EFFECT OF THE SAFENER DICHLORMID AND ANALOGUES ON THE HERBICIDE EPTC

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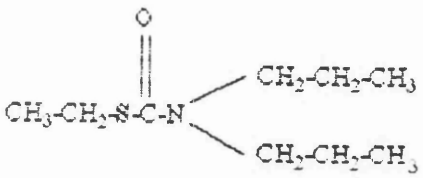
Contents

Chemical structures of EPTC, Dichlormid and Dichlormid analogues.



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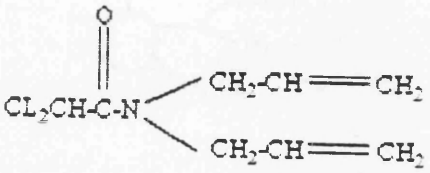

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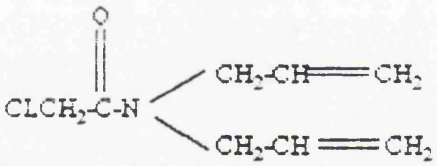

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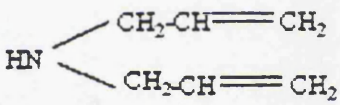

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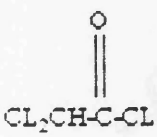

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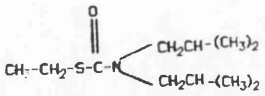

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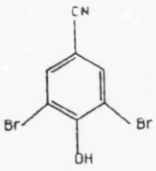

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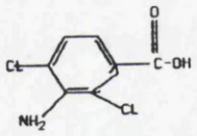

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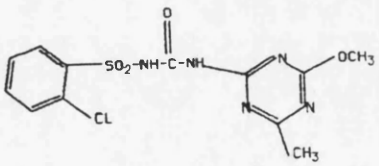

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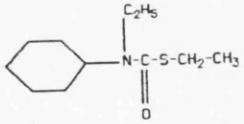

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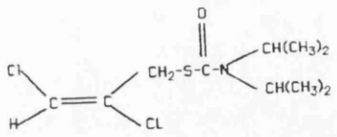

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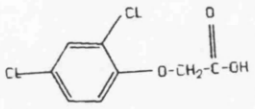

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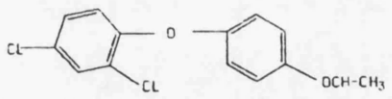

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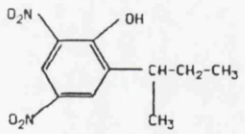

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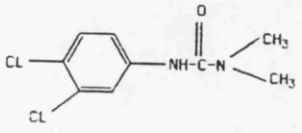

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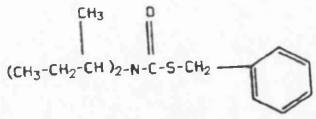

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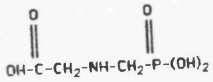

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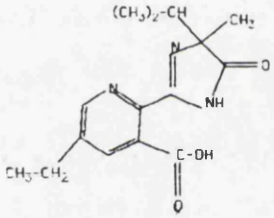

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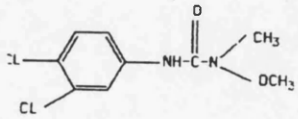

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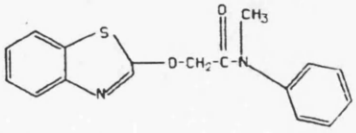

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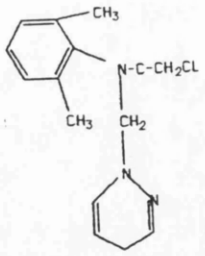

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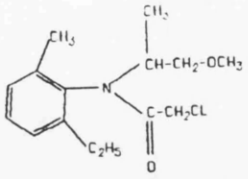

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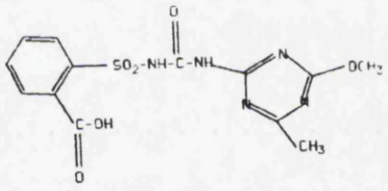

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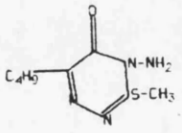

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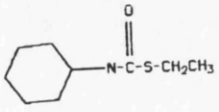

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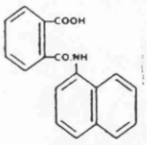

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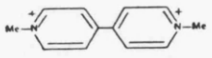

