported very interesting findings will be reviewed. Throughout this review, an attempt will be made to put light on this subject in order to emphasize the importance of pH and its effect on the chemistry of trace metals leading to changes in soil chemical composition. Soil pH influences the fertility of a soil and any significant changes in pH affects the solubilities of minerals in soil.

Many investigations have been carried out to study the pH as a major factor affecting trace metals in soils, their solubilities and also their availability to plants. Many suggestions and conclusions were drawn. Earlier in 1950, Christensen et al reported that as a result of soil liming to correct the pH from 4.6 to 6.5, exchangeable Mn decreased substantially. Others found that the toxicity of some trace metals was reduced as a result of increased pH as reported by Delas in 1960 who observed that Cu-toxicity was reduced at increased pH. In addition to this, many more researchers who had also investigated this matter came up with interesting finding such as: pH affecting the absorption capacity of trace metals in soil(Shuman, 1975) and their solubility as for instance that of Fe^{3+}, Mn^{2+} and also that of Zn^{2+} and Cu^{2+}(Lindsay,1979). In that respect, Fergusson(1982) suggested some chemical reactions involved in pH changes. He reported that pH can be affected by the cations adsorbed on to clays. These cations such as: Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} are predominant and tend to increase the pH when they are released from a clay as illustrated below.

$$Clay\_Na + H_2O = Clay\_H + Na^{+} + OH^{-}$$

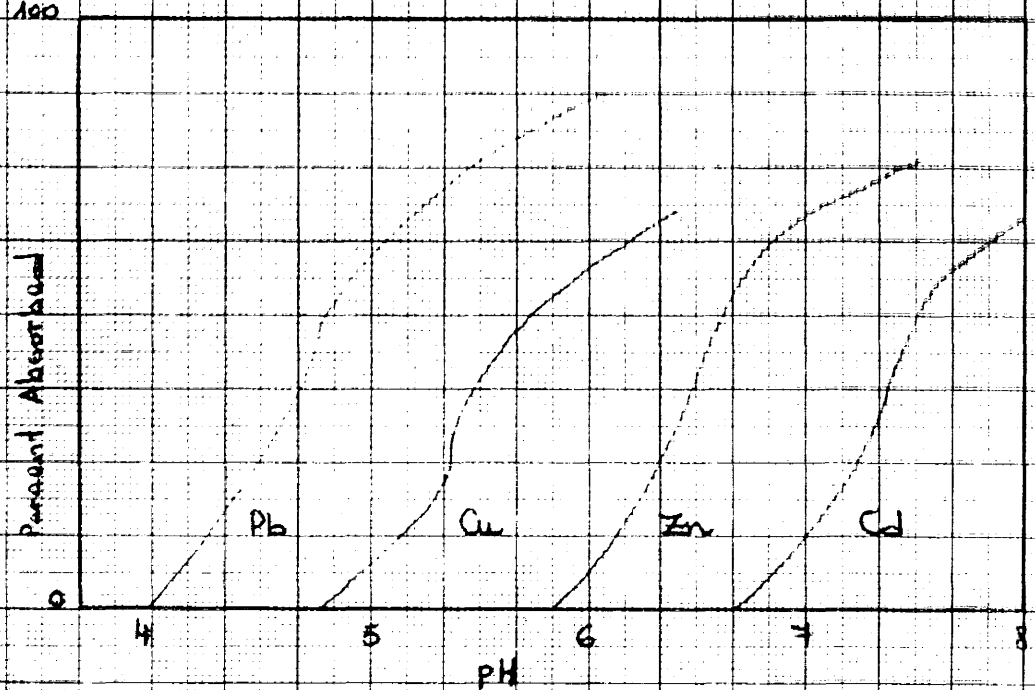
Other cations such as: Al^{3+}, Fe^{3+} and H^{+}, when desorbed from a clay produce soil solutions due to hydrolosis of the cations.

Fergusson also reported that soil pH can be elevated by liming and lowered by adding acid salts : eg, $(NH_4)_2\ SO_4$ or sulphur which is oxidized by soil-bacteria to sulfuric acid following the reaction:

$$2S + 3O_2 + 2H_2O \xrightarrow{\text{bacteria}} 4H^+ + 2SO_4^{--}$$

Laxen and Harrison(1982) added that pH was a major factor affecting the adsorption of metals. He suggested the following order of tendancy to bond to surface such as: clays and humic articles, Hg > Pb > Cu > Zn > Cd > Ni > Co and reported the diagram below (Fig. 2.1.1) which shows the adsorption of Cd, Cu, Pb and Zn on amorphous iron hydroxide as a function of pH. Lagerwerff,(1970) suggested in his study of the uptake of Cd, Pb and Zn by radish from soil that the accumulation of these elements was a function of pH. He also concluded that an increase in soil pH from 5.9 to 7.2 resulted in decreases in both yield and metal contents of radish. In addition to these suggestions, Mc.Bride and Blasiak(1979) added that, at low pH values, adsorption of zinc can be reduced by competing cations while at high pH (> 7.5), soluble zinc can increase as organic complexes in soil solutions. He also observed that zinc solubility of metal–zinc present in soil increased every unit increase in the pH range of 5 to 7 . Therefore, the zinc deficiency observed often in crops upon liming acid soils can be explained by the sensitivity of the metal to pH.

Diagram : 2.1.1.



Absorption of Cd, Cu, Pb and Zn on amorphous iron as a function of PH. (Lazen, L. & Hansen 1982)

2.1.2. Effect of pH on trace metals in plants.

Soil and plant are undissociable as far as metal uptake by plants is concerned. Nonetheless, some studies were carried out using culture solutions. As it was mentioned earlier, pH was found to affect the accumulation of toxic metals in the aereal part of plant tissue(Shuman, 1975; and Loganathan, 1977). Thus, by altering this parameter, the behaviour of some of these elements can be manipulated and therefore used to reduce their uptake by plants. Moreover, many contributions were made to explain the extent of the influence of pH on trace metal uptake by plants from culture solutions. Earlier in 1963, Hodgson concluded that responses of plant species to pH effects were different and the influence of pH varied with the amount and form of an element present in the plant. Solubility, availability and uptake of an element are the three major aspects to be affected by pH changes. At low pH values, effects are generally caused by toxic levels of soluble ions in the solution culture. Such effects may also arise from nutritional imbalance. consequently, under low pH conditions, an increase or decrease in the concentration of the nutrient is to be expected.

2.1.3. Effect of pH on Cd uptake by plants in soil.

Cd is relatively newly known toxic element compared with others such as: Hg, Pb, Se, and Co. However, great concerns were shown when investigating the element, its origins, its forms and its effects whether on soil, plants or animals. The element was also shown to be subjected to the effects of many factors among them and the most important, pH which does affect both the solubility and the uptake of Cd(John, 1972a; Andersson and Nilson, 1974, and Wallace et al 1977). Reddy and Patrick (1977), reported that Cd uptake increased with a decrease in pH by rice plants. Mosey(1974) added that the toxicity of Cd to anaerobic digesters was pH dependent when it was greater than 7 and independent when the pH was less than 7. This effect was found to be caused by the insolubility of $CdCO_3$. Some other workers suggested that the accumulation of Cd was increased by the wheat grain when sewage sludge was applied to a soil with a pH of 4.8 and reduced when lime was applied (Linnman et al, 1973). Additionally, John(1972a) and John(1975) observed that radishes grown in a soil with a pH of 7.2 accumulated less Cd than those grown in a soil with a pH of 5.4. A similar observation was made by Williams and David(1976) when the studied the growth of Subterranean Cloves in soil amended with $CaCo_3$ and $MgC0_3$ for correcting the pH to 5.1, 6.0 and 6.8.

Miller et al(1976) added that low pH values increased the solubilities of some other ions (Fe^{3+}, Mn^{2+}, Zn^{2+} and H^{+}) and suggested that this could lead to a great competition between trace metal for exchange sites than in soil with high pH values. This competition causes an increase in the Cd concentrations in soil solutions. There are many other suggestions and conclusions which could also be quoted(Allaway,1968; and Jastrow and Koeppe, 1980). An investigation was carried out to study the effects of pH on the behaviour, availability and uptake of Cd by plants. Beans (Phaseolus Vulgaris) was the model chosen for this purpose and the experiment was carried out in a hydroponic medium.

2.1.4 Culture solution

Most plants can grow perfectly with their roots immersed in a nutrient solution. To support the plant mechanically, roots are usually embeded in an inert material such as vermiculite (Hydrated Mg, Al, Silicate, Plastics) . For optimal growth, the nutrient solution must contain all the required elements in suitable form and in more appropriate proportions suitable for a normal growrth. Other factors such as, pH, temperature and light must also be kept at appropriate levels. These are some known nutrient solutions, Sach's solution 1860, Knop's solution 1865 and Hoagland's solution 1940.

2.1.5 Composition of the nutrient solution used for the investigation.

Much time was spent in preliminary experiment looking for the appropriate composition of a nutrient solution suitable for beans. Based on the definition of essential, beneficial and non-essential elements and using the concentrations of nutrients for a normal growth, their toxic levels and their deficiency as suggested by Bowen(1979). A final nutrient solution was made up with some modifications in its composition (as shown below) compared with that of Knop's 1865 used at early stage of the investigation.

Table 2.21. Composition of the nutrient solution.

Salt	g/l	ppm	
KNO_3	0.20	K(80);	NO_3(123);
$MgSO_4.7H_2O$	0.20	Mg(20);	SO_4 (78);
$FeSO_4.7H_2O$	0.05	Fe(10);	SO_4 (20);
KH_2PO_4	0.13	K(38);	PO_4(90);
$Ca(NO_3)_2.4H_2O$	0.88	Ca(150);	NO_3 (46)

The three important nutrients, K, Mg and Ca were added as KNO_3, $MgSO_4$ and $Ca(NO_3)_2$ respectively. Iron was added in small quantities sufficient for the plant requirement for a short period experiment and also sufficient to prevent chlorosis resulting from an iron deficiency. Evidence were obtained from control samples, some grown in culture solutions containing Fe(600μg) and some were grown in culture solution without Fe. After a period of 7 days, only control samples grown with no Fe shew signs of Fe deficiency which was presented as yellowing of the leaves and even death of bean plants. The pH range of the solution was maintained at about 5 to 6 considered to be adequate for the growth of several species. Cd was added as as $[Cd(NO_3)_2]$ and its addition was carried out at a range of 0 μg to 200 μg total.

2.2. Experimental

2.2.1. Plant growth

Seeds of beans were grown in a hydroponic medium for a period of 10 to 12 days to a mature stage of the plant(long stem and large leaves). Trays containing 24 seeds were used for the growth which took place in a growth chamber with a regulated temperature (20^oC + 4^oC) relative humidity (80%) and a photoperiodic regime of 16 hours light and 8 hours darkness. The moisture was kept by watering the seeds once in two days with deionised water.

After a maximum period of 12 days, a selected number of uniform plants were washed with tap and dionised water before being transferred to a 100 ml flask containing the nutrient solution and the appropriate amount of added Cd for a period of seven days.

2.2.2. pH buffering.

A citrate buffer was used for a range of pH from, 2.9 to 6.4 and low concentrations of citric acid [$C_6O_7H_8$:0.01M] and sodium citrate [$C_6H_5O_7Na_3.2H_2O$: 0.005M] were used to minimize any citrate effects on plants. Citrate effects are mentioned because at early stage of this experiment, solutions of 0.1M of citric acid and 0.1M of sodium citrate were used for the pH buffering and some anomalies in the bean growth appeared such as reduction in growth due mostly to root damage. The experiment was carried out over a period of more than six weeks and the same effects appeared in all plants grown in citrate-buffered nutrient solutions compared with the control sample (original nutrient solution with no citrate) and this resulted in several aborted experiments. However, this problem due to citrate was at least minimized by using lower concentrations of citric acid and sodium citrate as indicated above.

2.2.3. Plant analysis

a. Harvesting.

After a period of 7 days in nutrient solution during which a daily check of pH was carried out using a portable pH-meter. Whole plants were harvested, washed, cut in small parts and then put in silica basins to be dried overnight at 100 to 120 ^{o}C.

c. Digestion procedure.

Samples were ground in a silica mortar, weighed then transferred to 100 ml beakers ready to be digested. The digestion method was used to determine the total quantity of Cd in plant tissue samples. Each ashed quantity of the dried plant material was transferred with 10 ml of concentrated HNO_3 to a 100 ml beaker. The mixture was covered by a watch glass and put on a hot plate to start the digestion at a temperature ranging from 70 to 90 ^{o}C, then increasing it later to a temperature of 120 to 140 ^{o}C. After 20 to 30 minutes, the digestion was completed and solutions were clear. Samples were allowed to cool, filtrated through an ashless filter paper then diluted to 50 ml . The blank solutions were prepared the same way and analysed with the samples using atomic absorption spectrophotometry.

2.3. Description of atomic absorption spectrophotometry.

Atomic absorption spectrophotometry is considered along with other techniques as one of the most advanced for trace metals analysis. The technique has the advantages of simplicity, practicality and relatively low cost. It also has its disadvantages like the requirement that the sample must be introduced in the excitation source in the form of a solution at the time where most of the materials of interest such as, soils, plants, animal tissue are not directly soluble in common solvents. However, an extensive preliminary treatment is needed to transform these materials into solutions ready to be analysed. The sample, once ready is injected into a nebulizer then sprayed into a flame to produce an atomic vapour which contains most of the atoms in an unexcited state. The measurement of the absorption is made when the light selected is passed through the wavelength.

2.3.1. Description of the equipment.

The atomic absorption spectrometry equipement consists of:

a. Light source: The most commonly used light source is a hollow cathode lamp filled with neon or argon and has a cathode made of the element being determined.

b. Dispersive system: The so-called "nebulizer" is the common atomization device for atomic absorption spectrophotometry. It converts a liquid sample into a fine spray which is then atomized once passed through the flame.

c. Monochromator(or filter):To isolate the resonance line and protect the components from dust.

d. Detector: To detect the intensity of light energy by transforming it into readable pulses.

(This topic is very well documented, for more information see among many other authors, Christian, 1970; Alkemade and Herrmann, 1979, and Skoog, 1985.)

2.4. Interferences

Interferences in atomic absorption do exist and are classified as five types:

2.4.1. Chemical

This type occurs when foreign elements are combined with the element to be examined and this may affect the atomization. This type of interference can be overcome by addition of a releasing agent to form a more stable compound with the interfering element or by increasing

the temperature of the flame in order to break down the compound formed. (eg: releasing agents such as EDTA or Lanthanum).

2.4.2. Matrix

this occurs when there are differences in physical properties of the sample and the standard solutions. This type of interference can be corrected by diluting the sample solution to match the standard solution.

2.4.3. Spectral

The case of foreign elements present in sample solutions which absorb at wavelength nearest to the one of the element of interest. This kind of interference is rare in atomic absorption because only single element lamps are used.

2.4.4. Ionization

This happens when an appreciable fraction of the neutral atoms from the ground state is ionized as a result of the high energy of the flame. Corrections can be made by adding an excess of easily ionized elements: (e.g. Cs, Rb, Na,) to samples and standard solutions.

2.4.5. Background

The impurity of the flame can cause a background absorption. The presence of gaseous species or salt particles in the flame can disturb the elemental absorption. This can be eliminated by using a deuterium background corretor to correct for nonatomic absorption.

During the analysis, some chemical interferences were met and were mainly caused by sodium which could have originated from the deonised water used during the experiment. This interference did not cause any great problem for the analysis and was overcome by increasing the temperature of the flame. The use of nitric acid for the digestion caused a matrix interference due to the volume of nitric acid added to samples. This was corrected by adding the same volume of the acid to the standard solutions.

2.5. Part Two.

Transport and translocation of Cd within the plant.

The use of labelled Cd is of great importance to study the transport and translocation of Cd. By using the solid scintillation technique, much information was achieved. Before describing the method and discussing the results, this is a brief introduction.

2.5.1. Introduction.

2.5.1.1 Radiotracer application in soils.

The widespread use of pesticides and the increase in industrial waste created great concern and interests in investigating the extent of the effect of these contaminants and their influence on the environment. Therefore extensive research on the microbial metabolism of these contaminants was needed to preserve the environment. By using radioisotope-labelled compounds, information may be obtained concerning the transformation involved in the formation and degradation of natural soil molecules and the effect of these organic soil constituents on soil fertility. The other important advantage of the use of radiotracers is that this technique can help to make predictions about the biodegradability and possible toxicity of a given compound.

The use of radiotracers can be expanded to organic insecticides, herbicides, fungicides and other agricultural chemicals applied to soil systems.

2.5.1.2 In plants.

The use of labeled compounds is important to follow the uptake translocation and metabolism of very low levels of a pesticide or just an elements. This was made important by the increasing availability of pesticides labelled with radioisotopes such as: ^{3}H, ^{14}C, ^{35}S etc..(L'Annunziata, 1979). In addition to environmental concerns the use of radiotracers could make a great contribution to agriculture. An improvement in the efficiency of other organic compounds and plant growth regulators is predictible when studying their synthetic and metabolic pathway and the mechanisms involved.

2.5.1.3 In animals.

Radiotracers have the same objective and the same importance when used for animals. In addition to the determination of the biodegradability and metabolism of these compounds, a nutritional biochemistry can also be determined. The most commonly used radioisotopes are ^{14}C and ^{3}H mainly for their long half life of 5730 and 12.26 years respectively. Many others tracers can be used for the same purpose.

2.5.2. Description of the method

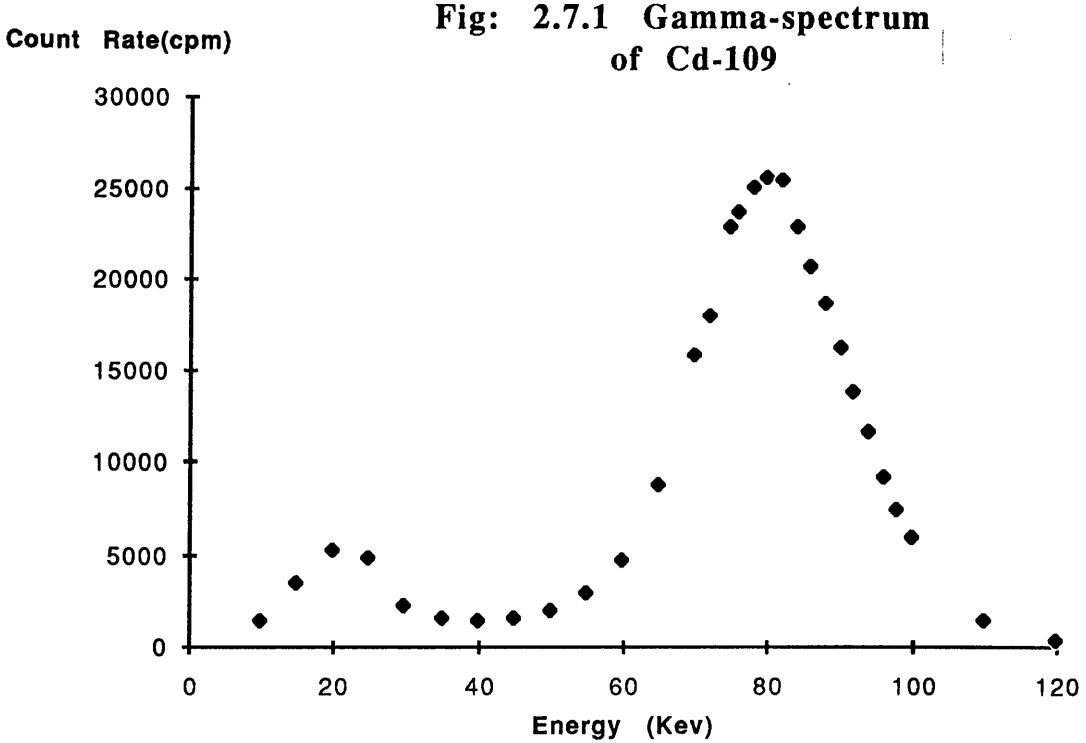
2.5.2.1 Procedure

Beans (Phascolus Vulgaris cv Canadian Wonder) seeds were germinated in vermiculite for 12 days. After this period, plants were transferred to 100 ml flasks containing nutrient solution and radioactive Cd-109 with gamma radiations and with a variable activity of: 0.05, 0.1 and 1.0 microcurie(μCi) respectively. The addition of radioactive Cd was carried out in a hot-lab with maximum safety and great precaution were taken when handling samples. Samples were transferred to a growth room particularly fitted for the experiment and left for periods of 4 days and 7 days, then later analysed by the solid scintillation method. The counting was carried out for roots, stems and leaves.

2.5.2.2 Description of the Technique

The solid scintillation technique is one of many used for radiotracer detection. It is a phenomenon whereby the absorption of bombarding alpha, beta or gamma radiation by certain crystalline inorganic or organic materials results in the emission of flashes of visible light from the solid absorbing detector (L'Annunziata, 1979). The one used for the experiment was a Thallium-activated sodium-Iodide crystal [Na(Tl)] which is commonly used for gamma detection because of its shorter decay time. Li(Eu) or Cs(Tl) can also be used.

Fig: 2.7.1 Gamma-spectrum of Cd-109



2.6. Results

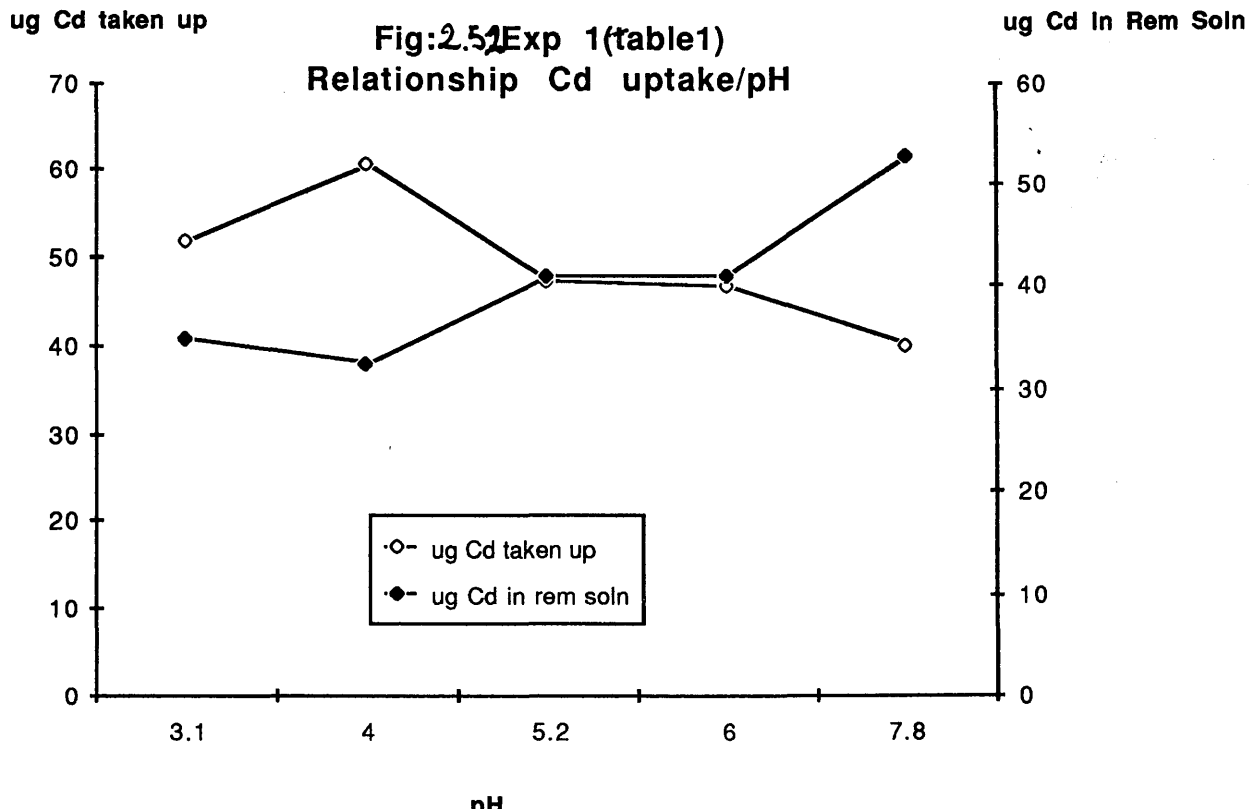
2.6.1. Results Part One.

Samples of all experiments were grown in nutrient solution for a period of 7 days. The results of all the following experiments are the means of 4 replicates.

Experiment 1. Carried out at a fixed levels of Cd of 100 µg with a range of pH: 3.1 to 7.8. The data below shows the effect of pH on Cd-uptake by beans.

Table 1

Sample pH	D.W g	Total µg Cd taken up	total µg cd in rem sol	Recovery percent %	µg/g D.W.
3.1	0.21	51.89±3.743	34.97±4.999	86.85	249.41
4.0	0.27	60.67±2.84	32.54±5.787	93.29	226.68
5.2	0.22	47.37±1.004	41.13±4.660	88.46	216.31
6.0	0.20	46.70±0.798	41.19±2.160	87.88	254.52
6.9	0.24	46.70±1.562	45.58±2.854	92.27	195.09
7.8	0.27	39.92±2.523	52.69±3.924	92.60	142.94


ug Cd taken up
Fig:2.5 Exp 1(table1)
Relationship Cd uptake/pH
ug Cd in Rem Soln
70
60
50
40
30
20
10
0
60
50
40
30
20
10
0
ug Cd taken up
ug Cd in rem soln
3.1
4
5.2
6
7.8
pH

Experiment 1: Carried out at a fixed pH4 using different levels of Cd ranging from 20 to 120 µg . The results shown below indicate Cd-uptake by beans as enhanced by pH 4.

Table 2

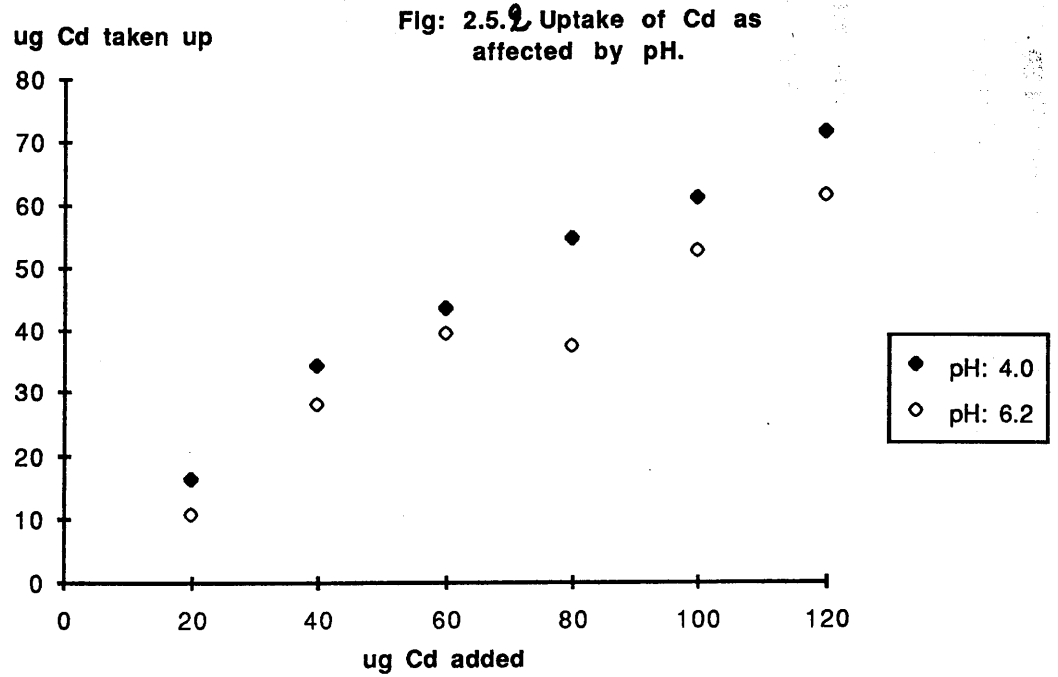
Sample µg Cd	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
20	0.31±0.03	15.98±1.26	2.91±1.40	63.17±23.38
40	0.28±0.03	34.53±1.14	1.85±0.82	123.90±8.50
60	0.30±0.05	43.91±2.91	10.55±0.90	149.70±27.60
80	0.22±0.03	54.54±6.27	16.48±1.44	268.70±53.50
100	0.19±0.02	61.20±2.80	23.83±2.52	324.10±32.50
120	0.15±0.01	71.84±1.75	34.75±2.50	482.40±51.00

Experiment 1. Carried out at a fixed pH of 6.2 with a range of Cd of 20 to 120 µg . The results indicate the uptake of Cd by beans as reduced by the pH 6.2.

Table 3

Sample µg Cd	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
20	0.27±0.01	10.79±1.12	6.48±0.60	39.90±2.08
40	0.25±0.01	28.29±1.50	8.83±0.98	113.20±3.47
60	0.26±0.01	39.92±1.62	12.29±0.85	153.50±1.08
80	0.21±0.03	37.52±2.61	35.40±1.30	181.0±30.0
100	0.21±0.02	53.08±2.76	38.55±1.94	253.9±21.1
120	0.19±0.02	61.61±2.41	48.24±3.03	318.2±33.4

Fig: 2.5.2 Uptake of Cd as affected by pH.



Experiment 2. Carried out at fixed Cd-concentration of 100µg and at a range of pH of 2.9 to 6.3. The results indicate the uptake of Cd by beans as affected by pH.

Table 1

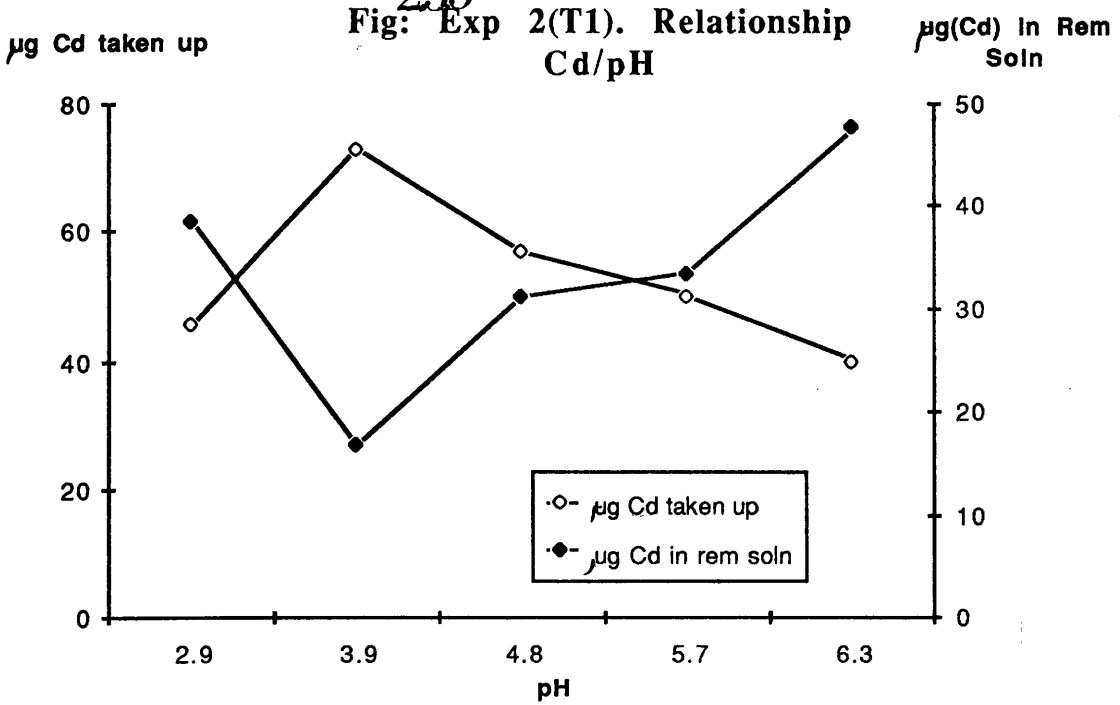
Sample µg Cd	D.W g	µg Cd taken up	µg Cd in rem sol	Recovery %	µg/g D.W
2.9	0.19	46.50±1.586	38.45±1.855	83.95	240.63
3.9	0.29	73.04±2.793	16.68±0.515	89.71	252.72
4.8	0.20	56.68±3.478	31.11±1.985	87.78	284.45
5.7	0.23	49.89±1.056	33.53±2.637	82.75	225.49
6.3	0.25	39.92±1.439	47.53±5.696	87.44	161.36

Experiment 2. Carried out at a fixed pH: 3.8 with a range of Cd of 20 to 140 µg .

Table 2

Sample µg Cd	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
20	0.15±0.03	15.18±1.82	2.75±1.00	102.5±13.8
60	0.18±0.03	51.22±0.60	7.18±0.80	289.4±49.5
100	0.12±0.02	70.24±1.05	14.76±1.20	596.4±101.1
140	0.14±0.02	83.01±1.38	46.38±6.26	601.1±85.7

Fig: Exp 2(T1). Relationship Cd/pH



Experiment 2. Carried out at a fixed pH: 6.0 and a range of Cd of 2o to 140 µg.

Table 3

Sample µg Cd	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
20	0.2±0.02	10.39±1.05	6.76±0.88	52.55±10.29
60	0.15±0.02	23.96±1.73	33.39±2.46	161.5±26.0
100	0.18±0.02	47.1±2.1	45.86±1.26	263.0±18.2
140	0.22±0.02	59.87±2.76	50.25±3.23	273.0±14.8

Experiment 3. Carried out at a fixed level of Cd of 100 µg and a range of pH, 3.0 to 6.6. The results express the effect of pH at low and high values on the uptake of Cd by beans.

Table 1

Sample pH	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
3.0	0.33±0.04	55.18±2.26	32.06±1.93	168.7±21.7
3.7	0.33±0.03	63.6±3.2	32.83±1.05	194.2±26.2
5.2	0.27±0.02	61.01±1.16	26.76±2.97	227.0±21.2
6.0	0.24±0.02	50.0±1.7	38.08±1.94	209.0±12.2
6.6	0.26±0.01	45.14±3.20	50.23±7.44	173.6±9.0

Experiment 3. Carried out at a fixed pH: 3.8 and at different Cd concentrations ranging from, 20 to 140 µg total. The data shows the uptake of Cd as affected by a pH of 3.8

Table 3

Sample µg Cd	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
20	0.34±0.02	12.76±0.64	5.95±0.52	37.57±2.70
60	0.27±0.03	38.60±1.37	17.8±1.5	144.1±18.0
100	0.20±0.02	60.36±1.41	28.18±0.78	303.5±29.7
140	0.16±0.02	87.57±0.90	34.34±2.54	551.5±58.1

Experiment 3. Carried out at a fixed pH: 5.9 and at a range of Cd levels of 20, 60, 100, and 140 µg.

Table 3.

Sample µg Cd	D.W Gr	µg Cd taken up	µg Cd in Rem soln	µg/g D.W
20	0.32±0.04	7.89±0.65	10.72±0.75	24.74±1.01
60	0.28±0.05	30.89±2.75	24.59±1.37	111.3±9.4
100	0.25±0.02	50.65±0.97	41.82±3.77	203.4±18.3
140	0.30±0.02	68.46±1.97	49.93±2.83	228.6±10.3

Experiment 4. Carried out at a fixed level of Cd of 100 µg and at a range of pH of 2.9 to 6.1. The results indicate the uptake of Cd by beans as a function of pH.

Table 1

Sample pH	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
2.9	0.28±0.02	32.75±2.15	53.76±2.20	117.6±14.9
3.6	0.23±0.05	61.08±1.11	27.92±1.63	272.3±50.7
5.3	0.25±0.02	64.16±2.40	11.30±2.43	257.3±15.8
5.6	0.28±0.02	58.31±2.44	28.18±0.70	208.4±4.5
6.1	0.31±0.03	43.84±1.34	43.87±4.30	142±12

Experiment 4. Carried out at a fixed level of Cd of 100 µg with a range of pH of 2.6 to 5.2.

Table 2

Sample pH	D.W g	µg Cd taken up	µg Cd in rem sol	µg/g D.W
2.6	0.31±0.02	40.73±1.64	41.0±1.2	132.7±5.7
3.0	0.35±0.03	56.25±2.70	28.37±3.19	161.6±18.3
3.9	0.22±0.02	65.38±0.92	26.63±2.82	289.5±24.0
4.3	0.33±0.01	61.68±1.11	34.29±2.70	187.0±2.5
4.9	0.28±0.02	47.37±0.92	41.19±1.46	169.7±13.0
5.2	0.31±0.02	43.88±1.50	38.50±3.40	141.8±8.0

2.6.2 Results. Part ~~two~~ 2 .

Experiment 1. The experiment was carried out over a period of 7 days. The data below shows the counting of Cd-109 used at different activities for the three different parts of the bean plant; Roots, Stems and Leaves. The counting time was fixed to 3 minutes.

Table 1. Number of observations: 3

Sample µg Cd	Activity µCi	Roots counts	Stems counts	Leaves counts
10	1.00	7553	1180	113
10	0.10	3071	230	71
10	0.05	1849	232	39
30	1.00	11458	1289	578
30	0.10	2645	280	60
30	0.05	1632	237	77
0	1.00	13163	1390	138

Experiment 1. Carried out the same way as above with a counting time of 5 minutes.

Table 2. Number of observations: 3

Sample µg Cd	Activity µCi	Roots Counts	Stems counts	Leaves counts
10	1.00	12677	1989	198
10	0.10	4935	378	122
10	0.05	2954	414	67
30	1.00	18875	2046	912
30	0.10	4483	546	111
30	0.05	2646	461	116

Experiment 2. Carried out over a period of 7 days with an additional Cd- activities of: 0.25, 0.5 and 1.0 μCi. The counting time was fixed to 3 and 5 minutes.

Table 1. Number of observations: 3

Sample μgCd	Activity μCi	Roots counts	Stems counts	Leaves counts
		Counting time: 3 minutes.		
30	0.25	3277	590	217
30	0.50	5408	862	192
30	1.00	11128	1382	319
		Counting time: 5 minutes.		
30	0.25	5244	953	333
30	0.50	9675	1498	340
30	1.00	18318	2499	518

Experiment 3. Carried out in a shorter period of 4 days and using only one Cd activity value of 1 μCi. The counting was determined at two different time of 3 and 5 minutes as shown respectively in the following tables.

Table 1. Number of observations 3.

sample μg Cd	Activity μCi	roots counts	Stems counts	Leaves counts
		Counting time: 3 minutes		
30	1.00	9763	891	228
30	1.00	12425	1104	267
30	1.00	11952	1177	201
		Counting time: 5 minutes.		
Sample μg Cd	Activity μCi	Counts Roots	Counts Stems	Counts Leaves
30	1.00	23723	1692	522
30	1.00	23083	1166	420
30	1.00	18782	1721	574
30	1.00	20263	1778	496

The three experiments were carried out and analysed in order to obtain more results confirming the favorable localization of Cd which was shown to be in the order: Roots > Stems > Leaves as indicated in the results of all the experiments .