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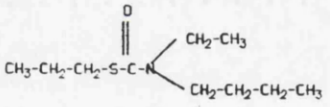

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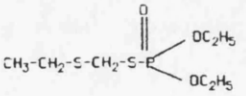

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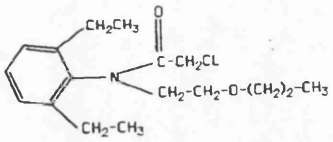

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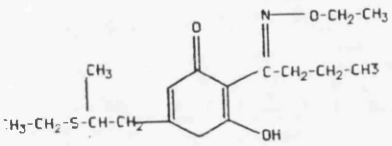

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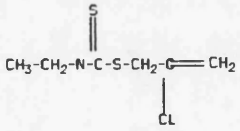

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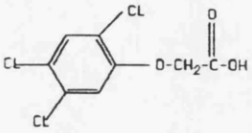

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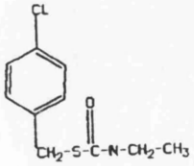

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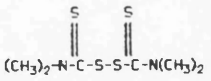

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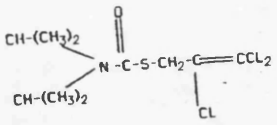

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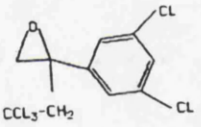

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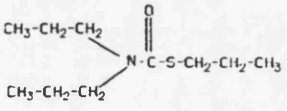


List of Tables :

Page No

List of Figures :

List of Plates :

SUMMARY

This project set out to examine the effects of the safener Dichlormid and analogues on the herbicide EPTC and the implications for safening action.

Their effects on EPTC were explored through several angles e.g absorption by maize roots, volatility from nutrient solution, metabolism in maize tissues, injury to maize plants under greenhouse conditions and susceptibility to phototransformation under different conditions.

EPTC primarly dissipates from nutrient solution through volatilization. Almost 93% disappeared within five days and only 7% of the applied dose was uptaken by maize roots within 5 days. Dichlormid had no significant effect on EPTC uptake nor on EPTC volatility. These results were obtained using a new experimental technique and HPLC as a basis of the analytical method.

Synthesis of EPTC metabolites in maize tissue (EPTC-Glutathione and EPTC-Cysteine conjugates) revealed that EPTC sulfoxidation is a vital step for its detoxification through conjugation with plant thiols. EPTC-Sulfoxide was the true form to conjugate with Cysteine, while EPTC-Sulfone was the form able to conjugate with Glutathione (GSH) in vitro as shown from the identification of the conjugates using mass spectrometry. HPLC was an effecient method for the separation of EPTC, Dichlormid, EPTC-cysteine conjugate

and EPTC-glutathione conjugate standards. The attempt to extract the metabolites from maize tissue prior to their identification either by HPLC or TLC was not successful.

Dichlormid analogues (Diallylamin, Dichloroacetylchloride and N,N-dially-chloroacetamide) were examined as proposed safeners against EPTC injury to maize under greenhouse conditions. Various degrees of safening activity were demonstrated either in the form of counteraction of injury symptoms or as a reduction of the inhibition of maize height caused by EPTC. The results indicated that more than one biochemical or/and physiological process is involved in EPTC and Dichlormid action. No specific functional group is responsible for the full safening activity and Dichlormid as one complete is required to exert complete safening action.

Phototransformation of EPTC, Dichlormid and a mixture of both has been studied in water and in methanol via irradiation with UV light at 254nm and >290nm. Both EPTC and Dichlormid underwent rapid phototransformation at 254nm. EPTC half-life was 14.14 ± 0.93 and 37.22 ± 5.16 min in water and in methanol respectively, Dichlormid photolyzed more rapidly than EPTC with a half-life of 10.22 ± 0.76 and 5.32 ± 0.72 min in water and in methanol respectively. At >290nm, which reflects artificial sunlight conditions, negligible degradation of EPTC and Dichlormid in water and methanol took place. Dichlormid had no significant effect on

EPTC rate of photolysis either via irradiation at 254nm, or at >290nm in both media.

A complex mixture of products was formed from the photolysis of EPTC and Dichlormid via irradiation at 254nm in water and methanol. Three methods were used for their separation and identification. On that basis, the routes of EPTC phototransformation were suggested to be: hydrolysis ,dealkylation, sulfoxidation and yielding radicals from which a variety of dimers formed. Dichlormid phototransformed through dechlorination, dealkylation and hydrolysis. At >290nm, although negligible transformation has taken place, several compounds were formed which were different from those formed at 254nm. Dichlormid did not interfere significantly with the nature of EPTC photoproducts either in water or in methanol.