2.7. Discussion

2.7.1. Discussion of Part ~~one~~ I .

Interesting observations were made as a result of experiments carried out to investigate the effect of pH on Cd uptake by plants.

1. Experiment 1

During this experiment it was observed that at the two lowest pH levels of 3.1 and 4.0, Cd uptake by plants and the total quantities of Cd per gram of dry matter for the same added amounts of Cd were significantly higher than for plants grown in high pH solutions. It was also observed that for the same high levels of Cd (100, 120 and 140 µg total) and the lowest pH values (3.1 and 4.0) plants responded quicker in showing the first Cd effect symptoms. The first signs of Cd toxicity were observed at the third day of the experiment. A complete yellowing of the leaves(chlorosis) was shown by plants grown in low pH solutions containing 100 µg of Cd and more. Necrosis was also observed in stems of all samples containing 60 µg of Cd and more in the increasing order of the brown colour at the end of the stems. The same observations were made for the other three experiments. Although the growth of the bean plants was carried out in the same type of medium and at the same conditions, uniformity in plant growth was difficult to obtain and this created significant differences in Cd uptake and total Cd in dry matter. The overall pattern of the results is still valid for all experiments in terms of Cd effects on yield and the effect of pH on Cd uptake by plants.

After studying all the figures shown in this chapter, the following conclusions were drawn:

(i) By extrapolating the three curves one may suggest that it is clear that quantities of Cd taken up by bean plants were higher at pH 3.8 and pH 4.0 than at pH 6.0 This indicates that the optimum pH value ranged between 3.0 and 4.0 and this range may be extended from 3.0 to 5.0 according to the results achieved in experiment 3 and experiment 4.

(ii) As far as the quantities of Cd remaining in solutions were concerned and as one may expect, there was more Cd retained in solutions at pH 6.0 than the two others, 3.8 and 4.0. This could be explained by a possible formation of an insoluble Cd salt at pH6.0. Supporting evidence was also obtained when plotting the total micrograms of Cd remaining in solutions against pH. The highest quantity of the remaining Cd was obtained at the highest pH values of 6.0, 6.9 and 7.8. It was observed that at high pH values, the uptake of Cd by plants was less and the appearance of Cd toxicity symptoms was delayed for plants treated with high levels of Cd. Considering the composition of the nutrient solution used, there is a possibility of formation of a Cd-phosphate salt which may retain Cd in solution. The equilibrium reaction could be as suggested by Street et al. (1978).

$$Cd_3(PO_4)_2(C) + 4H^+ = 3Cd^{2+} + 2H_2PO_4^-$$

This suggestion of the formation of an insoluble $Cd_3(PO_4)_2$ was consolidated when four experiments were carried out using nutrient solution and Cd as $Cd(NO_3)_2.4H_2O$. An amount of Cd of (180 µg) was added to 5 samples buffered at different pH values of 3.4, 4.5, 6.5, 7.3 and 8.2. One hour later a gray-white precipitate appeared in samples at pH 6.5, 7.3 and 8.2, whereas the two others were clear. Later, after seven days, samples were filtrated and analysed by atomic absorption spectrometry . The filters(ashless) containing the precipitate were first digested with HNO_3 then analysed as samples. Results obtained are as illustrated in table 2.7.1.

Table 2.7.1

pH	Total µg Cd in sol	Total µg Cd in filter	Recovery %
3.3	171.37	4.11	97.48
4.5	160.95	12.85	96.55
6.5	106.42	67.44	96.58
7.3	52.77	117.62	94.66
8.2	37.24	131.23	93.59

From these results, it was suggested that the quantity of the insoluble $Cd_3(PO_4)_2$ increased with the increase in pH. To confirm the formation of the insoluble salt, an ultra violet spectrophotometer was used to determine the clarity of each sample by using an absorption wavelength of 700nm.

As was expected, the highest absorption was obtained at high pH values at which precipitates were also obtained(6.5, 7.3, and 8.2). The best chemical explanation to confirm this matter was to study the solubility product of Cd-phosphate by carrying out an experiment using only Cd and phosphate salts. Analytical and X-ray techniques are often used to determine the formula and the solubility product of the precipitate (Jurinak and Inouye, 1962). By making an analogy between Cd and Zn, the dissociation of cadmium orthophosphate could be written as shown below.(Jurinak and Inouye, 1962)

$$Cd_3(PO_4)_2.4H_2O = 3Cd^{2+} + 2H_2PO4^- + 4OH^-$$

and the pKsp will be written as follows:

$$pKsp = 3pCd^{2+} + 2pH2PO^{4-} + 4pOH^-$$

2.7.2. Discussion of Part two.2.

For all the experiments of part 2 , the radioactive cadmium(Cd-109) was added through the plant roots. This technique was very appropriate when studying the absorption and translocation of any element or compound. From the results achieved, information was obtained concerning the translocation of Cd and its preferable localization in the plant. It was observed that labelled Cd accumulated more in roots, stems and leaves respectively. This suggestion may join that of Cunningham et al(1975) and Cutler and Rains(1974) who investigated barley plant and reported that Cd was preferably accumulated by roots and stems than by leaves. This finding was in agreement with the suggestions of Wallace and Romney(1977) who after dividing several trace metals into three groups according to their distribution between the different part of a plant, classified Cd among trace metals which usually accumulate more in roots compared with other trace metals[Fe, Cu, Co, Mo,...]

From the results presented in Part One, it would appear that leaves responded faster to Cd toxicity than the two other parts. This may explain the chlorosis symptoms and suggest that Cd metabolism occurs mostly in the leaves which appeared to be more sensitive to Cd than the other parts of the bean plants. However, this would later possibly serve as a basis for the explanation of the effect of Cd on chlorophyll and the causes of chlorosis. In addition to this, one may mention again that plant species do differ in their response to trace metals as they differ in their type.

2.8. Conclusions

After looking at one aspect of the role of pH in Cd uptake by plants and also Cd transport, the following conclusions were drawn.

(i) pH did affect the uptake of Cd by plant beans by increasing it at low pH values (3.0 and 4.0) and decreasing it at high pH values (6.0 and 8.2).

(ii) pH suppressed the solubility and availability of Cd to plants by possibly forming insoluble Cd compounds such as $Cd_3(PO_4)_2$.

(iii) Plant yield(expressed as dry weight) decreased with decreasing pH and increasing Cd concentrations.

(iv) Reduction in growth due to root damage caused by Cd toxic levels[70 μg and above]

As a result of this investigation, more emphasis was given to pH as an important factor affecting both the behaviour of trace metals, their uptake by plants and also the medium they are present in culture solution or soil. John(1972a) carried out an experiment in soil with a range of pH values and using three plant species, radish, lettuce and soybeans.

CHAPTER 3

Study of cadmium and zinc interactions

3.1. Introduction.

Interaction may be defined as an influence, a mutual or reciprocal action of one element upon another in relation to plant growth process. It is also the differential response of one element in combination with varying levels of a second element applied simultaneously. The presence of a number of heavy metals creates interactions between them in soil or within the plant. This interaction is, as was mentioned earlier, a major factor affecting the behaviour of an element to be considered. Interactions can take place during absorption or within the plants or during their injection into animals. These interactions are sometimes described as antagonisms and are sometimes seen as competition for sites between one heavy metal and another in some compounds such as enzymes or those species involved in translocation. Very little is known about the mechanisms involved in the antagonism-type of interactions. But, *some chemical answers have been suggested concerning the competition for this type of interaction. This aspect of interaction between elements has been a matter of interest and has been investigated for many years by numerous research workers. Haghiri(1974) investigated the interactions between Cd and Zn in soybean grown in soil and John(1976) studied the interactions between Cd and K in lettuce grown in a hydroponic medium.

John observed that as a result of decreasing of the pH, Cd concentrations in all three species increased and he also observed that by adding lime to the soil,the pH increased from 4.1 to 5.5 causing a decrease in Cd concentrations in radish. Thus pH correction was always a positive method used to tackle the effects of some heavy metals including Cd on plants and soils. The use of the labelled Cd consolidated several reports concerning Cd effects and its toxicity to plants, its transport and distribution. Haghiri(1973) and Cunningham et al (1975) among many others used labelled Cd (Cd-115) and autoradiography techniques respectively for their investigation on soybean. They both suggested that Cd transport followed the sequence, roots > stem > leaves and this was in agreement with the results of this investigation. John(1972b); Page et al (197); Cutler and Rains(1974); also dealt with this matter. The technique also created a relationship between the effect of Cd and its favourite localization in bean plants(leaves), chlorosis and chlorophyll content. This would serve to correlate the results of the in vivo and in vitro experiments which can lead to the evaluation of the extent of Cd effects on plant species.

Through this review emphasis will be put on the work carried out by Olsen(1971) believed to be a complete study of the matter.

Olsen studied the interaction between zinc and phosphorus because of the widespread use of phosphatic fertilizers and emphasized the complex nature of the relation between plant growth, nutrient concentration in solution and within the plant. He reported that plant species and varieties vary in their susceptibility to iron and zinc deficiencies,to interactions of phosphorus with iron and zinc and interaction of the latter with iron and other micronutrients. Most of Olsen's emphasis was concentrated on zinc-phosphorus interactions about which he concluded that zinc deficiency was caused by high levels of available phosphorus or its application to soil. These interactions may constitute a positive contribution in protecting plants against injury from some heavy metals when there is a high supply of phosphate. The Zn deficiency caused by P was explained by Olsen in 1971 as an excessive concentration of P interfering with the metabolic function of Zn at certain sites in the cell. He also explained it as an interaction between P and Zn. The cause of this interaction was suspected to be the formation of an insoluble salt with the composition, [$Zn_3(PO_4)_2$]. This causes a reduction in available zinc and therefore creates a deficiency in Zn. This suggestion was mentioned earlier by Boawn and Viets(1954) and by Kalyanasundaram and Mehta (1970). They confirmed that this precipitation was indeed a mechanism causing zinc-deficiency and concluded that there was an antagonistic relation between zinc and phosphorus in soils.

Loneragan(1975) also reported that the concentration of other micronutrient or macronutrient ions in solution may strongly influence the absorption of other trace metals. Later in 1978, Leeper reported that Fe or Mn deficiency symptoms were induced in plants given large amounts of Zn, Ni or Cu metals. As far as interactions of heavy metals in animals are concerned, many workers contributed with major suggestions like Bunn, and Matrone(1966) who studied the interaction of Cd, Zn, and Fe in the mouse and rat, Mills (1974) and Petering(1974) studied the effect of other heavy metals on the metabolism of other heavy metals such as for instance the effects of Cd on Cu and Zn metabolism in ruminants and rats. Many more suggestions and conclusions can be cited concerning the inter-relations between heavy metals and between these elements and other major macronutrients in soils, plants and animals(Tiffin, 1967; Allaway 1968; and also Kawasaki, and Wallace 1980; Wallace and Romney 1980, who carried out their studies using corn (Zea mays) as a model.

3.2. Interaction of Cd with other elements

With the growing interest of the effects of cadmium on plant systems, emphasis on studying its behaviour, effects and its interelation with other elements was also growing. Thus, investigations and studies on cadmium-interaction with other elements were developed to investigate cadmium response to the presence of other elements in soils, plants and animals.

Experiments undertaken for this purpose gave significant results and suggestions which made significant contribution in solving many problems related to Cd and Cd-toxicity in the three systems quoted above. The most important conclusion was the discovery of an antagonism-type of effect between Cd and some other elements (Lagerwerff and Biersdorf, 1971, and Jarvis et al, 1976). This helped to minimize and inhibit in some cases Cd-effects on plants and animals. The review below will give us more details about some major suggestions and conclusions.

3.2.1. In animals:

Many tests and experiments were carried out using different animals with the main objective to investigate some suspected Cadmium-induced injuries (testis injury, kidney, liver, etc...). Earlier, after experimenting on chicks, Hill et al(1963) reported that the administration of large amounts of Cd interfered with the metabolism of copper and iron as well as with zinc. To explain this biological interference it was necessary to go back to 1960 when Kagi and Vallee invoked thioneine (or metallothioneine) involved in resistance to heavy metals toxicity and the fact that it also served to sequester Cd as a major factor in its biological effect . Anke et al (1971) reported that high levels of cadmium block the protein which is necessary for the Cu-absorption and decrease the amount of Cu in maternal and foetal tissue. They also noticed that feeding of Cd caused low liver-Cu and death of infant goats due possibly to a competition between Cd and Cu resulting in a displacement of Cu by Cd.

Evans et al(1970) confirmed earlier suggestions made by Powell et al (1964) and Hill et al (1963) when they studied the interactions between Cd, Zn, and Cu present in bovine liver and they reported that Cd and Zn decreased Cu and antagonized its metabolism by interfering with the -SH binding sites on a thioneine-protein type in the intestinal lumen. Evans added that the antagonism of Cu by Zn and Cd was due to interferences with the binding of Cd to sites on a thioneine-protein type in the intestinal lumen. Later Petering (1974), after studying the effect of Cd on the metabolism of Cu and Zn in the rat reported that Cd interacts with Cu by perturbing and interfering with its metabolism. Investigating the effect of dietary composition on the development of imbalance and toxicity in ruminants (sheep and calf), Mills (1974) confirmed the existence of an antagonistic effect of cadmium upon copper and zinc (see also Parizeck, 1957) and concluded that Cd strongly depressed the concentration of copper. Gabbiani et al (1967) observed that increasing levels of dietary cobalt does show effectiveness in decreasing Cd effects after adding Co as a mean to minimize the detrimental effect of Cd. Additionally, Mason and Young (1967) reported that the same effect of Se on Cd was observed when levels of dietary Se were increased in rats and shown to reduce Cd content in them. The work of Johns et al(1923); Banis et al (1969); Parizeck(1974) and Matrone(1974) also dealt with this matter.

3.2.2. In soils and plants

The important findings and conclusions on Cd interelation with other elements was as mentioned earlier a positive contribution to study the effect of Cd on animals as well as on soils and on plants. It was positive in the sense that the addition of some elements was shown to be effective in minimizing Cd effects on animals, soils and plants by interaction. Thus, in addition to the studies carried out on animals, experiments and investigations were also carried out on soil and plants using a large range of plants species. Working with beans, Hawf and Schmidt(1967) reported a decrease in Zn concentration in beans when Cu^{2+} was added to the solution and suggested that the competing ions exerted their effects mainly at the site of uptake into the plant and not in translocation process (Figure 3.2.1) Hawf and Schmidt also observed interactions between Zn^{2+} and Cd^{2+} and between Zn^{2+} and Mn^{2+}. Jarvis et al (1976) observed that, in the short-term experiments, the uptake of Cd by living roots of ryegrass was considerably depressed by Ca^{2+}, Mn^{2+} and Zn^{2+} competing for sites at the root surface. The interaction of Cd with Zn, Cu and Ni respectively was also reported by Wallace and Romney(1977) who experimented with bean plants. They observed synergistic effects between the Cd and Ni concentrations and they also observed that the concentrations of P and Zn were decreased as a result of Cd and Ni interactions. The effects were mostly noticed in the stems of the plants presented as a brown colour across the stems. They also reported that Zn and Cu decreased Cd in roots when added together.

Later in 1977, after studying the growth of the American Sycamore(Plantanus Occidentalis) , Carlson and Bazzaz (1977) concluded that there was a synergistic effect of Cd and Pb which reduced the root growth and the photosynthesis. The synergistic effect of Cd and Pb was also reported by Miller et al (1974) suggesting that Cd and Pb had more effects on corn seedling radicles when added together even at low concentrations compared with their added separate effects (see also Hassett et al 1976). Koeppe(1977) came up with different suggestions and reported that the interaction between Pb and Cd was rather additive than synergistic as it was observed in corn root elongation investigated by Miller et al in 1974. The answer to these differences between both suggestions could be correct because two completely different models were used, a tree (American sycamore) on the one hand and a herbaceous plant (corn) on the other. Differences in the behaviour of both elements and the responses of the two models are to be expected. The type of interactions depends mainly upon the concentrations of elements involved, the type of plant and the conditions under which experiments were carried out. More observations were made on soil by Haghiri (1974) who reported that addition of potassium to a silt loam soil suppressed Cd uptake by soybean and reduced Cd uptake by lettuce when added to solution (see also John, 1976).

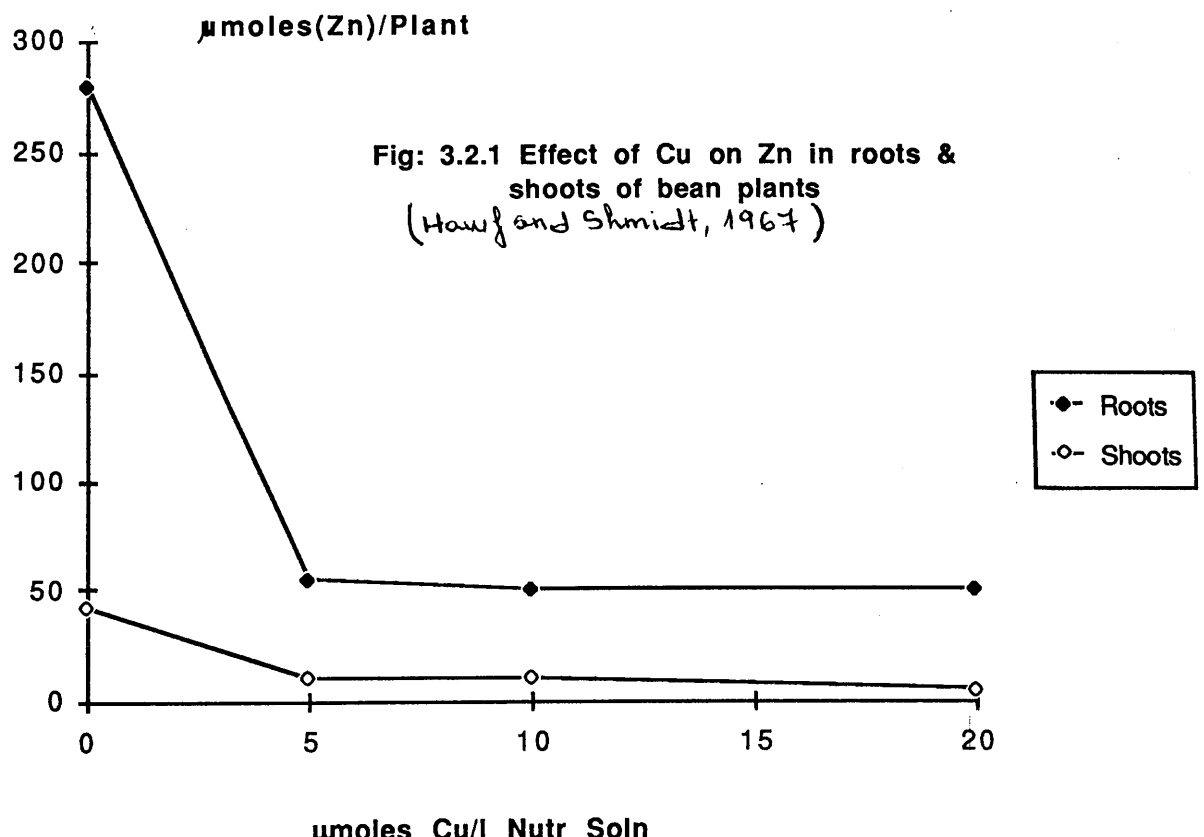


Fig: 3.2.1 Effect of Cu on Zn in roots & shoots of bean plants
(Hau∫ and Shmidt, 1967)

3.3. Interaction between Cd and Zn.

3.3.1. Introduction.

Throughtout the work and suggestions already reviewed, one conclusion is to be drawn and that is the importance of the findings and their positive contribution in solving many problems especially in agriculture, (Boawn, 1954 and Olsen, 1971). The study of interaction between any two or more trace metals was carried out but the Cd-Zn interaction was of particular interest. The investigation on the interelation of these two elements attracted great attention and was a subject of research for many years because of the importance of Zn in soil, in biological systems and also because of the closest chemical similarities between them. Thus, the investigation of these two elements became the major concern of many workers. Some of the investigations carried out on soil, plants and animals will be reviewed and the following table is a list of some Zn-enzymes known to be important for both plants and animals.

3.3.2 In animals.

Dietary Cd is known to be toxic to several species of monogastric animal (Johns et al 1923 and Byerrum et al 1960), because it is a strong antimetabolite for zinc (Parizeck and Zahor,1956 and Cotzias et al 1961). The primary objective of studying the interaction of Cd and Zn was to obtain some information on the Cd and Zn relationship in animals and to

determine whether additional Zn would alleviate the Cd toxicity shown in many cases. Working with rats, Parizeck in 1957 demonstrated in addition to what was suggested by Elcoate et al in 1955, that chronic nutritional Zn-deficiency causes testicular damage and that Cd leads to the same effect since both elements have similar physico-chemical properties. He also reported that the addition of large quantities of Zn was shown to be effective in preventing any eventual damage of testicles by Cd suggesting competitive inhibition between the two elements. He consolidated his findings by making a comparison with what was suggested by White and Munn, in 1951, who found that the inhibitory effect of Cd on yeast growth was antagonized by the addition of Zn to the medium. What Parizeck demonstrated regarding the Zn-protection against Cd injury was shared by many others. Kar et al(1960) and Gunn et al(1961) showed that the effect of Cd on rat testis can be prevented by administrating a large dose of zinc.

Experimenting with turkey, Supplee (1961) reported that addition of Zn to a turkey poultry diet containing Cd prevented the appearance of most Cd-toxicity symptoms. Hill et al(1963) later added that besides the preventative aspect of Zn, Cd showed a tendency in replacing Zn and Cu at active sites such as enzymes, thereby transforming them into inactive sites. The work carried out by Powell et al(1964) experimenting on calves was a great contribution in giving details about the important role played by Zn and its interaction with Cd . After feeding calves with a range of different concentrations of Cd from 0 ppm to 2560 ppm, Powell observed that at the highest Cd levels of 2560 ppm and 640 ppm, the calves exhibited some clinical symptoms similar to those attributed to Zn

deficiency (Miller and Miller, 1962). Therefore, the explanation for this can be associated with the interaction between Cd and Zn. He also observed that those animals fed with 2560 ppm and 640 ppm of Cd had higher Zn concentrations in both livers and kidneys. This increase of Zn in these two organs can be attributed to Cd acting as an antimetabolite rendering certain Zn-enzyme molecules inactive. Thus a deficiency of Zn can be expected due to the competitive action of Cd. Finally, Powell et al concluded that the supplemental Zn had a tendency towards reducing the toxic effect of Cd. The interaction of Cd and Zn ions was also confirmed by Banis et al(1969) who, after studying rats concluded that liver-Zn was increased by Cd (see also Ferm et al 1968). After experimenting on rats, Webb(1972) reported that Cd added to the animal was mainly accumulated in the liver or the kidney and it was found to be bound to a soluble protein identified as metallothionein. He also reported that animals containing Zn-protein in the liver showed an increased resistance to Cd toxic effects.

In another experiment with rats, Webb(1972) concluded that the pre-injection of male rats with 20 mmoles of zinc 24 hours before the administration of 4 mmoles Cd protected the animal from Cd injury. Webb suggested that this pre-treatment with Zn caused in the livers of the rats the synthesis of a protein with a high binding affinity for Cd. The Cd ions are consequently immobilized more rapidly than in the livers of rats without Zn pre-treatment because of their chemical similarities by inducing the same protein. The metallothionein or the metallothionein type of protein was mentioned in many experiments as being a key explanation for Cd and Zn interactions in the biological system.

The important role played by this type of protein in retaining some heavy metals including Cd and Zn was emphasized by Mills(1974). Mills stated that the metallothionein-type sequesters Cd and other heavy metals from sensitive sites and therefore, the protein creates antagonistic interaction between them which could eventually causes an excess or deficiency of one or more trace metals. As a result of this experiment, he concluded that increasing levels of Cd increased the concentration of Zn in the liver of the sheep and young calves and that Zn depresses the retention of Cd. Additionally, Bremner(1974) found that the addition of Cd and Zn to the animal diet may induce the formation in the kidney and the liver of a soluble cysteine considered to be the main amino acid of the thioneine.

Table 3.3.1. List of some zinc-enzymes: From Ochiai, 1977.

Enzyme	Definition and Role
Carbonic anhydrase	It catalyses the formation of carbon dioxide from hydrogen carbonate in the blood vessel of lung. Zinc is present as co factor. It is present in animal and in plant tissue.
Carboxy peptidase	A pancreatic enzyme which detaches terminal amino-group of peptides. Zinc is a cofactor.
Alcohol dehydrogenase	Enzyme that catalyses the oxidation of ethanol to acetaldehyde. It has zinc as cofactor and it is present in animal and plant tissue.
Aldolase	Catalyses the cleavage of fructose aldolase 1,6-diphosphate to glyceraldehyde 3-phosphate. It is present in muscle. Aldolase in plant does not contain zinc.
Arginase	Catalyses the splitting of urea from the amino-acid arginine. It is found in liver.
Alkaline phosphatase	Hydrolyses the many different combinations of phosphatic acid with organic substances.It is present in blood.
Catalase	Catalyses the decomposition of hydrogen peroxide into molecular oxygen and water.It is present in plant and animal. It is used in food preservation (removing O_2).

Peroxidase	Found particularly in plants and some animal cells.It catalyses reactions in which hydrogen peroxide is an electron acceptor.
Pyruvate decarboxylase	Enzyme that hydrolyses the carboxy radical -COOH. It is present in living cells that carry the function of removing CO_2.
Glutamic Oxaloacetic Transmitase	Enzyme catalysing the transfer of amino group. It is found in all organisms.

The selected Zn_enzymes listed above are some among many others considered to be important for plant and animal tissues.

3.3.3 In plants and soils

The increasing danger caused by the high toxicity aspect of Cd attracted the attention of a great number of research workers who, as a result investigated Cd behaviour in soils and plants especially when present with other heavy metals. This matter became important and of concern since it was found that Cd interacts with other elements. Being aware of the dangerous consequences caused by Cd effects, investigators took a step forward in tackling this problem by closely investigating the relationship between Cd and other major heavy metals. Zn was the first choice which received most interest because of its chemical similarity with Cd. This suggested possible approaches by using Zn to reduce the Cd content of plants, therefore impeding its effects.

The close chemical association of Cd and Zn created a considerable interest for many workers to study the interaction between the two elements by using many different plant species.

First a review of some previous conclusions and suggestions. Working with bush beans, Hawf and Schmidt(1967) observed that Cd had an inhibitory effect on zinc uptake similar to that of copper. He also added that all ions used as competitor ions exert their effects on the site of transport into the plant and not in the translocation mechanism, except when high concentrations are used(See Figure 3.2.2 by Hawf and Schmidt, 1967). Lagerwerff and Biersdorf(1971) studied the interaction between Cd and Zn in radish plants. He concluded that increasing Zn concentration suppressed Cd uptake at the two lower Cd concentrations in the solution. But at high Cd levels (100 ppb), increasing the amount of Zn increased Cd uptake as illustrated in the following tables. Investigating a range of plants (carrots, tomatoes, etc), Turner, (1973) found that Cd treatment caused an increase in Zn concentration and uptake in plant tops. This hypothesis was not shared by Koshino(1973) who studied Cd and Zn interactions in rice and wheat. He reported that Zn enhanced Cd uptake and addition of Cd decreased Zn uptake by the plants causing its deficiency. Later, John(1976) also found that the uptake of Cd by ryegrass grown in solution was depressed by adding Zn^{2+}, Ca^{2+}, and Mn^{2+} and suggested that the competition for exchangeable sites between Cd and the other metals takes place on the root surface. It is believed that when different species are investigated, differences in responses and conclusions are to be expected.

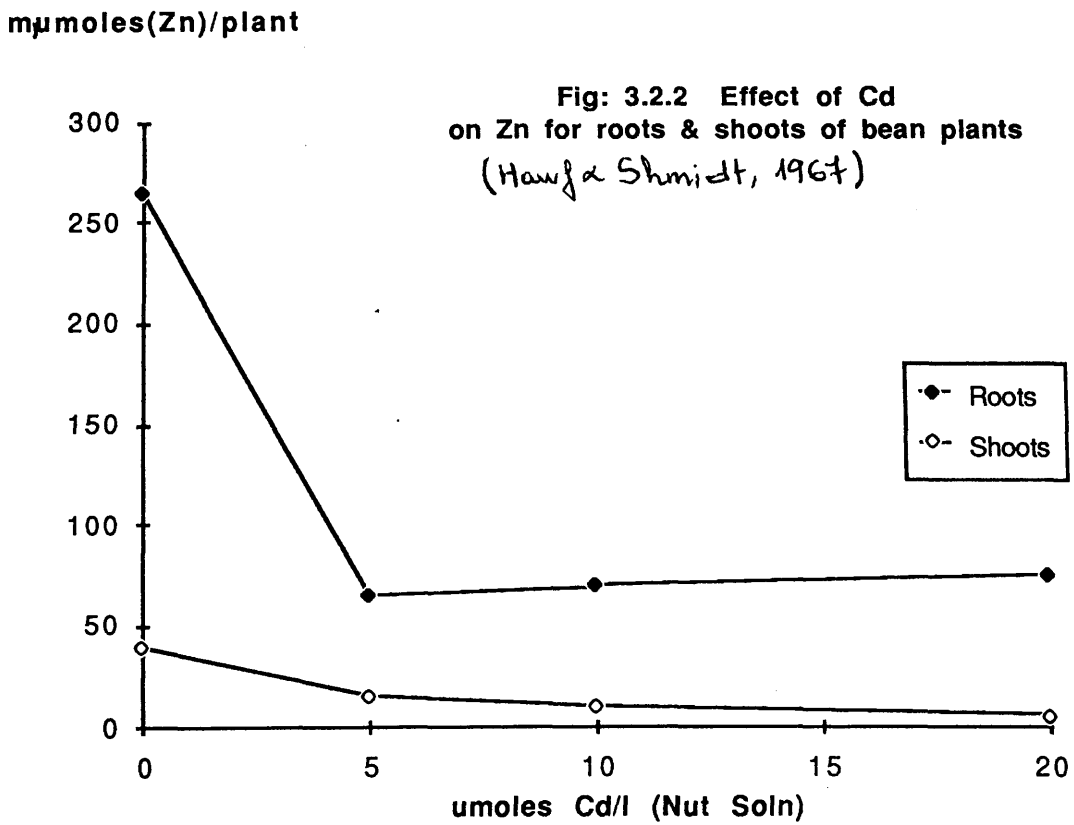


Fig: 3.2.2 Effect of Cd on Zn for roots & shoots of bean plants

(Hau & Shmidt, 1967)

Table 3.3.2. From Lagerwerff and Biersdorf, 1971.

Amounts of Cd and Zn in roots and leaves of radish grown in an open aerial environment on nutrient solutions variable in those metals.

	Zinc			Zinc		
Cd	ppb in soln(µg/g D.W)			ppb in soln(per µg/gD.W)		
in soln	20	100	400	20	100	400
(ppb)		Cd			Zn	
			Root			
2.0	2.1	0.8	1.0	82	119	267
20.0	6.1	5.3	4.3	76	112	243
100.0	16.1	17.8	22.5	65	106	210
			Leaves			
2.0	6.6	4.9	5.4	78	125	271
20.0	25.4	22.9	18.0	110	150	287
100.0	81.2	87.1	92.4	113	177	310

Zinc concentrations are expressed in µg/g of plant tissue.

From this table, one may observe that the concentration of Cd in the roots and leaves of the two lower levels of Cd [2.0 ppb and 20.0 ppb] decreased as the concentrations of Zn increased for the highest Cd concentration[100ppb] as those of Zn increased. Another indication of Cd and Zn interactions was obtained and presented on the table above as Zn uptake by the leaves increased at all levels of Cd with the increase of Cd

concentrations suggesting that the synergistic effect may be due to root damage caused by Cd. The competitive aspect of Cd and Zn interactions was indicated through the results of Zn concentrations of the roots which appeared to decrease slightly as Cd increased.

Table 3.3.3. From Lagerwerff and Biersdorf, 1971

Distribution of Cd and Zn in roots and leaves of radish grown in an opened aerial environment on nutrient solutions variable in those metals.

	Zinc			Zinc		
Cd	ppb in soln(per µg/g D.W)			ppb in soln(Per µg/g D.W)		
ppb in soln	20	100	400	20	100	400
		Cd			Zn	
			Roots			
20	36.5	23.9	23.0	66.6	66.0	61.2
20.0	34.3	33.7	30.4	60.0	62.1	60.7
100.0	27.3	27.3	27.4	62.3	52.3	51.2
			Leaves			
2.0	63.5	76.1	77.0	33.4	34.0	38.0
20.0	65.7	66.3	69.6	40.0	37.9	39.3
100.0	72.7	72.7	72.6	37.7	47.7	48.7

The experiment was mainly aimed at determining the distribution of Cd and Zn between the roots and the leaves. From the data shown above, one may observe that the accumulation of Cd was greater in leaves than in roots in a range (in %) of 63 to 77and 24 to 37 respectively. On the other hand, the accumulation of Zn was higher in roots than in leaves in

a range (in %) of 51 to 67 and 33 to 49 respectively.

After studying soybeans, Haghiri(1974) found that addition of Zn from 5 to 50 ppm greatly increased the concentration of Cd in soybean shoots causing a damage of plants which leads to a decrease in their growth. An increase of the displacement of Cd into the soil solution from the soil exchange complex was observed as a consequence of the damage of the plant. He also reported that addition of large amounts of Zn depressed Cd. He explained this as a dilution of Cd in the soil solution rather than a direct effect of Zn. The suggestions made by Haghiri(1974) were shared by many others among them, Jarvis(1976) who investigated ryegrass and reported that the uptake of Cd by roots was reduced when Zn was added. Both synergistic and competitive effects were observed by Lagerwerff and Biersdorf (1971) and Haghiri (1974). More about Cd and Zn interactions can be cited in addition to what was mentioned earlier in the previous chapters. (John, 1976; Maclean, 1976; Cunningham et al 1977, and Wallace and Romney 1980). Before starting the investigation on Cd and Zn interactions, a preliminary experiment was first set up using only Cd to evaluate its degree of toxicity, its symptoms and plant responces. Then a further experiment was set up using a combination of Cd and Zn concentrations. The main objective was to study the interaction between the two elements and its repercussions on plant growth.

3.4. Experimental.

3.4.1. Procedure.

The method of growing beans was the same as described in chapter 2. The only changes were the addition of Cd separately and also combined with Zn. The range of Cd levels was in total μg 0, 20, 60, 100, 140 and 180. That of Zn: 0, 2000, 6000 and 10,000 μg. When plants reached a mature stage (usually after 12 days in vermiculite), they were transfered to separate 100ml flasks containing the nutrient solution and different concentrations of Cd and Zn. The culture of beans lasted 7 days before being harvested and analysed. Furthermore, daily check and observation were carried out during the experiment.

3.4.2 Analysis.

After the seven-day fixed period, plants were harvested, cut into parts, weighed then dried, (see method in Chapter 2). The dried plants were then wieghed a second time, digested, filtered and analysed for Cd, Zn and also Fe using atomic absorption spectrophotometry. The remainders of the nutrient solutions were filtered, their volume measured then analysed the same way as the plant tissue described earlier.

3.5. Results (+ SD). The statistical calculations were carried out using the Macintosh Plus Cricket program.

Experiment 1. (Number of observations, 4). It was carried out at a fixed Zn level of 2000 µg total added to the nutrient solution combined with a range of Cd levels of 20 to 180 µg total. The data below shows the uptake of Cd by bean plants as affected by the addition of Zn.

Table 1.

Sample µgCd	D.W g	µgCd taken up	µg Cd in rem sol	µg/g D.W
20	0.27±0.03	10.05±0.98	6.98±2.42	56.75±16.61
60	0.26±0.01	23.70±3.03	30.99±3.28	9.06±1.33
100	0.23±0.04	22.22±0.41	65.50±3.33	99.29±14.07
140	0.18±0.02	24.46±3.70	96.67±8.31	134.60±20.62
180	0.21±0.01	29.94±1.87	126.00±5.57	174.70±57.12

Experiment 1. (Number of observations 4). It was carried out the same way as the one above with an addition of 6000 µg total Zn to the nutrient solution.

Table 2.

20	0.22±0.02	8.16±1.17	9.83±1.23	36.59±4.65
60	0.22±0.03	14.68±1.25	40.70±4.18	67.28±14.04
100	0.18±0.04	19.67±0.78	76.21±3.36	111.7±18.82
140	0.20±0.03	18.64±1.42	109.70±5.14	93.25±14.75
180	0.14±0.03	32.93±2.39	124.10±14.83	233.0±44.8

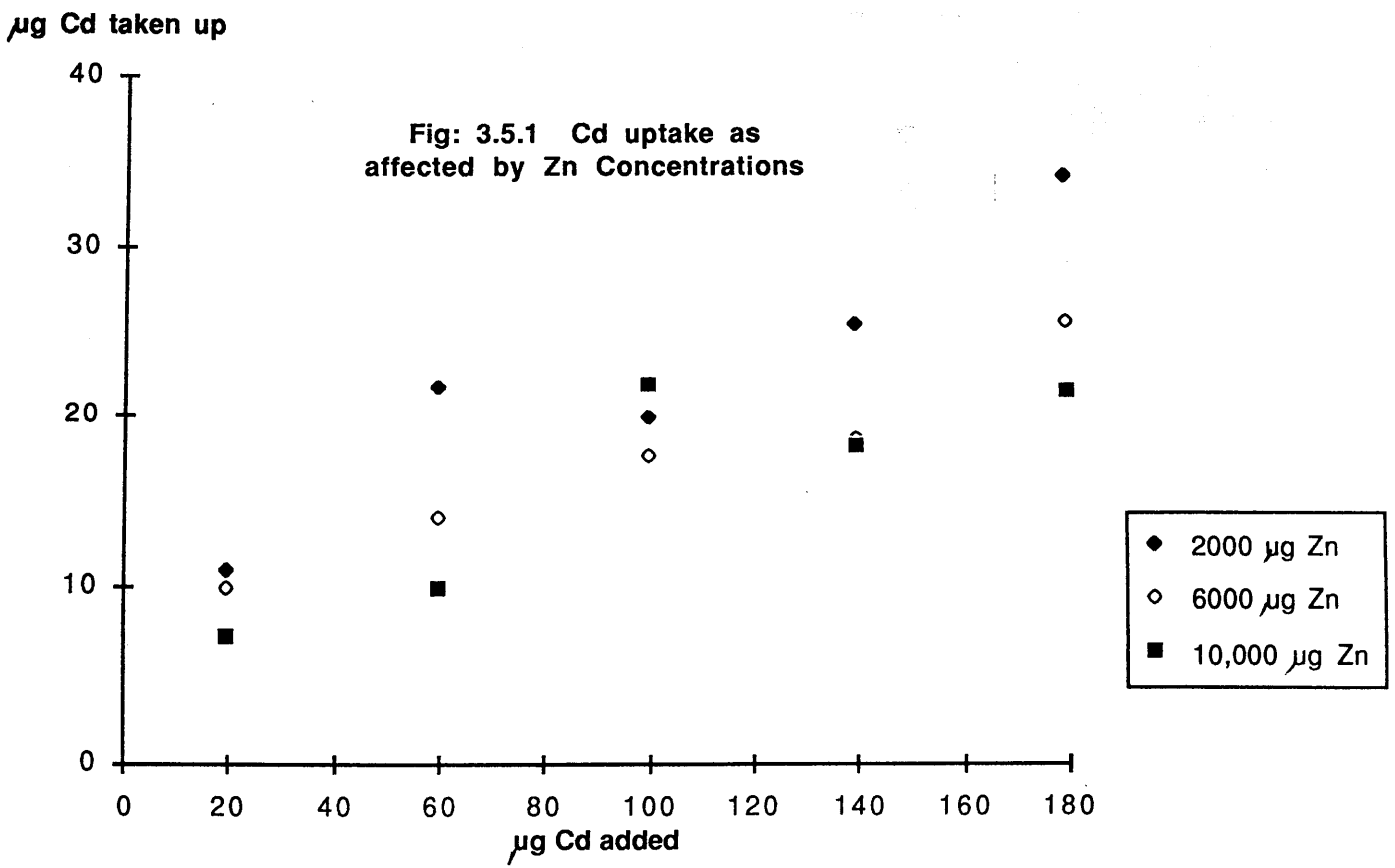


Fig: 3.5.1 Cd uptake as affected by Zn Concentrations

Experiment 1. (Number of observation: 4). It was carried out as previous ones with an addition of 10,000 μg total Zn to the nutrient solution.