Further work using a different approach is required toexplore the above effects more closely and accurately and other possible targets for studying the joint effects of both EPTC and Dichlormid need to be carried out.

Chapter one

Introduction and Thesis objectives

1.1 Introduction :

The great increase in the world population, with the resulting cases of starvation that we see today means that adequate methods to improve food production are required. Weeds are one of the obstacles that hinder the achievement of this goal. To overcome weed problems, different methods and techniques have been proposed.

The chemical control of weeds by herbicides is the most effective and adequate method. It is the miracle of our technological age according to Ashton and Crafts.(1973),and it has become an integral part of man's strategy to maximize food production.

Unfortunately, like other beneficial methods, herbicides have their limitations and shortcomings that reduce their effectiveness.

Applying more than one agrochemical in the same formulation with the resulting synergistic, or antagonistic effect has overcome some of these problems and acheived more effective weed control.

The marginal selectivity of herbicides has encouraged researchers to explore methods and ways of achieving better selective weed control. Among these techniques is applying chemicals with the herbicide to increase the crop's tolerance to the herbicide without reducing its effectivness against the weeds. These

chemicals, called safeners, protect the crop from injury by the herbicide.

This chapter introduces basic concepts related to chemical weed control with particular emphasis on thiocarbamates and chloroacetanilides as herbicides, Also herbicide combinations with their practical applications as well as their shortcomings and limitations will be clarified. The methods proposed to achieve more selective chemical control will be briefly described, and at the end of the chapter, the objectives of the work that was carried out will be outlined.

1.2 Weed control methods(Non-chemical) :

Weeds are a natural hazard to the activities of man (Mortimer, 1990), or according to the European Weed Research Society " any plants or vegetation interfering with the objectives of the people", while Stephens.(1982) defined them as plants in the wrong place that compete with cultivated plants and interfere with man's legitimate objectives.

Weeds compete for light, water and nutrients during establishment and in the established crop. They interfere with harvesting operations, act as hosts for pests and diseases, provide shelter to insects and compete for space above and below the ground. Some are parasitic to crop plants and some are poisonous to livestock (Fletcher and Kirkwood, 1982; Gwynne and Murray, 1985).

Non-chemical methods for weed control have been implemented; crop rotation, hand pulling, hoeing, flooding,burning, soil sterilization, biological control using insects and fungi, stimulation and exhaustion, desiccation, and mulching(Stephens, 1982; Muenscher, 1980; Gwynne and Murray, 1985; Lockhart et al., 1990).

1.3 Chemical weed control (Herbicides) :

The introduction of the herbicides marked a major development in the concept of weed control, and has been one of the most important advances in agriculture. The earliest use of chemicals for weed control included inorganic compounds(e.g Aluminium sulphate, Copper nitrate, sodium nitrate). The initial use of organic chemicals to control weeds was in 1932 with the introduction of nitrophenol compounds, followed by the growth regulator analogues Chlorinated phenoxy acetic acids, and then a wide range of chemicals.

The basic idea behind the early herbicides was that you sprayed a stand of plants with a compound and the weeds were killed leaving the crop unharmed. This type of herbicide was applied post-emergence, but it was found that for certain crops such as maize, cotton and soybean it was hard to find compounds that killed the weeds without injury to the crop. The second generation of the herbicides included those requiring application to the soil before the crop and weeds emerged. In this way many of the troublesome weeds could be eliminated

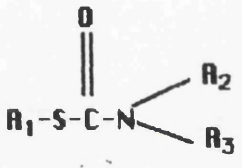
selectively in crops. With the advent of these second generation compounds came the finding that many different steps in plant biochemistry are susceptible to chemical exploitation. Those pathways that are different from other forms of life are prime targets for attack in the design of new herbicides. Toxicologically safer compounds are more likely to be found by this approach.

Herbicides are classified by their site of application as foliage or soil applied. The soil applied ones generally affect germinating weeds, so must persist in the soil for a period to be effective, while those applied to foliage are classified as contact or translocated. Both the foliage and soil applied herbicides may be categorized according to the time of application as related to the growth stage, as pre-sowing, preemergence, or postemergence herbicides (Stephens, 1982; Hance and Holly, 1990).Chemical classification of herbicides was also considered; Phenoxyalkalonic acids, Plant growth regulator analogues, Quaternary ammonium compounds, Ureas, Triazines, Uracils, and Pyridizines, Nitroanilines and Nitrophenols, Carbamates, and Thiocarbamates (Hassall, 1990).