Table 3.

20	0.20±0.04	7.44±0.91	10.61±0.43	38.01±5.42
60	0.21±0.02	13.27±2.20	43.93±2.50	61.22±8.80
100	0.19±0.02	19.93±1.60	68.63±2.55	125.50±37.23
140	0.22±0.06	17.21±1.56	111.10±6.84	83.82±25.99

Experiment2. (Number of observation: 4) It was carried out as experiment 1 and also aiming at determining the effect of Zn on Cd uptake by bean plants with a first addition of 2000 μg total Zn to the nutrient solution.

Table 1.

20	0.30±0.04	10.90±0.75	8.41±1.36	37.27±7.97
60	0.25±0.03	21.69±2.53	34.16±5.37	89.60±5.90
100	0.26±0.03	19.78±3.68	76.95±4.68	78.11±19.92
140	0.26±0.03	25.31±2.31	107.00±3.71	96.68±16.98
180	0.29±0.07	34.12±3.85	122.30±5.41	124.40±17.83

Experiment 2. (Number of observations: 4). It was carried out at a range of Cd levels of 20 to 180 μg with a 6000 μg total Zn added to the nutrient solution.

Table 2.

20	0.21±0.03	10.03±0.98	9.01±1.41	49.36±9.72
60	0.26±0.02	13.98±0.79	39.46±2.05	53.39±6.41
100	0.24±0.02	17.55±0.94	71.24±9.64	72.53±4.29
140	0.20±0.01	18.44±2.82	112.2±5.9	91.24±15.17

Experiment 2. (Number of observations: 4). It was carried out at a range of Cd levels of 20 to 180 μg with a 10,000 μg total Zn added to the nutrient solution.

Table 3.

20	0.25±0.05	7.13±0.87	10.79±1.24	29.96±6.57
60	0.24±0.05	10.04±0.66	44.72±3.13	42.90±11.02
100	0.28±0.03	21.72±1.77	69.01±2.55	78.18±10.29
140	0.23±0.03	18.07±2.37	115.00±5.73	78.29±11.13
180	0.24±0.03	21.34±2.53	142.40±11.74	92.38±20.43

Experiment 3. (Number of observations: 4). It was carried out at a range of Cd levels of 20 to 180 µg with a 6,000 µg total Zn added to the nutrient solution

Table 2.

Sample µgCd	D.W g	µgCd taken up	µg Cd in rem sol	µg/g D.W
20	0.31±0.034	7.79±0.47	9.99±1.39	25.10±3.21
60	0.27±0.03	17.68±2.19	38.82±3.69	65.98±10.33
100	0.26±0.02	19.98±1.07	72.18±2.89	73.32±3.35
140	0.24±0.02	15.61±0.84	109.70±10.68	64.64±4.33
180	0.23±0.02	26.19±0.82	134.80±10.86	115.00±13.71

Experiment 3. (Number of observations: 4). It was carried out at a range of Cd levels of 20 to 180 µg with a 10,000µg total Zn to the nutrient solution.

Table 3.

20	0.26±0.03	8.71±0.66	10.01±1.05	33.61±5.83
60	0.24±0.02	13.91±1.40	42.13±1.88	57.79±9.02
140	0.24±0.03	14.84±1.47	93.85±5.36	61.32±1.81
180	0.20±0.03	17.14±1.41	145.40±4.11	85.94±1.53

Experiment 4 differs from the previous ones by fixing the amounts of Cd [100, 140, and 180 µg total] and varying those of Zn [2000, 6000, and 10,000 µg total]. The experiment is aimed at demonstrating the mutual effect of the two elements and its inplications on their uptake by bean plants.

Experiment 4. . (Number of observations: 5). It was carried out at a fixed level of Cd of 100 µg combined with a range of Zn levels of 2000, 6000, and 10, 000 µg respectively.

Table 1

Sample µg Zn(x 10^3)	D.W g	µg Zn taken up	µg Zn in rem sol	µg/g D.W
2	0.20±0.03	348.3±31.66	1530.0±149.6	1752.0±305.5
6	0.24±0.01	1025.0±103.7	4305.0±366.1	4310.0±391.0
10	0.29±0.04	1446.0±59.5	7049.0±397.0	5078.0±596.0

Experiment 4. (Number of observations: 5). It was carried out at a fixed level of Cd of 140 µg total combined with a range of Zn levels of 2000, 6000, and 10,000 µg.

Table 2

Sample µg Zn(x 10^3)	D.W g	µg Zn taken up	µg Zn in rem sol	µg/g D.W
2	0.18±0.03	141.3±19.48	1727.0±69.9	782.0±143.6
6	0.20±0.03	547.6±40.1	4620.0±211.0	2737.0±505.7
10	0.23±0.02	675.0±39.5	7983.0±384.0	2895.0±198.0

Experiment 4. (Number of observations: 5). It was carried out at a fixed level of Cd of 180 µg combined with a range of Zn levels of 2000, 6000, and 10,000 µg.

Table 3

Sample µg Zn(x 103)	D.W g	µg Zn taken up	µg Zn in rem sol	µg/g D.W
2	0.18±0.02	74.02±3.22	1776.0±78.0	422.7±63.7
6	0.18±0.03	380.64±39.5	5199.0±244.0	2117.0±419.0
10	0.24±0.04	887.6±88.9	8088.0±455.0	3746.0±840.0

3.6 Discussions.

1. Experiment 1

During the 7-day period of the experiment daily pH checks were carried out and the following observations were made:-

a. Considerable decrease in plant growth especially those containing more that 60 µg of total Cd added. Compared with the control plants, plants containing less that 60 µg of Cd did not show any obvious sign of Cd effects.

b. Cd toxicity was confirmed visually and by determining levels of Cd in bean plants shown to be affected by Cd. The effect of Cd was presented as yellowing of the leaves and also its effect on the growth of the plants but the symptoms were less pronounced than in the earlier experiments where no zinc was added. (see Chapter 2). In this experiment Cd toxicity symptoms were presented as grey spots on the leaves and strong yellow ones on the leaves of plants containing higher concentrations of cadmium,(100 µg and 140 µg). The percentage recovery was over 80%, the rest could be expressed as losses in Cd retained in filters or errors caused by mishandling.

2. Experiment 2

In this experiment Cd effects were clearly observed and Cd toxicity symptoms were more pronounced than those observed in experiment 1. Yellow spots were observed on the leaves of plants containing 100 µg of Cd and those containing the highest levels, 140 µg and 180 µg presented a complete chlorosis. Roots did not appear to be greatly affected by these Cd levels whereas stems showed the same kind of effect observed earlier in Chapter 2. The stems were dry at the middle and stunted with a slightly brown colour at the root level and this may have been a symptom of necrosis.

3. Experiment 4

The effect of Zn on Cd was investigated and plant responses to the interaction were studied. However, a further experiment was carried out with the main objective to investigate the effect of Cd on Zn uptake by plants. It was observed that addition of Cd had a double effect.

a. Cd behaved as a major toxic element by delaying the growth of plants which exhibited various symptoms due to Cd toxicity. Dry matter was also decreased at all levels of Cd (100,140 and 180 total µg), combined with the 2000 µg and 6000 µg total of zinc.

The effect of Cd was minimized in plants containing the following combined concentrations:-

Cd(μg)	100	140	180
Zn(μg)	10,000	10,000	10,000

b. Cd had also a second effect on Zn by decreasing its uptake by plants. As far as experiment 5 was concerned, its main objective was to investigate the mutual effect of Cd and Zn and its repercussion on Fe which is known to be important in chlorophyll biosynthesis. Therefore the addition of sufficient iron (600 μg) comfirmed that chlorosis was due to Cd toxicity rather than Fe deficiency.

3.7. Mechanisms involved in the interaction between Cd and Zn.

The interaction between the two elements may be seen as competition for sites on the surface of the roots. The interaction could also be antagonistic due to the chemical and stereochemical similarities between them. Therefore, these similarities will be used to explain the action of Cd and Zn and the type of bonding occuring. The elements in group IIB (Zn, Cd and Hg) are known to have an electronic configuration $(n-1)d^{10}ns^2$ with a full (d) shell . This means that they involve only the ns^2 electrons for bonding as shown below.

a. $_{48}Cd: 4d^{10} 5s^2$

b. $_{30}Zn : 3d^{10} 4s^2$

Cd and Zn are diamagnetic and their highest normal oxidation state is +2 gained by losing the two electrons of the 5s becoming Cd^{2+} and Zn^{2+} respectively. By losing the two electrons, Cd and Zn present a state of forming dative or coordination bonds with ligands called also donors, with an sp^3 type of complex and sometimes an sp^3d^2 especially for cadmium ion (Cd^{2+}). This leads to the introduction of the concept of Lewis acids and Lewis base. Lewis classified Cd and Zn as soft and borderline acids respectively, playing the role of acceptors and ligands donors. In the coordination chemistry there are at least two mechanisms which could be involved in forming complexes; a substitution of a metal by another or a ligand by another and electron transfer. In the case of Zn and Cd, a substitution type of reaction would be predicted and more likely to take place because of their chemical similarities. The substitution reactions are not as simple as shown in the reaction below:

$$A + :B \_\_\_\_ A:B$$

forming a dative (or coordinate) bond: A _ B or $A^{\partial-}$ $B^{\partial+}$ but involve several steps including ionic dissociation and reactions of coordination (Hughes and Ingold). As zinc is present in many biological systems and plays a great role in various enzyme metabolism, however, its replacement by another metal, Cd for example, is to be expected. In that respect, it became important to look for some approach to explain some kind of mechamisms involved in an eventual substitution of Zn by Cd in some zinc-enzymes as a direct effect or indirectly through an intermediate, iron for example. (See table 3.3.1. of some zinc-enzymes as quoted earlier by Ochiai, 1977).

The following table illustrates some amino acid which can be linked to zinc enzymes as ligands and are present in most of the enzymes and in protein structure chains. (From Ochiai, 1977).

Table 3.7.1

Amino acid	Abreviation	R
Aspartic acid	Asp	$HOOCCH_2$
Glutamic acid	Glu	$HOOCCH_2CH_2$
Aspargine	Asn	H_2NCOCH_2
Glutamine	Gln	$H_2NCOCH_2CH_2$
Histidine	His	-CH2-C=CH-HNNH-CH
Cysteine	Cys	$HS\text{-}CH_2\text{-}$

From table 3.3.1 of Zn-enzymes, three major ones were selected because of their importance and also because Zn plays the role of the active group or the co-factor in their structure. Therefore they are the most likely to be affected as a result of Zn interactions with any other trace metals. They are carbonic anhydrase, alcohol dehydrogenase and carboxypeptidase.

1. Carbonic anhydrase (CA): It is mostly known for the catalysis of the following reaction. From Ochiai,1977 and Bertini et al, 1983.

$$CO_2 + H_2O \_\_\_\_\_ HCO_3^- + H^+$$

Carbonic anhydrase contains one zinc atom per molecule. The active site of this enzyme is constituted of 3 histidine moieties, see Fig 3.7.1.

2. Alcohol dehydrogenase: It catalyses the reaction below:

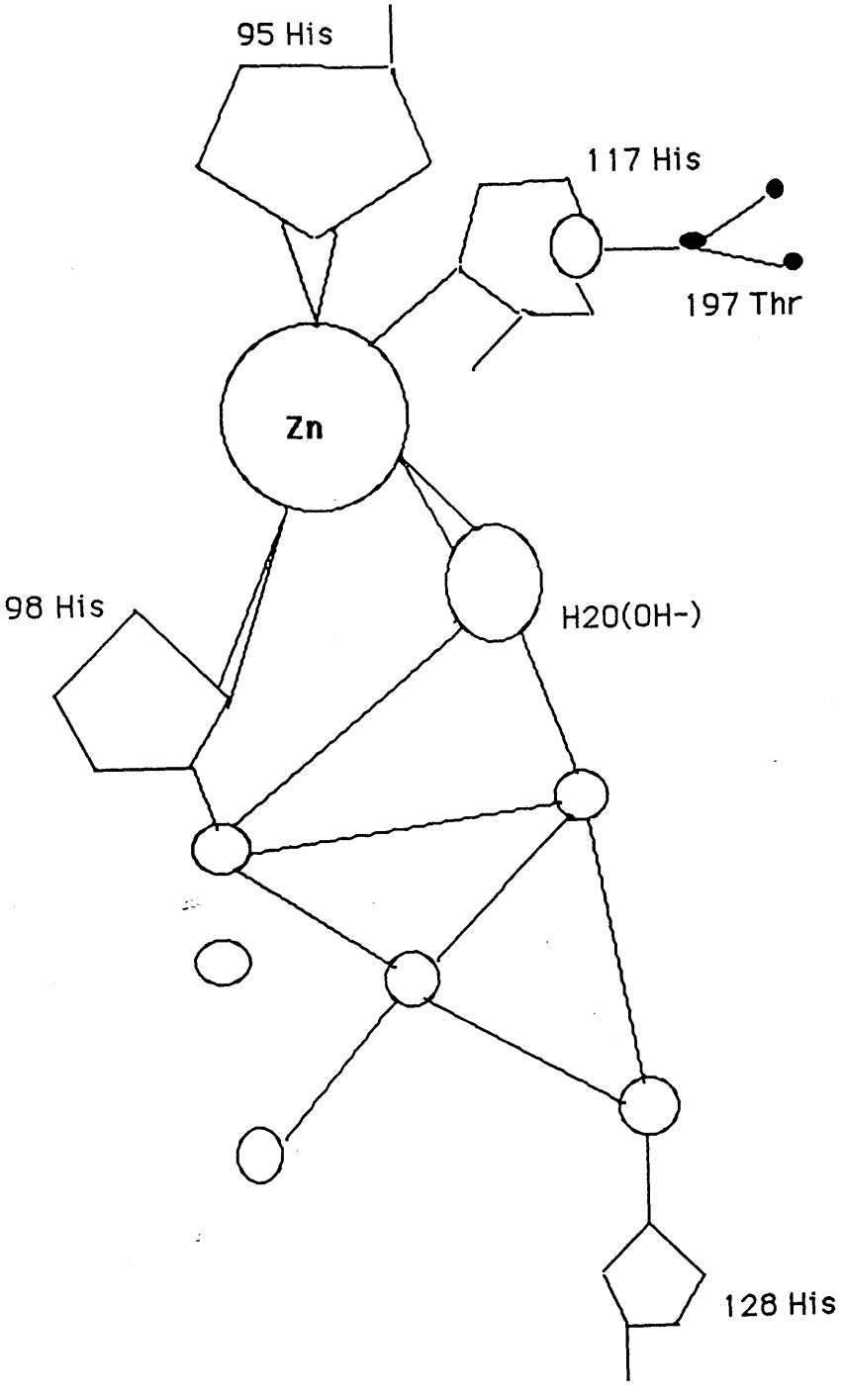
$$NAD^+ + C_2H_5OH \_\_\_\_\_ NADH^+ + CH_3CHO + H^+$$

The enzyme contains four zinc atoms per molecule and has 1 histidine and 2 cysteine to form its active site, see Fig 3.7.2.

3. Carboxypeptidase: The protein residues of the carboxypeptidase active site are 2 histidine and 1 glutamic acid moieties as shown in Fig 3.7.3.

Thinking in terms of Zn substitution by Cd in bioligical systems, the amino acids, histidine, cysteine, glutamic acid and aspargine play the role of ligands and they are known to be suitable donors. However, Cd may be attracted to form complexes with them and possibly will be held in an octohedral environment although its favourable one is tetrahedral considered to give more stable complexes(Basolo and Pearson, 1967).

Fig: 3.7.1 **Structure of the active site of the Carbonic Anhydrase.**

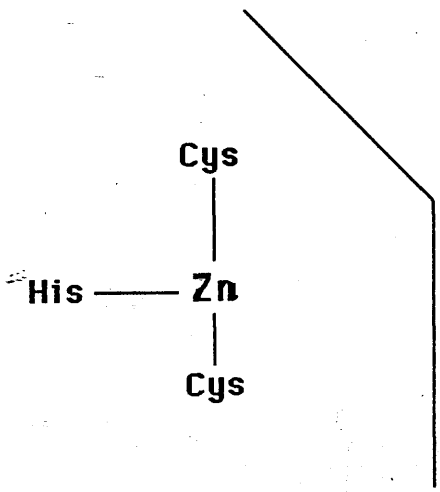


Coordination Sites of Zn(2) of Carbonic Anhydrase.(From: Kannan et al, 1972)

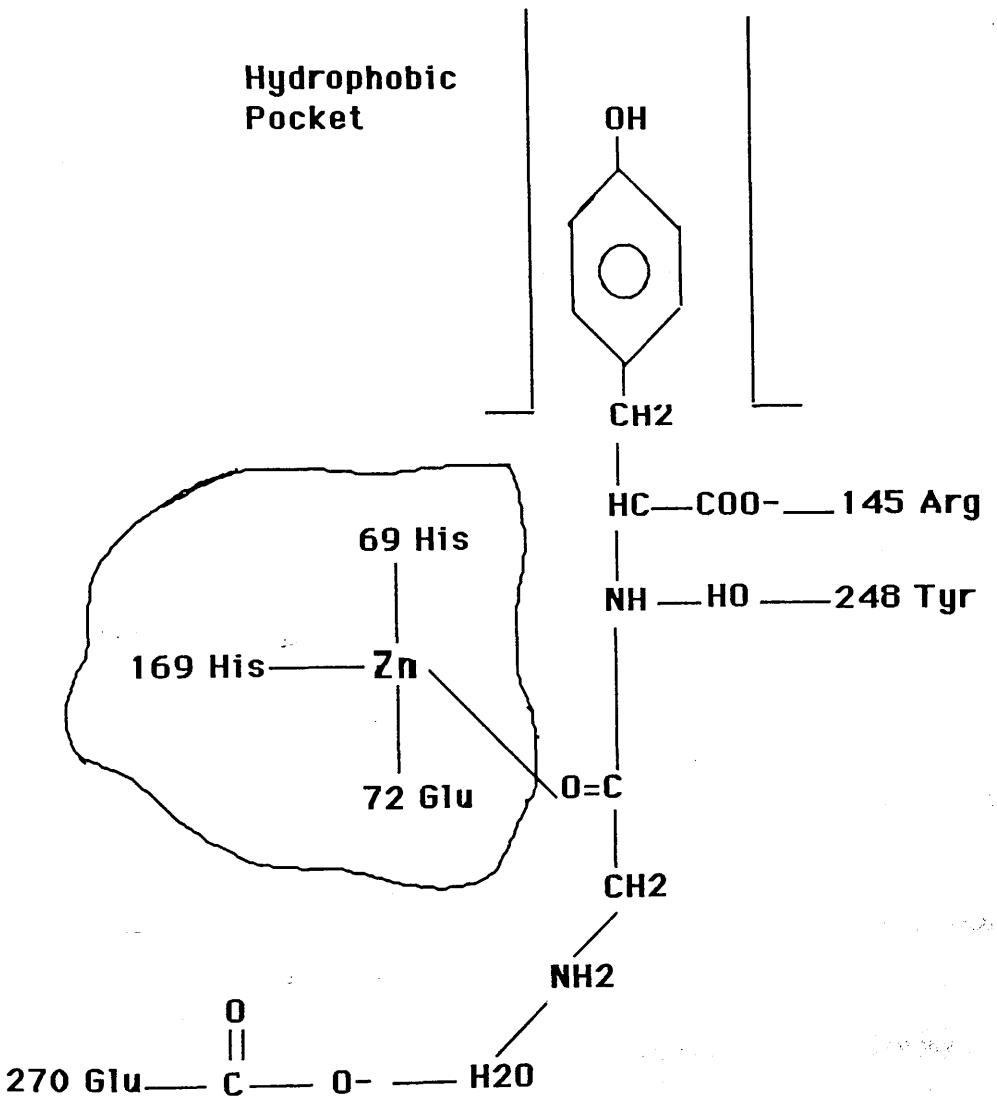
Fig: 3.7.2

Structure of the active site of the Alcohol dehydrogenase

From: Ei.Ichiro Ochiai, 1977.



37.3 Stucture of the active site of the Carbopeptidase
From: Quiocho and Lipscomb, 1971.



Because of the chemical similarities between the two elements, the possibility of Cd replacing Zn in the active sites in the structure of some exzymes would be suggested, thus rendering them inactive. After developing the concept of Werner of secondary valence, Lewis (1923) introduced the concept of the effective atomic number (EAN) to explain some aspects of coordination in the chemistry of coordinates. The effective atomic number was used to explain the favourable tetrahedric form of Cd^{2+}. The ion ligands tend to donate as many electrons as possible till the metal ion (Cd^{2+}) of the resulting complex gets the electonic configuration of the next noble gas. The most likely EAN for Cd^{2+} is +4 taking a total of 54 electrons, the atomic number of the next noble gas Xenon. In addition to the sp^3 complexes which may be formed by Cd^{2+}, octahedral (or sp^3d^2) complexes may also be found by bonding to six ligands. The mechanisms of the coordination complexes are very well documented and explained by Basolo and Pearson(1967) and Ochiai(1977). The main basis for the explanation is the coordination chemistry principle including three major theories, the valence bond theory, the crystal field theory and the electrostatic theory. Emphasis was put on the effect of Cd on Zn-enzyme metabolism as a result of their mutual interaction which may take place during their absorption and transport. All three aspects of interaction are resulting from the similarities between the two elements.

3.8. Conclusions

The investigation carried out in this experiment has enabled to a better understandings to be obtained about Cd effects on beans and their responses to Cd. It also demonstrated and confirmed the interaction between Zn and Cd and its implications on plant responses. Knowing that Cd was far more toxic to plants than Zn, it could be suggested that the addition of Zn was a positive contribution in minimizing the effects of Cd on beans. As a result of this investigation, some answers were reached concerning several questions related to plant responses to Cd and the mutual effects of Cd and Zn on their respective uptake by plants. The following conclusions may be drawn accordingly:-

1. The addition of Zn had an effect in depressing Cd uptake by plants and protecting them from Cd-injury. The opposite effect of Cd on Zn uptake was also observed when high levels of Cd were added (100 µg, 140 µg and 180 µg total). Therefore, this leads to suggest that there is an antagonism between the two elements which is positive for plants, soils and also animals due to the preventing role played by zinc. This comes in agreement with what has already been acheived by other investigators as reviewed previously (Powell et al 1964). The antagonism can also be explained by the fact that some trace metals bind to a single amino-acid (Cysteine) and proteins(metallothionein) present in the biological system. Cysteine can be found as ligand of a Zn complex with an -SH radical favouring the binding to Cd.

2. As a result of the effect of Zn on Cd uptake, plants showed resistance to high levels of Cd at all Zn levels. Plants containing 100 μg of Cd and more showed signs of Cd-toxicity at the fourth and sometimes at the fifth day of the experiment rather than the second or the third day as found in Chapter 2 where no Zn additions were made. The effect of Cd on Zn could cause Zn deficiency, but this problem was not met in the experiment carried out here. Firstly, this was because amounts of Zn added were sufficient for a short period of growth, secondly, the 7-day period experiment was short in terms of absorption and transport of nutrients and plants were not expected to manifest the requirement for deficient nutrient during this period. The Zn-deficiency problem could have been complicated if the experimental period had been 3 weeks or more. The most likely mechanism could involve a substitution of Zn by Cd in Zn-containing compounds (eg. enzymes). Therefore, a chemical approach to explain this aspect of Cd and Zn interactions is the more valid to explain the type and nature of their interaction which could be a competition for sites between Cd and Zn or a substitution of Zn by Cd in many Zn-enzymes. The main basis of this chemical explanation are the similarities between the two elements in respect to the substitution principles and coordination chemistry. Further details will be added in the following chapter where the effect of Cd on chlorophyll content and the possible interaction between Cd and Mg will be investigated.

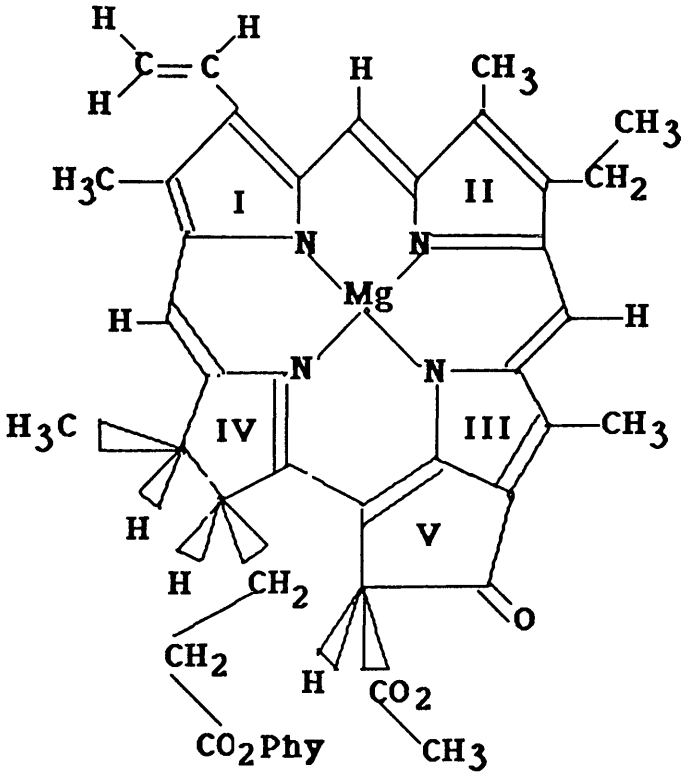
CHAPTER 4

The effect of Cd on chlorophyll

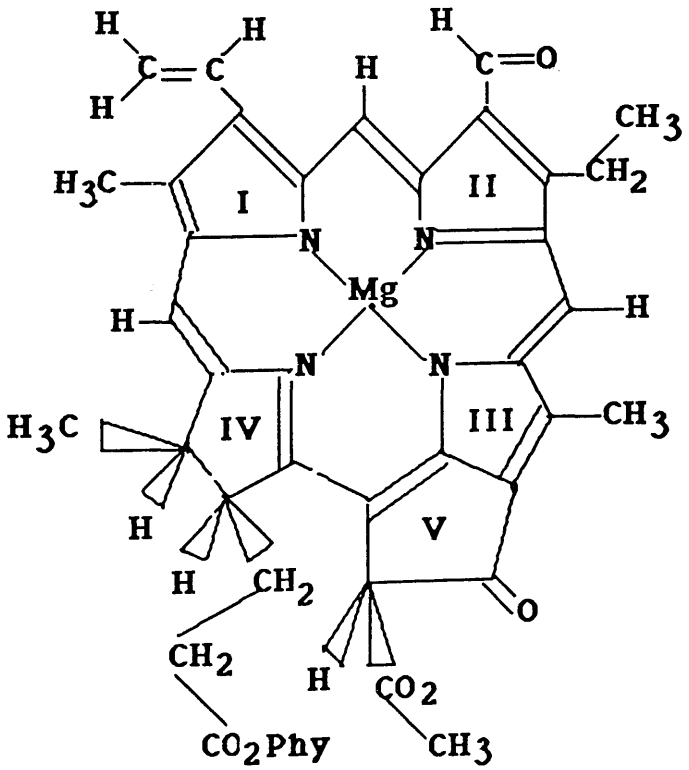
4.1. Introduction

Chlorophylls are located in the chloroplast and are important pigments known to give plants their characteristic green colour (Halliwell, 1981). Chlorophyll consists of a porphyrin part to which is attached a pyrol. The synthesis of the porphyrin can be considered in three stages: Synthesis of G-aminolaevulinic acid, its conversion to protoporphyrin IX, and the conversion of this molecule to protochlorophyllide, which becomes reduced to chlorophyllide when chloroplasts are illuminated(Givan and Harwood, 1976). They are involved in the light reactions of photosynthesis by initiating electron transport. There are four groups of chlorophylls: a,b,c and d. Chlorophyll a is present in all autotrophic plants, Ch b in land plants and Ch c and Ch d in certain algae. Chlorophyll a and chlorophyll b absorb light in the blue(420nm-435nm) and red(660nm-680nm) and the shape of the absorption spectrums of chlorophyll molecules depends on the polarity of the environment in whicht hey are placed(Halliwell,1981). Chlorophyll is essential to the life of the plant because it acts as a catalyst in the photosynthesis of carbohydrate from carbon dioxide and water. Chlorophylls were shown to be a Mg complex (Katz et al 1976) and their Structures are not completely known apart from chlorophyll a and chlorophyll b and bacteriochlorophyll a.

Structure of Chlorophyll a
Katz et al 1976

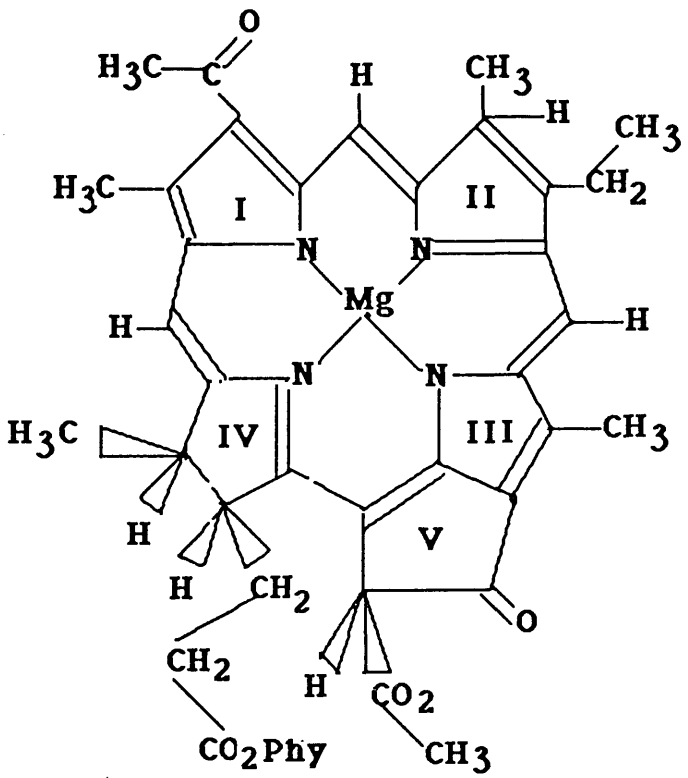


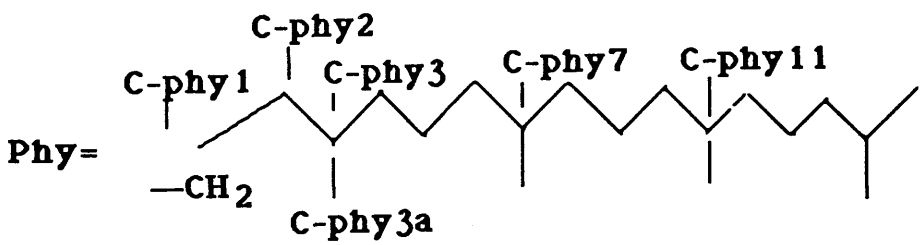
Structure of Chlorophyll b



Structure of bacteriochlorophyll a (Bchl a)

Katz et al 1976.





Phy= $C_{20}H_{39}$

They are insoluble in water but dissolve in organic solvents(acetone, hexane, ether, benzene and alcohols). Chlorophyll is also known to be the principal energy transfer agent and the primary electron donor in photosynthesis(Katz et al 1976). As chlorophylls are important for the photosynthesis mechanism, therefore to the life of any plant species, steps were taken in that sence to protect plants and chlorophyll content against any external substances. Trace metals are known to affect the photosynthesis rate and the chlorophyll content. They can also be toxic and cause the deficiency of other major elements resulting in the appearance of some disease symptoms such as chlorosis, necrosis and other symptoms of trace metal toxicity. Thus the determination of chlorophylls becomes important to clarify the mechanisms involved in causing chlorosis which is expressed as losses in chlorophyll. However it became interesting to investigate the effect of Cd on chlorophyll content in vivo and in vitro. Going back to Chapter 2 and Chapter 3 and to the conclusions drawn as a result of these two investigations, one may ask the question about the cause of chlorosis and its origins. In that respect, experiments were carried out to investigate the effect of Cd on chlorophyll and its biochemical aspect in plants. As known, chlorosis is a yellowing of the leaves which is in fact a loss in chlorophyll. This loss could be an iron or other trace metal deficiency or Cd toxicity effect or even light effects. Iron was eliminated as being the cause for chlorosis by adding a sufficient amount for normal growth which 600 µg total added to the plant grown in the nutrient solution.

By doing so, it was confirmed that chlorosis and also necrosis were rather caused by Cd toxic level than iron deficiency. This led to an increase in interest by looking closely at the Cd effect and its relation to chlorophyll content. Consequently, experiments were carried out in vivo and in vitro to find out about the mechanism of Cd involvement in causing chlorosis and reducing thus the chlorophyll content. Historically Cd was shown to affect many parts of the plants, alter their metabolisms and disturb some enzyme activity(Miller, 1973; and Li and Miles, 1975). Cd was also shown to affect some plant compartments such as chloroplasts, cytosol and mitochondria (Bazzaz et al 1974, and Ernst, 1980). Looking at the biochemical aspect of Cd in plants, Ernst (1980) stated that the distribution of Cd^{2+} was likely to take place within the cell of higher plants or algae. He made this observation when analysing the leaves of two trees (Tilia platyphylos and Aesculus hippocastanum) grown in the vicinity of a Cd smelter. The results of this experiment are shown in the table 4.1.1.

Table 4.1.1.

Compartmentation of Cd in the leaves of trees growing in the vicinity of a cadmium smelter.(Ernst, 1980)

	Tilia Platyphyllos	Aesculus Hippocastanum
Total amount mmoles/kg D.W	0.79	1.21
Distribution %		
Cell wall	72.80	87.60
Plasm, vacuole sap	19.20	4.40
Plastids nuclei	8.00	8.00
Mitochondria,ribosomes	0.01	0.01

Below is another example showing the distribution of Cd within green and chlorotic leaves of two plant species.

Total amount	Agrostis tenuis		Cardaminopsis halleri	
mmol/kg D.M	Green	Chlorotic	Green	Chlorotic
Fe	1.4	1.4	3.8	2.2
Cd	0.01	0.03	0.08	0.50
Distribution of Cd(%)				
Cell wall	45.6	31.6	59.4	56.4
Chloroplast and Mitochondria	13.8	9.3	17.3	10.2
Plasm, Vacuole Sap and Microbodies	40.6	59.1	23.3	32.9

One information can be obtained from these results is that chloroplast accumulated less Cd than the other compartments. This may explain the sensitivity of both chloroplast and mitochondria where Cd was found to affect the most in plants leading to reduction of photosynthesis(Sempio et al 1971, and Miller et al 1974). The data also suggested that sometimes the increase in Cd can be negatively correlated with the amount of Fe as in the case of Cardaminopsis halleri, and sometimes not as in the case of Agrostis tenuis(Ernst, 1980). Ernst also reported that an excess of Cd affects the metabolism of a plant rendering it chlorotic. This loss in chlorophyll causes a reduction in photosynthesis and plant growth. He later added that Cd may have effects on enzymes by transforming them inactive by blocking the active site by binding to the -S.H groups. Furthermore, Ernst added that Cd may substitute for other divalent cations and interact with the electron transport in chloroplasts. The functions mentioned above (enzyme activity, electron transport) are both important for photosynthesis. Thus, both should be protected from any external effect induced by toxic metals or other factors. Many investigators took interest in investigating this matter which increased in importance especially when dealing with heavy metals. After studying sunflower and corn leaves, Bazzaz et al (1974 a) and Bazzaz et al (1974 b) reported that Cd treatment of the two species caused substantial reduction in net photosynthesis (up to 76%). This led to a loss in chlorophyll content as well.

Later in 1975, Cunningham et al confirmed that the chlorophyll content of soybean decreased greatly when treated with a range of Cd concentrations of 4.4 to 27 μM of Cd^{2+}. Root et al (1975) also reported that Cd caused metabolic alterations of corn when studied over a 12-day period in solution containing 1to 40 mg/l of Cd . Root et al also observed a conspicuous change in the colorour of the leaves. This means a decrease in chlorophyll content due to an increase in Cd concentrations. These suggestions and findings led to investigate the mechanisms involved in Cd effect on chlorophyll. One of those hypothesises was the replacement of Mg^{2+} by Cd^{2+}in the chlorophyll structure. In that respect Hurwitz et al (1956), stated that in in vitro experiments, Mg can be replaced by the same amount of a divalent metal when studying spinach. This suggestion was supported by Pakshina and Krasnovskii (1975) who reported that Cd depresses chlorophyll and may replace Mg as the central cation. They also suggested that, as a result of the pheophytization reaction (removal of magnesium) a Cd-complex called Cd-pheophitinate may be formed. Consequently, an investigation was carried out experimenting on beans in vivo and in vitro. From the previous experiments carried out, one of the major observations related to Cd toxicity was the production of a chlorotic aspect of the leaves. Similar experiments were carried out in order to find out about the origins of the chlorosis and its relation to chlorophyll loss.