1.3.1 Thiocarbamates :

Thiocarbamate herbicides are clear liquids, with a sharp and aromatic odour, miscible with most organic

solvents. Their solubility in water ranges from 4-900mg/L. They are quite volatile compounds with vapour pressure range from 0.1- 0.001mmHg at 20^{o}C. Their toxicity to mammals is relatively low and does not include acute or chronic effects at low concentration. They fit the following chemical formula :



They are usually applied to annual and perennial grasses and sedges. They are effective against broad-leaf weeds, They kill weed seeds at the time of germination, and attack troublesome grasses such as wild oat, black grass, and barnyardgrass (Fang, 1969; Fletcher and Kirkwood, 1982; Hassall, 1986; Wilkinson, 1988).

Well known examples of thiocarbamate herbicides are EPTC, Butylate, Cycloate, Benthiocarb, Vernolate, Diallate, and Triallate.

Thiocarbamates have their limitations; in spite of their low mammalian toxicity, mutagenic or carcinogenic characteristics of some members have been detected (Schuphan and Casida, 1979a; Schuphan et al., 1979, 1981; Woo and Acros, 1989). Their marginal selectivity and the resulted damage to the crops are a disadvantage (Hoffmman, 1978; Wilkinson, 1988; Hatzios, 1989a), while recently a remarkable reduction in their biological

activities due to their enhanced degradation in soil previously treated with thiocarbamates has been demonstrated (Obrigawitch et al., 1982; Tal et al., 1989; Harvey, 1990; Skipper, 1990).

1.3.1.1 Methods of application :

Due to their high volatility, thiocarbamates are incorporated into soil pre-planting or preemergence, in the form of emulsifiable concentrates or as granular formulations.

The objective of achieving effective and selective weed control was the motive behind the research carried out to improve their methods of application; using granular formulations (Hott et al., 1962), coulter injection into soil (Wooten et al., 1966), starch encapsulated formulation (Schreiber et al., 1978), sub-surface soil line injection (Dawson and Dell, 1978), Starch-Borate encapsulating (Trimnell et al., 1982), or their application jointly with liquid or dry fertilizers (Buhler, 1987).

1.3.1.2 Absorption and Translocation :

For thiocarbamates to exert their biological activity, they should be absorbed and translocated in a sufficient amount to reach their site(s) of action.

Their pattern of absorption and/or translocation depends upon the herbicide structure. Some are very mobile , while others are poorly translocated (Dutka et

al. , 1978), Some are taken up through roots and translocated apoplastically to leaves, while others are absorbed through coleoptiles and translocated both symplastically and apoplastically(Wilkinson, 1988). In general their absorption is initally rapid and then slows (Dutka et al, 1978).

The significance of underground or emerging shoots to absorption and subsequent biological activity of herbicides has been considered (Gray and Joo, 1978; Caseley and Walker, 1990; Hance and Holly, 1990). This subject is covered in greater detail in chapter three.

1.3.1.3 Mode of action :

More than one biochemical and/or physiological processes have been suggested as site of action for thiocarbamates.

Their biochemical site(s) of action has not yet been clearly elucidated (Georgy et al., 1988; Wilkinson, 1988), Lipid biosynthesis was considered as the most sensitive process (Ashton et al., 1977; Abulnaja and Harwood, 1991_b).

Various morphological responses of plants to thiocarbamates have been demonstrated; stunting, leaf growth inhibition, failure of leaves to penetrate through the coleoptiles, unfurled, wrinkled, looped, twisting, disorted, brittle, and hard leaves, young leaves failing to unroll, abnormalities and deformation of growth, and dark-green leathery leaves (Ashton and

Crafts, 1977; Harvey et al., 1975; Sagral, 1978; Wilkinson, 1978; Donald, 1981; Barta et al., 1983; Wilkinson, 1983, 1988; Hance and Holly, 1990).

Thiocarbamates have been shown to interfere with more than one biochemical and/or physiological process; inhibition of fatty acid elongation, hence epicuticular wax formation, which caused an increase in transpiration rate and then increased plant susceptibility to environmental stress (Gentner, 1966; Wilkinson and Hardcastle, 1969; Still et al., 1970; Wilkinson and Karunen, 1977; Karunen and Wilkinson, 1975; Karunen and Eronen, 1977; Leavitt and Penner, 1979; Bolton and Harwood, 1976; Kolattukudy and Brown, 1974; Ezra et al., 1982; Dodge , 1983; Fedtke, 1987; Harwood et al., 1979, 1987; Abulnuja and Harwood, 1991a; Bata, 1991). Thiocarbamates enhanced lignin deposition and stimulated peroxidase activity (Harvey et al., 1975; Wilkinson, 1988), they inhibited nucleic acid synthesis (Beste and Schreiber, 1972), their action resulted in a decrease in gibberellic acid content (Wilkinson and Ashley, 1979), and they exerted some effect on other processes including respiration, oxidative phosphorylation, and protein synthesis (Ashton and Crafts, 1973; Ashton et al., 1977; Wilkinson, 1988).

1.3.1.4 Degradation :

Thiocarbamates undergo transformation in soil, plant, water, and mammals. Their rate of transformation and the nature of their transformation products depend on the herbicide structure, the plant species, and the environmental conditions.

Thiocarbamates are relatively stable chemically, hence biological processes are the dominant route of their transformation either in plants, soil, or mammals. In general, their degradation leads to less phytotoxic products, although in some cases carcinogenic or mutagenic products have been detected (Woo and Arcos, 1989).

Various transformation processes have been identified; hydrolysis, sulfoxidation, N-oxidation, dealkylation, and hydroxylation of the aromatic ring.

1.3.1.4.1 Soil :

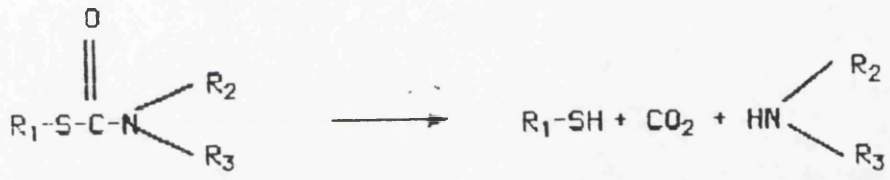
Microorganisms are the main agents responsible for thiocarbamate breakdown in soil as in sterilized soil significant inhibition of their degradation was demonstrated (Wilkinson, 1988).

The behaviour of thiocarbamates in the soil (e.g volatility, adsorption into soil colloids, and absorption by plants), and soil characterstics(moisture, organic content, texture) are factors affecting the rate and pathways of transformation.

Hydrolysis of the ester linkage with the

release of CO_2, dealkylation, and sulfoxidation are general pathways proposed for the degradation of thiocarbamates in the soil (Kauffmman, 1967; Georgy, 1988).

The general pattern of their degradation was proposed as the following :



Reports dealing with the degradation of indiviual thiocarbamates in soil have been published; the herbicide Derpamon underwent transformation to its sulfoxide and sulfone (Santi and Gozzo, 1976); Benthiocarb was degraded by hydrolysis, sulfoxidation, and dealkylation (Ishikawa et al., 1976; Nakamaura et al., 1977), and with the thiocarbamates Diallate and Triallate degradation was correlated to soil moisture (Anderson, 1981, 1984).

1.3.1.4.2 Water :

In water , the degradation pathways are similar to those in soil; the herbicide Benthiocarb was transformed via hydrolysis, sulfoxidation, dealkylation, and ring hydroxylation (Nakamura et al., 1977; Chen,

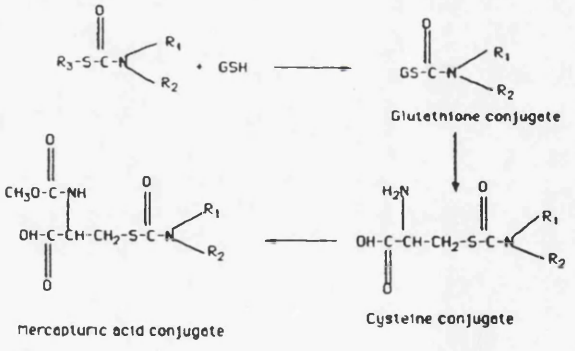
1990), while the thiocarbamate Drepamon was converted to its sulfoxide and sulfone (Santi and Gozzo, 1976).

1.3.1.4.3 Mammals :

Conjugation with mammalian constituents is an additional pathway to those previously detected in soil and water.