4.2. Method and Material

4.2.1. In vivo

Seeds of beans (Canadian wonder) were grown exactly the same way as in previous experiments. Seeds were germinated in vermiculite for 10 to 12 days. The germination took place in a growth chamber under a photoperiod regime of 16 hours light and 8 hours in darkness with an approximate 80% RH. The temperature ranged between 24°C and 26°C during light and about 20°C during darkness. After 12 days growth, the seedlings were transferred to a nutrient solution containing different levels of Cd ranging from 0 to 180 µg. After 7 days, plants were washed and weighed and half of the lot was analysed for Cd and Mg using atomic absorption spectrophotometry. The other half was used for chlorophyll extraction and determination by using UV spectroscopy. Chlorophyll extracts were then digested with HNO_3 and analysed for Cd and Mg by atomic absorption spectrometry after being dissolved in diethylether and dried with nitrogen.

4.2.2. In vitro Experiments

During the experiment Cd was added directly to chlorophyll. Samples were kept stored in darkness and analysed daily for 7 days.

4.3 Chlorophyll extraction

a. Total chlorophyll

For both *in vivo* and *in vitro* experiments, extraction of chlorophyll from plant beans was carried out using an acetone-hexane solvent(4:1 v/v) (Vernon, 1960).

b. In Vivo Experiments

After a 7-day period, plants grown in nutrient so.lution containing different levels of Cd were washed with water and then dried with paper towels. Later whole plants were put in a blender to be disintegrated using a mixture of acetone and hexane(4:1) as extracting solvents. The mixture was then filtered and 50 ml of water was added to the clean filtrate to form two separate phases. 5 to 10 grams of sodium sulphate were added to remove most of the water from the organic phase. After a good shake, the organic phase was filtered, separated in a conical flask and hexane evaporated to dryness at about 70^{o}C. As a precaution, all extractions were carried out under dim light to minimize the loss of pigments. The solid chlorophyll was dissolved in purified ether and then analysed using UV spectroscopy.

c. Extraction of chlorophyll a and b

The chlorophyll extraction was carried out the same way as described earlier. Thin layer chromatography (TLC) was used for the separation of the different pigments. The adsorbant substance silicagel was used to transform the chlorophyll into a solid phase easier to remove. Chlorophyll a and chlorophyll b can be removed, dissolved in diethylether, filtered and then analysed. TLC plates were put in a tank containing a mobile phase was selected after preliminary screening using other solvents.

Petroleum (bp 60 to 80) : 90%

Acetone : 10%

n-propanol : 0.45%

4.4. Chlorophyll digestion:

4.4.1 Description of the method

After extracting the total chlorophyll using 80% in volume of acetone and 20% of hexane, the extract was transfered to a separatory funnel to be mixed with 50 ml of water. Then, acetone and water were recovered and 5 to 10 grams of sodium sulphate were added to the organic phase to remove water. The organic phase was filtrated in a conical flask to evaporate the hexane. The solid chlorophyll was then dissolved

in diethylether and dried by nitrogen. Ten ml of HNO_3 were added to the remaining solid chlorophyll and digested for about 10 minutes at 100°C to 120°C. After the digestion, samples were filtrated through an ashless filter paper and then diluted in a 50 ml to 100 ml flask before being analysed by atomic absorption spectrophotometery. As for the water phase samples, they were directly analysed through the atomic absorption after the evaporation of acetone using a rotary evaporator at a temperature of 40 to 60 °C. Below is a compiled list of some pigments and their occurence and their absorption maxima (From Hall, 1981)

Table 4.1.1

Pigment	Characteristic Abs maxima(nm)	Occurence
Chl a	420, 660	All higher plants and algae
Chl b	435, 643	All higher plants & green algae
Chl c	445, 625	Diatoms and brown algae
Chl d	450, 690	Red algae
Bchl	365, 605, 770	In all bacteria
β Carotene	425, 450, 480	Higher plants and most algae
α Carotene	420, 440, 470	Most plants and some algae
Pheo a	409, 667	

4.5. Chlorophyll estimation

Interests in using analytical methods to determine chlorophylls in plant species increased with the years. Many techniques may be used. The most widely used are colorimetry, spectrometry and fluorimetry. For this investigation, the ultra-violet spectrometry was used to estimate the chlorophyll content and determine its UV spectra. Each chlorophyll extraction was put in 1cm glass cell and put together with the second cell containing ether as a blank. The absorbance was then measured at different appropriate wavelength, from 260 nm to 800 nm.

For the estimation of chlorophyll, several empirical equations were suggested. The following equations were used for calculations (Strain, and Svec, 1966).

$$\text{Chl a(mg/l)} = 11.63\,(A665) - 2.39\,(A649)$$

$$\text{Chl b(mg/l)} = 20.11(A649) - 5.18(A665)$$

$$\text{Total chl(mg/l)} = 6.45(A665) + 17.72(A649)$$

A665 and A649 are the absorbances of chlorophyll at 665nm and 649nm.

The estimation of chlorophyll was carried out daily for a period of seven days. The main objective in doing so was to confirm that the effect of cadmium on chlorophyll was a function of time. The values of the two wavelengths mentioned in the equation are the two values giving the maximum absorbance ,therefore the two highest peaks. An extra measurement at 700 nm was carried out for all samples to check their optical clarity.

Assuming that the free-Mg chlorophyll was a pheophytin, however, its calculation became important . This may at least give a clearer idea about the extent of the effect of Cd on chlorophyll and lead to greater understanding of the relationship between trace metals and chlorophyll.

The equations to be used for the calculation are those suggested by Strain and Svec, 1966.

Pheo a (mg/l) = 20.15 (A666) - 5.87 (A655)

Pheo b (mg/l) = 31.90 (A655) - 13.40 (A666)

Total Pheo (mg/l) = 6.75 (A666) + 26.03 (A655)

The A666 and A655 are the absorbances of the pheophytins at 666nm and 655nm.

4.6 Results. (+SD)

Control samples were used during all experiments, but only few are reported.

Experiment 1 (in vivo) . This experiment was carried out using thin layer chromatography method to separate the different pigments and to isolate chlorophyll a and chlorophyll b. Cd was added on the first day of the transfer of plants to the nutrient solution.

Sample µgCd	D.W g	Chl a mg/l(solvent)	Chl b mg/l(solvent)
30	2.89±0.01	0.412±0.02	10.00±0.0001
100	2.64±0.02	0.412±0.01	10.00±0.0001
150	2.45±0.01	0.405±0.02	10.00±0.0002
200	1.70±0.03	0.366±0.02	10.00±0.0002
250	1.57±0.02	0.374±0.01	10.00±0.0003
300	1.46±0.04	0.336±0.01	10.00±0.0002
350	1.45±0.03	0.305±0.01	10.00±0.0001

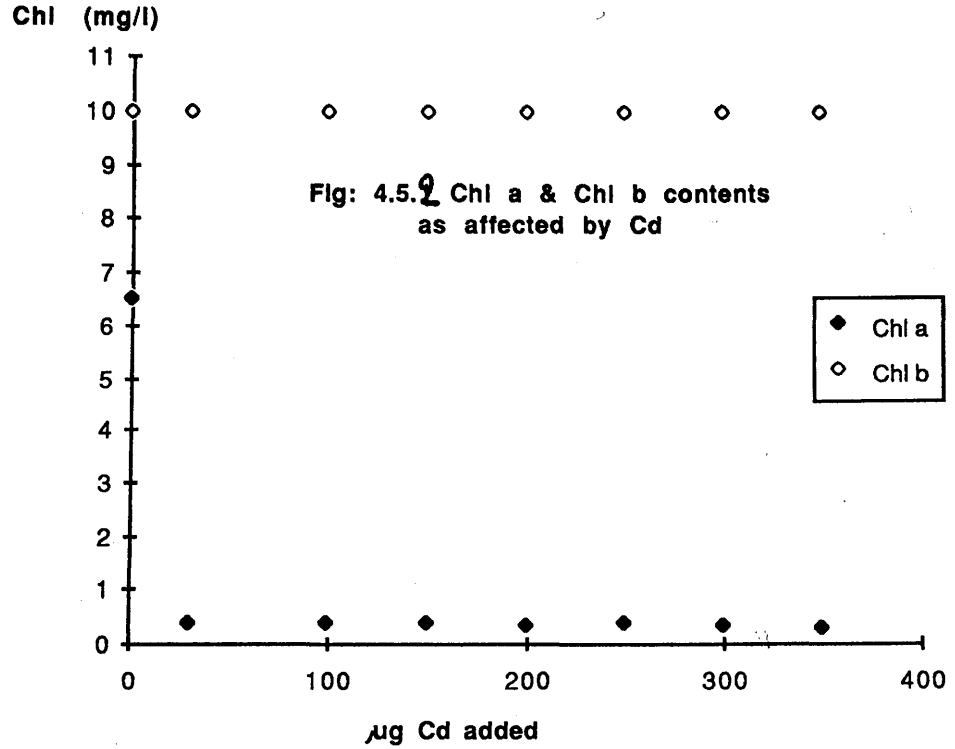


Fig: 4.5.2 Chl a & Chl b contents as affected by Cd

Experiment 2. (In vivo). During this experiment Cd was added on the first day of the growth of the plant at levels ranging from 30 to 350 µg. The experiment was carried out to determine Cd and Mg in bean plants using both atomic absorption and UV spectroscopy.

Atomic absorption analysis for Cd and Mg.

Table 2. Cd results for plant tissue

Sample µgCd	µg Cd in plant tissue	µg Cd in rem. Sol
30	19.05±0.80	8.38±0.65
100	24.42±0.63	68.28±1.10
150	30.50±0.63	91.99±2.04
200	127.4±6.0	34.55±1.07
250	28.88±1.10	177.1±3.1
300	26.95±1.06	194.2±6.3
350	36.27±0.76	265.90±3.34

Table 3(in vivo). Cd results for chlorophyll extract. The data below show the increasing of Cd concentrations in chlorophyll extracts as Cd levels increased.

Sample µgCd	Total µg Cd in Chl extract	Total µg Cd in rem sol	Recovery %
30	10.14±1.264	9.27±1.535	88.69
100	15.41±0.926	66.54±2.783	89.78
150	22.09±0.931	99.25±4.229	87.16
200	25.34±0.931	141.00±5.108	84.58
250	20.07±1.610	192.40±4.893	86.81
300	30.20±0.926	233.00±7.994	95.42
350	32.83±0.610	_	94.86

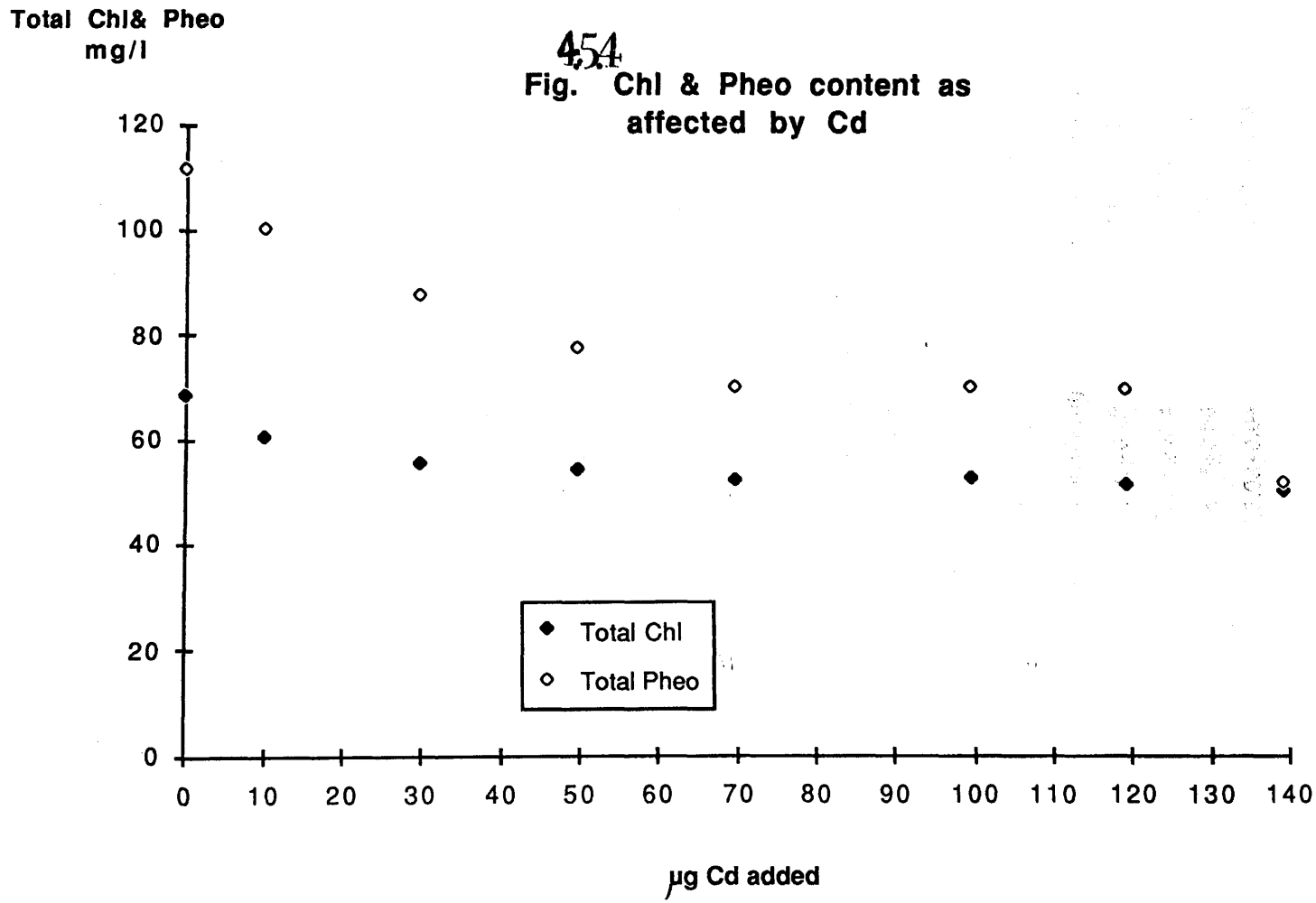
Table 4 (in vivo). Mg results in plant tissue. The aim of the experiment was to determine the effect of the added Cd on the concentrations of Mg in chlorophyll extracts

Sample µgCd	µg Mg in plant tissue	µg Mg in rem. Sol
30	879.6±4.3	1019.0±160.2
100	887.1±3.6	727.0±127.2
150	798.9±3.2	966.8±32.4
200	650.8±5.2	1220.0±62.6
250	148.0±6.6	1639.0±23.11
300	371.0±3.6	1398.0±94.42
350	348.6±15.8	1592.0±19.2

Table 5. (in vivo). Mg results for chlorophyll extract. Chlorophyll of samples of the second lot was extracted, dried, digested and analysed with atomic absorption. Levels of Cd were added at a range of 30 to 350 µg.

Sample µgCd	Total µg Mg in Ch ext	Total µg Mg in rem sol	Recovery %
30	774.3±35.43	942.8±71.89	87.52
100	466.8±16.73	1316.0±72.73	89.16
150	401.6±9.737	1471.0±40.57	93.62
200	322.9±6.246	1575.0±35.201	94.89
250	148.0±6.595	1639.0±23.11	89.34
300	371.0±3.593	1398.0±97.42	88.46
350	348.6±15.8	1592.0±19.20	97.03
Control	868.76	1097.19	96.91

Fig. Chl & Pheo content as affected by Cd



In vitro experiments.

Experiment 3. Estimation of total chlorophyll and the free-Mg chlorophyll was carried out using UV spectroscopy and emperical equations for the calculations. The results shown below indicate the decrease of the chlorophyll content as Cd levels increased.

Table 5

Sample µg Cd	Total Chl mg/l(solvent)	Total free Mg Chl mg/l(solvent)
10	60.52±0.180	100.60±1.980
30	55.58±0.159	87.81±0.011
50	54.15±1.300	77.52±2.125
70	50.87±1.961	70.00±0.579
100	52.21±0.173	69.94±0.282
120	51.08±0.410	69.58±1.093
140	49.94±0.304	51.53±0.147
control	68.6112	111.9125

Experiment 4. Total Chl and free Mg Chl estimation.

Table 6

Sample µgCd	Total Chl mg/l(solvent)	Total free Mg Chl mg/l(solvent)
10	80.85±0.42	119.7±0.7
30	76.99±0.22	109.9±0.07
50	74.15±0.58	103.5±1.4
70	73.08±0.04	99.16±0.23
100	67.86±0.16	91.84±0.51
120	63.91±0.31	90.00±0.1

Experiment 5. The experiment was carried out the same way as previously and samples were analysed for day 1, 2, 3, and day 7. It was aimed at demonstrating that the effect of Cd was a function of time.

Table 7. Day one

Sample µgCd	Total Chl mg/l(solvent)	Total Free Mg Chl mg/l(solvent)
100	41.17±0.05	55.49±0.10
200	37.85±0.08	50.73±0.09
300	32.57±0.10	44.01±0.09
400	31.91±0.09	42.34±0.10
500	28.84±0.10	38.35±0.20
600	27.63±0.12	35.79±0.20
700	24.77±0.14	34.91±0.07
800	22.08±0.11	29.24±0.32

Experiment 5.

Table 8. Day 2

Sample µgCd	Total Chl mg/l(solvent)	Total free Mg-Chl mg/l(solvent)
100	34.24±0.60	45.82±0.70
200	30.59±0.46	39.60±0.25
300	29.19±0.08	38.01±0.09
400	24.90±0.15	31.63±0.18
500	23.04±0.07	30.05±0.27
600	21.07±0.07	27.27±0.30
700	20.00±0.13	25.63±0.22
800	19.11±0.30	25.58±0.52

Experiment 5.