Three phases were distinguished in the metabolism of thiocarbamates in mammals: sulfoxidation, conjugation with the reduced form of Glutathione(GSH), and catabolism of the conjugate with the ultimate formation of a mercapturic acid conjugate (Casida et al., 1975; Hubbell and Casida, 1979; Chen and Casida, 1978; Dutka et al., 1978; Hatzios and Penner, 1982; Shimabukuro, 1985; Lamoureux and Rusness , 1989).

The proposed pathway of metabolism is :



Other transformation pathways are possible; hydrolysis, dealkylation, hydroxylation of the aromatic

ring, and alkyl oxidation (Casida et al., 1975b; Hubbell and Casida, 1977; Fang et al., 1964; Lay et al., 1979; Cashman and Olsen, 1990).

The formation of haloacroleins as degradation products of some thiocarbamates and the demonstration of their carcinogenic and mutagenic properties in mammals has been confirmed (Schuphan and Casida, 1979a; 1979b; Sikka and Florczyk, 1978; Schuphan **et al**, 1979; 1981 ;Rosen and Casida, 1980; Sandhu et al., 1988; Woo and Arcos, 1989).

1.3.1.4.4 Plants :

Metabolism of thiocarbamates in plants follows almost the same pathways as in mammals. A difference in the terminal product results from the catabolism of the Glutathione conjugate; in plants, the terminal product was characterized as either the Cysteine conjugate (Casida et al., 1975a; Hubbell and Casida, 1977), or the Malonyl-Cysteine conjugate (Lamoureux and Russen, 1989), or the Thiolactic acid conjugate (Lamoureux and Russen, 1987).

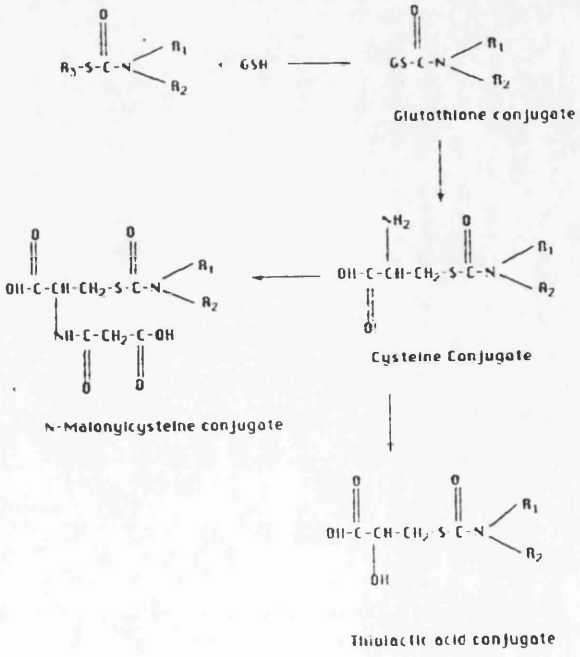
Plant species, plant age, and herbicide structure are factors affecting thiocarbamate metabolism in plants. In soybean, the thiocarbamate Vernolate was degraded with the release of CO_2 and the formation of four unidentified products. Plant age and time of herbicide exposure affected their distribution (Bourke

and Fang, 1968). The herbicide CDEC metabolized rapidally with CO_2 liberation and the formation of naturally occuring products (Jaworski, 1964).

In rice, the thiocarbamate Derpamon underwent conversion to its sulfoxide and sulfone (Santi and Gozzo, 1976).

In maize, the metabolism involves sulfoxidation , conjugation with reduced Glutathione(GSH), and catabolism of the conjugate, while hydrolysis, dealkylation, and alkyl oxidation as minor pathways are possible (Lay and Csida, 1975; Hubbell and Casida, 1977; Chen and Casida, 1978; Shimabukuro et al, 1978; Carringer et al., 1978a; Chang et al., 1974; Lamoureux and Russens, 1980; Hatzios and Penner, 1982; Shimabukuro, 1985; Lamoureux and Frear, 1987; Fedtke and Trebst, 1987; Komives and Dutka, 1989; Lamoureux et al., 1989).

Their pathways of degradation were suggested to be



The degree of transformation of thiocarbamates by light varies according to the herbicide structure, the medium in which the herbicide is examined, and the presence of other ingredients that facilitate or retard the photodegradation.

Thiocarbamate photoproducts are similar to those obtained through biological transformation. The formation of dimers and polymers as a result of free radical coupling is an additional route (Casida et al., 1975; Ishikawa et al., 1977; Demarco and Hayes, 1979; Draper and Crosby, 1981, 1984a, 1984b). This subject is covered in greater detail in Chapter six.