Table 9. Day 3

Sample µgCd	Total Chl mg/l(solvent)	Total free Mg Chl mg/l(solvent)
100	35.67±0.10	46.52±0.26
200	29.82±0.15	38.25±0.18
300	27.10±0.40	34.03±1.14
400	22.76±0.44	30.91±0.46
500	22.20±0.15	29.75±0.09
600	21.96±0.06	29.36±0.12
700	19.94±0.45	22.91±0.10
800	17.68±0.20	22.05±0.23

Experiment 5

Table 10. Day 7

Sample µg Cd	Total Chl mg/l(solvent)	Total free Mg-Chl mg/l(solvent)
100	32.01±0.18	43.14±0.13
200	26.76±0.22	36.35±0.06
300	22.96±0.05	30.24±0.02
400	21.28±0.23	29.21±0.34
500	18.83±0.09	26.06±0.14
600	17.31±0.10	25.24±0.05
700	17.84±0.09	24.87±0.10
800	17.38±0.05	23.80±0.23

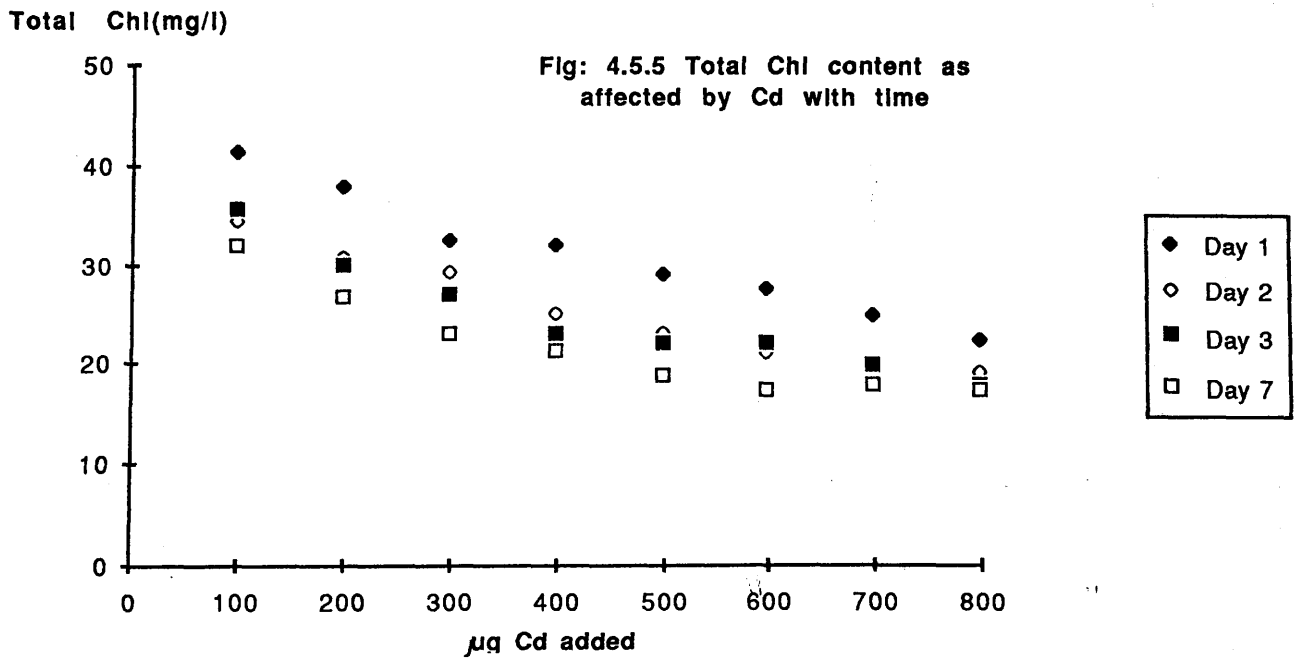


Fig: 4.5.5 Total Chl content as affected by Cd with time

4.7 Discussions

Acetone was used for the extraction because of its miscibility with water and it is also regarded as one of the most suitable solvents for chlorophyll known to be effective in extracting most of the pigments present(see, Harborne, 1973). Acetone was selected after preliminary work using hexane which was shown to give ~~less~~ poorer ~~better~~ results in terms of quantity of chlorophyll extractable. Ether was used for the UV analysis because it is considered to be a good solvent and also because it can be used for short wavelengths above the infra-red limit. Acetone and hexane were thought to be inappropriate because of the miscibility of the first with water and the short wavelength of the second which could ~~lead into~~ encroach the infra-red zone. Problems were met when evaporating acetone present in the water phase which was due to be analysed. Acetone is known to be miscible with water and can be evaporated only at warm temperatures (60°C to 70°C). The monitoring of the temperature was not particularly easy especially when using a flame. As a result, drops of acetone were left in the sample and this eventually alters the Atomic Absorption readings.

1. In vivo Experiment

a. Atomic Absorption Analysis for Mg and Cd. Atomic absorption was used because of the practicality of the technique, its cheap price and also because its instrumentation is accessible. Samples of this experiment were analysed for the determination of chlorophyll content and also Cd and Mg in chlorophyll.

Two sets of samples were used, one for chlorophyll extraction and the other for atomic absorption analysis. The purpose of this experiment was to assess the extent of Cd effect on chlorophyll content and to understand the causes of chlorosis observed earlier as a result of cadmium toxicity. It was thought apropriate to use atomic absorption spectophotometry to determine Cd and Mg in both plant tissue and chlorophyll extract, and also the relationship between the two elements and its repercussion on chlorophyll formation resulting from the alteration of Mg or Mg compounds by Cd. The most important observation to be made according to data shown in tables 3,4 and 5 and represented in Figures 4.5.3, was the decrease in Mg content of both plant tissue and chlorophyll extract due to the increase in Cd concentration. This loss in Mg expressed a loss in chlorophyll content occurring at all added levels of Cd. The opposite result was obtained for the amount of Mg and Cd in the remaining solutions. These observations led to suggest the removal of Mg from the chlorophyll structure and its displacement by Cd as a result of its effect This was suggested on the basis of observations made during the experiments and were prsented a changes in the colour of the chlorophyll from dark green to olive green and to yellow for samples containing the highest levels of Cd(400 to 800µg). The total Mg expressed in µg present in chlorophyll extract was calculated using the original amount of Mg present in a whole plant (1009.32 µg) deducted from each calculation of Mg.

b. Ultra-Violet Spectroscopy. This technique was also used for its practicality, its low cost and the accessibility to its instrumentation.

The use of TLC method enabled the separation of the different pigments and permitted the determination of the two major ones – Chl a and Chl b. From the results presented above, the same observation was noted as previously found. There was a clear indication of a Cd effect on the content of Chl a and Chl b and the total chlorophyll while assuming that Chl a and Chl b form the total chlorophyll. As a result of the effect of Cd, a new spectrum appeared for all samples containing Cd when analysed by ultra violet spectroscopy. The form of the spectrum became increasingly clearer when Cd levels were increased; thus, its similarity to a Mg-free chlorophyll also increased. Concerning the results of chlorophyll b and its constant level, one may suggest that chlorophyll b was less affected by Cd than chlorophyll a. The latter is considered to be the major pigment occurring in all plants that produce oxygen by photosynthesis, whereas chlorophyll b is considered to be a minor chlorophyll (Strain and Svec, 1966). Therefore, Chl a is the most likely to be subjected to Cd effects than Chl b which is present in about one third the concentration of chlorophyll a in green plants(Strain, 1958, and Bonner and Varner, 1976).

2. In vitro Experiments

Experiments 1,2,3 &4

Results of these experiments gave confirmation about the effect of Cd in decreasing the amount of chlorophyll at all Cd levels. At high Cd levels (300 to 800 µg total) the effect on chlorophyll content was greater compared with the amount of chlorophyll present in control plants. As far as the Mg-free chlorophyll was concerned, there was an increase in its amount compared with the amount of chlorophyll at the same corresponding Cd level. Experiment 3 was carried out during a period of seven days equal to the period used for the in vivo experiment. From the results obtained, there was a decrease with time of the amount of total chlorophyll starting from the third day and from which a certain constancy of the decrease was noticed. This could be a sign of Cd and Mg concentration balance necessary for a possible substitution besides all the reserves concerning complications related to chemical approaches. The same argument can be used about the total pheophytin (or the Mg-free chlorophyll). As for experiment 4, the determination of Cd and Mg in both water phase and chlorophyll extract brought more evidence of the effect of Cd in suppressing Mg content by decreasing the amount of chlorophyll.

4.8. Mechanisms involved.

It was mentioned earlier that a chemical approach could be used to explain some mechanisms involved in cadmium relationship with other elements. From the results of the in vivo and in vitro experiments, the following conclusions were drawn.

(i) Cd disturbing the biosynthesis of chlorophyll by possibly interfering with intermediate elements(or precursors) involved in chlorophyll formation. Iron-proteins such as, ferredoxin and cytochrome are the best known for their role in the biological system including chlorophyll formation. They both contain Fe with which Cd can compete and displace by binding to the -SH group.

(ii) Cd displacing Mg as the centre atom in the chlorophyll structure.

a. Hypothesis one.

The synthesis of chlorophyll requires iron for its formation and any iron deficiency causes a loss in chlorophyll which is expressed as chlorosis. It is known that the Fe-sulphur protein plays a role of electron carrier between molecules of hydrogen and various electron acceptors. (Van Tamelen, 1978). Being aware of this, one may expect an interference of Cd with Fe because both elements belong to the transition group and also because Cd and Fe are both divalent metals susceptible to compete with each other especially when knowing that

cysteine is part of the active site of the protein [$Fe_nS_n(Cys)_p$ units]. As mentioned in the previous chapter, cysteine contains an -SH group known to bond preferably to Cd. Thus, displacement of iron in the protein structure is to be expected. As a result of this, the iron and the iron-sulphur protein will be prevented from fulfilling their metabolic role as mentioned above. Cytochrome which contains Fe may also be affected by Cd, thus preventing it from playing its role as a catalyst of respiration and its involvement in biological electron transport (Slater, 1958).

b. Hypothesis two.

From all the data illustrated earlier, there was a clear indication of Mg losses due to Cd additions. It is early to suggest that there is a possible substitution of Mg by Cd as the centre atom of the chlorophyll structure. Neverthless, one can speculate on the basis of some evidence obtained during experiments. Starting from the results obtained by atomic absorption spectrophotometry used for the analysis of the chlorophyll extract for Cd and Mg; the results obtained indicated a decrease in the amount of Mg in the chlorophyll extract and Cd concentrations were increased. This information may lead to suggest that besides the direct effect of Cd on Mg, substitution of the latter by Cd may also occur . This suggestion was supported by spectroscopic evidence. Spectra of a free Mg-chlorophyll were obtained for all samples of chlorophyll extracts containing Cd.

By studying the highest peaks of the spectra and by comparison, the spectra of the complex pheophytin suggested by Vernon and Seely(1966) looked to be the most similar at the respective absorbance of 666nm and 655nm. However, if Cd replaces Mg in the structure of the chlorophyll, the formation of a Cd chlorophyll complex is not to be ruled out. Cd among other divalent metal chlorophyll complexes have been isolated by several investigators among them, Fleischer(1969); Pakshina and Krasnovskii(1975); Clarke(1975) and Clarke and Frank(1977). Therefore, spectra of the Cd-complex are more likely to be obtained as a result of the effect of Cd on chlorophyll. A further experiment involving the use of a Gringnard reagent was carried out to confirm the removal of Mg and the recovery of the green colour when this was added to Cd treated extracts. Four attempts were carried out and the green colour was fully recovered after about thirty minutes. Although, the speculative aspect of the explanation is valid on the basis of the evidences mentioned earlier it is too early to suggest a definite answer to this matter. When trying to find an appropriate chemical explanation, some differences between the two elements emerged and a direct substitution of Mg by Cd looked more complicated than first thought. Firstly, because there are several biological reactions involved in the biosynthesis of chlorophyll and Cd is more likely to act rather on an intermediate than directly on Mg. Secondly, the two elements are different in their nature, size and chemical properties. Therefore, and without ruling out this hypothesis, the first one seemed to be the most plausible at this stage.

4.9 Conclusions

Despite some evidence concerning the effect of Cd on chlorophyll and Mg content, much more is still to be achieved to suggest that the direct effect of Cd was a displacement of Mg . This may be realised when the matter is a subject of a very extensive investigation. In addition to this a competition may occur between Cd and Fe and Cd and Mg in the process of chlorophyll formation, forming possibly a Cd complex called Cd-pheophytinate (Pakshina and Krasnovskii, 1975).

CHAPTER 5

5.1 Conclusions

Trace metals are undoubtedly recognised to be very important for all kind of life systems, soils, plants human life and animals . Great efforts have been made by many workers and many questions related to the behaviour, uptake and most of all the effects of trace metals have been answered. However, questions are still remained unanswered and problems related to trace metals still persist. This investigation was aimed at highlighting some of the problems related to Cd and trying to answer some of the questions that have arisen as a consequence of Cd behaviour and its toxic nature to plant species. In that respect, a study was carried out with the main objective to investigate the uptake of Cd and its effects on beans. Two major factors among others known to affect the behaviour of Cd were included in this study to give the investigation a more consistent background. pH was shown to affect the solubility, availability and transport of Cd. The use of Zn was selected for this purpose because of the chemical similarities existing between the two elements. It was also chosen for its advantageous use as a competitor metal because Zn is essential to plants and its toxicity can be prevented if it is added at appropriate proportions.

Chlorosis was the primary symptom caused by Cd toxic levels and this led to the study of its causes by investigating the effect of Cd on Chlorophyll formation and its content in plants. Suggestions were obtained concerning the role of pH in the uptake and availability of Cd, its interactions with Zn and its interference in chlorophyll synthesis.

It was shown from the results obtained in Chapter 2 that pH played a great role in affecting the behaviour of Cd. It was also found that Cd uptake by beans increased at low pH value (3.0 to 5.2) and as it was predicted a grey-white precipitate was formed at high pH values (5.7 to 7.8). This precipitate which is thought to be Cd- phosphate became clearer with time. Although, pH is known to affect the solubility of trace metals, the information obtained is still important and useful to practical uses in laboratory and also in the fields. The adequate monitoring of soil pH can prevent many soil hazards and environmental problems especially when treating waste waters.

Trace metals interelationship has been a major issue for investigations for many years. This matter has been growing in interests especially since it has been found that the interaction of metals had a positive impact in minimizing and also preventing the effects of many trace metals on soils, animals and plants. Gabbiani et al(1967); Kar et al(1960); and Matrone (1974). The introduction of Zn in this investigation was mainly aimed at studying the response of Cd to Zn and vice versa and also to study its usefulness to beans in preventing or minimizing Cd injury. Indeed some conclusions were obtained concerning the competitive role of Zn . It was shown that Zn suppresses Cd uptake at all small levels of Cd (20 and 60 µg). It was also found that at high Cd levels combined with all Zn concentrations, beans showed a certain resistance to high levels of Cd (120 µg, 140 µg and 180 µg) found to be toxic when they were used previously in Chapter 2 without any Zn addition.

This resistance was expressed as a delay of the appearance of Cd toxicity symptoms. This led to the suggestion that a competition for exchangeable sites between the two elements was taking place likely at the root surface, thus preventing the transport of Cd into the plant. A substitution of Zn by Cd in many Zn-enzymes was not ruled out in respect of the chemical similarities between the two elements. The method of competitor metals has already been successfully used by many workers among them, Hawf and Schmidt (1967) who used Cu and Cd to prevent Zn problems; Haghiri(1976) used K, Ca and ,any other metals in soil to suppress Cd uptake by soybeans, and John(1976) used K to reduce Cd uptake by lettuce and oats. This was to confirm the great impact which the addition of metals to tackle complications due to be caused by others. The pretreatment of beans with Zn and its use as a competitor ion to Cd is valid to prevent the effects of Cd by possibly enhancing the resistance of beans to Cd. Due to time constraints, this pretreatment was not carried out . However, it is believed that such pretreatment would have this predicted effect. The conclusions of this investigation confirmed the fact that chlorosis is a loss of chlorophyll due to the loss of Mg caused by Cd as shown in the results. In that respect, one question appeared to be important and this was about the nature of the effect of Cd on chlorophyll and the mechanisms involved. Cd decreasing chlorophyll content and Mg concentration was the first conclusion obtained on the basis of evidence achieved from analysis by atomic absorption and ultra violet spectroscopy.

Although some results were obtained indicating the formation of a free-Mg chlorophyll suggesting the removal of Mg and the increase of Cd concentration in the chlorophyll, but more still to be achieved to suggest a possible displacement of Mg by Cd. Meanwhile a speculative view was taken to explain the matter by suggesting that Cd was rather interfering with other divalent elements such Fe which are known to be involved in the chlorophyll synthesis than Mg directly. Although some investigations confirmed that Cd suppresses chlorophyll content, this investigation went one step further and attempted to explain the mechanism involved on the basis of the evidence. This work lays the foundations for further research aimed at investigating whether Cd can directly displace the Mg atom from its cental position in the chlorophyll structure.

5.2 Prospects for future research

In addition to several effects due to Cd-toxicity such as decrease in growth and inhibition of the photosynthesis process, chlorosis is considered the most apparent symptom of Cd-toxicity. After investigating the behaviour of Cd, its interaction with Zn and its effects on beans , the main results and suggestions converged towards one aspect of Cd-toxicity and this was chlorosis. In that respect, attempts were made to find out about the origins of chlorosis and assess possible mechanisms involved. Two hypothesises were suggested.

Hypothesis one. In Vivo experiment.

Cd may interfere with Fe by preventing it from fulfiling its role as an electron-carrier or causing its deficiency, therefore impeding the formation of chlorophyll. The displacement of Fe^{2+} by Cd^{2+} in the ferredoxin chain possible and could be enhanced by the presence of an -SH group present in cystein and favorable to bind with Cd.

Hypothesis two. In Vitro experiment.

This is more complicated because it involves at least two suggestions; Cd disturbing the structure of the chlorophyll by possibly binding to one of the rings, and a possible displacement of Mg^{2+} in the central position of the chlorophyll structure and its replacement by Cd^{2+}.

Besides evidence obtained in Chapter 4 concerning the decrease of Mg concentration in the chlorophyll complex and spectra of a free Mg-chlorophyll were also obtained. However, hypothesis one is favoured for the time being without ruling out the second. Pakshina and Krasnovskii (1975) suggested the formation of Cd and Zn complexes called respectively Cd-pheophytinate and Zn-pheophytinate resulting from the reaction of pheophytinization of Mg^{2+} by Cd^{2+} or Zn^{2+}. Other workers had successfully formed these complexes (Clarke and Connors 1975; Clarke and Frank 1977) when studying the triplet state as affected by the substitution of Mg^{2+} by Cd^{2+} or Zn^{2+}. Alongside the evidence already obtained during this investigation, extensive experiments on chlorophyll using the same techniques (i.e. U.V. and A.A. Spectrophotometry) should be carried out in order to obtain more results. These results will be backed up by the use of Grignard reagent on a regular basis to confirm the removal of Mg^{2+} from the chlorophyll structure. Other techniques could also be used such as polarography and chromatography. Thus one ~~can~~ may obtain ~~have~~ more ~~material~~ endara in hands to suggest a replacement of Mg^{2+} by Cd^{2+} by suggesting a more valid chemical appraoch to explain the mechanism involved. However, this chemical approach to explain the mechanism responsible for the substitution of Mg^{2+} by a divalent metal is far from simple because of the complicated biological process of chlorophyll synthesis and the biochemistry involved. Nevertheless, one can still consider the possibility of a displacement of Mg^{2+} by a divalent metal by using the coordination chemistry and the notion of the ionic radius to determine the distance of the $N\text{-}M^{2+}$ (N= nitrogen of the pyrolle ring) and also to determine whether a given divalent metal can fit in the center of the porphyrin ring or not

as suggested by Fleischer(1969) with emphasis on the nature of the N-M^{2+}.

As far as Cd^{2+} is concerned , the distance N-Cd is estimated to be bigger than those of Ni^{2+}, Cu^{2+}, Zn^{2+} etc... with the opposite order of stability; Ni^{2+} > Cu^{2+} > Zn^{2+}> ...>Cd^{2+}. Thus Cd^{2+} is thought to slightly bigger to be accommodated in the center of the porphyrin structure and adopting a square-planar geometry. But instead, Cd^{2+} will adopt a more complicated geometry, most probably a pyramidal with four(4) nitrogens forming its basis, as shown below. See Structure. The stability of a metalloporphyrin complex depends mainly upon the size of the metal and the geometry of the corresponding complex (Fleicher, 1969). Knowing that Cd^{2+} like others, Ni^{2+}, Cu^{2+} and Zn^{2+}(Fleischer, 1969) can fit in the porphyrin ring, one may carry in the future a more extensive investigation mainly concentrated on studying the kinetics of the mechanism involved in the formation of Cd-Chlorophyll in in vivo and in vitro experiments.

X-ray diffraction could be used for more understanding of the chemistry of the metalloporphyrin complexes especially their geometry. Complications concerning a definite mechanism responsible for the formation of metal-chlorophyll complexes(mainly with transition metals) are still arising. Only speculations are being put forward baring in mind that a dative bonding could be formed between a ligand playing the role of a Lewis base and an empty hybridized orbital on the metal ion.

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