1.3.2 Chloroacetanilides :

Chloracetanilides are one of the most important groups in the family of amide herbicides. Substituted anilides of the Chloro family are well known as preemergence soil applied selective herbicides. They are liquid or crystalline solids with a pungent odor, slightly soluble in water. They inhibit the germination of grass weeds, and are effective against broad-leaved weeds (Georgy et al., 1988; Sharp, 1988).

Chloroacetanilides are chemically reactive because of their chlorine atom which makes them liable to electrophilic attack by nucleophiles such as the thiol group in the reduced form of Glutathione(GSH).

Examples of chloroacetanilide herbicides are CDAA, Propachlor, Metolachlor, Acetolachlor, Butachlor, and

Pretilachlor.

1.3.2.1 Absorption and Translocation :

It seems that emerging shoots and the coleoptile region are the main site of uptake of chloroacetanilides (Jaworski, 1969; Ashton and Crafts, 1973). Their absorption was shown to be rapid (Arm stng , 1973; Ashton and Crafts, 1973; Breaux, 1986). Their uptake through the roots has also beendemonstrated (Chalendr et al., 1974; Dixon and Stoller, 1982).

1.3.2.2 Mode of action :

They inhibit root elongation, cell division, and cell growth. Various biochemical targets for their action were proposed: protein synthesis (Jaworski, 1969; Ashton and Crafts, 1973; Duke et aL., 1975; Deal et al., 1980), Lipid synthesis (Mann andPu, 1967; Hassall, 1986; Fedtke, 1987; Weisshear and Boger, 1990), Gibberellin biosynthesis (Wilkinson, 1985), and membrane integrity (Mellis et al., 1982).

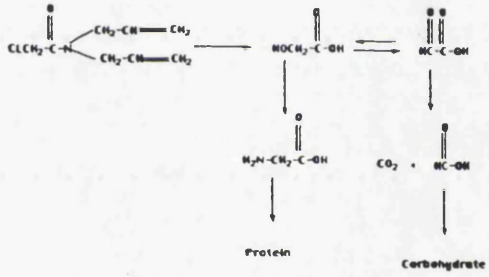
1.3.2.3 Transformation :

Chloroacetanilides undergo degradation in soil, water,mammals, and plants. Herbicide structure, plant species, plant age andenvironmental conditions are factors affecting their degradation.

Soil moisture, temperature, and organic content influenced their degradation in soil (Hargrove

and Merkle, 1971; Zimdah and Clark, 1982; Peterson et al., 1988). Microorganisms are the main cause of their degradation. Their degradation involves dealkylation, hydroxylation of the aromatic ring, and hydrolysis with the libration of CO_2 (Jaworski, 1969; Krause et al., 1985; Novick et al, 1986; Sharp, 1988; LeBaron et al., 1988; Sun et al., 1990).

In ground water, the chloroacetanilide Propachlor underwent degradation by microorganisms, and four organic products were detected (Novick et al., 1986).
In plants, Jaworski(1969) has drawn the following diagram for CDAA degradation :



while their conjugation with Glutathione (GSH) in maize, and with Homoglutathione (hGSH) in soybean and legumes has been demonstrated (Breaux, 1986, 1987).

In mammals, conjugation with Glutathione(GSH) is the main route (Larson and Bakke, 1983), the conjugate being catabolized in the following manner:
Glutathione conjugate——— Cysteine conjugate———N acetylcysteine conjugate———S-oxide of N-acetylcysteine conjugate———mercapturic acid conjugate.
Alteration as a result of light exposure has been studied. The herbicide CDAA was transformed into unidentified products via irradiation with UV light at

254nm on filter paper (Mitchell, 1961), while the herbicide Metolachlor was degraded to unknown compounds via irradiation with UV light (LeBaron et al., 1988).

1.4 Herbicide combinations :

Herbicide mixtures are used increasingly on all major crops to improve efficacy and/or reduce costs. Simultaneous or sequential application of agrochemical mixtures such as herbicides with adjuvants, fertilizers, fungicides, insecticides, and other herbicides has become a part of modern pest management practice, these combinations have advantages in reducing costs by saving time and labour, reducing soil compacting by eliminating multiple field operations, increasing the spectrum of weed control, improving chemical weed control under variable weather or soil conditions, reducing crop and/or soil residues by using the minimum dose of each chemical, delaying the appearance of herbicide resistance in weeds, and improving crop safety by using minimum doses of selected agrochemicals applied in combinations rather than a single high dose of one chemical.

Interaction between chemicals occurs when there is a modification in the biological activity of one agrochemical brought about by the other one. The nature and magnitude of the interaction depend upon dose, time of application, time of observation of the response, and the nature of target organism of the

mixture. Interaction might occur inside or outside the target species, or even before application of the mixed formulation. To study the interaction an experimental design for the study, a parameter to measure plant response, and a statistical method to predict the interaction are required (Hatzios and Penner, 1985; Green, 1989; Flint et al., 1988).

Several methods have been proposed to predict the nature and/or the magnitude of interactions (Gowing, 1960; Colby, 1964, 1967; Nash, 1981; Flint et al., 1988; Green, 1989). The earliest methods dealt with predicting the type of the interaction, not its magnitude, based on a comparison between the expected response of plant growth to the chemicals and the observed response to them in combination (Gowing, 1960; Colby, 1964, 1967).The latest methods deal not only with the existence of the interaction but also with its nature, its magnitude, and its significance (Nash, 1981; Green, 1989).

The combinations may be herbicide-herbicide or herbicide-non-herbicide. The interaction between herbicides and non-herbicides may be either enhancement, antidote activity (safening), or zero, while that between herbicide and herbicide might increase, decrease, or have no effect on the biological activity of a particular herbicide i.e. synergism, antagonism, and additive effects respectively (Green, 1989).

1.4.1 Additive Activity :

When the cooperative action of two agrochemicals is such that the observed response of a test organism to their joint action is equal to the response to both separately as predicted to occur by an appropriate reference method (Hatzios and Penner, 1985), or when the two herbicides are applied in combination over a range of rates and ratios, and the dose-response curve of each is unaffected by the other (Akobundu et al., 1975), the resulting interaction is additive.

When the total effect is equal to the effect of the most active component alone, the resulting interaction is independent.

The significance of the additive interaction comes from the fact that it has practical value in indicating the most economical mixture of agrochemicals that can accomplish the same job, and it requires that there is no phytotoxic incompatibility in mixture of herbicides with other chemicals (Hatzios and Penner, 1985).

The interaction between 2,4-D and Diclofop-methyl when applied to wild oat roots was additive (Todd and Stobbe , 1980), while Hatzios and Penner.(1985) reviewed several cases in which additive interactions between herbicides occurred.

1.4.2 Synergism :

Synergistic interaction is the simultaneous action of two or more herbicides in such a way that the total response of the organism to the mixture is greater than the sum of its response to the indiviual components (Nash, 1981), or such that the total effect is greater or more prolonged than the sum of the effects of the two components taken independently (Hatzios and Penner, 1985).

Synergistic interactions can be between a herbicide and another herbicide, e.g. between EPTC and Dinoseb in maize (Genter, 1966), and between EPTC and Bromoxynil in Alfalfa (Hartzler, 1991) or between herbicide and non-herbicide as between Metolachlor and Ozone in maize (Mersie et al., 1989).

Various mathematical methods have been suggested to evaluate the synergistic interaction (Richer, 1987). Synergism might occur as a result of an increase in uptake and translocation of a herbicide (Lichtenstein et al., 1973; Acosta-Nunez and Ashton, 1981b), or due to interference with herbicide detoxification (Mersie et al., 1989), or as the result of an effect on physiological process(s) such as between pre-emergence thiocarbamates that cause alteration in the epicuticular wax layer and post-emergence herbicides (Davis and Dusbabek, 1973).

1.4.3 Antagonism :

When the response of an organism to the combination is less than the sum of its response to the indiviual chemicals (Nash, 1981), or when the total effect of a mixture is smaller than the effect of the most active compound alone (Tammas,1964), an antagonistic interaction exists.

The degree of the antagonism is critical in determining whether a mixture is agrochemically useful or not. Thus the best combination is to achieve a synergistic effect on weeds, and an antagonistic one on crops (Green, 1989). Herbicide application rate, mode of action, plant species, time of application, stage of growth, and environmental conditions affect the magnitude of antagonistic interactions (Hatzios and Penner, 1985; Green, 1989).

Early studies on antagonistic interactions lead to the development of chemicals (safeners) that protect crops from herbicide injury without reducing the efficacy of the herbicide against weeds (Hoffmman; 1953; 1962; 1962). These studies simultaneously helped to elucidate sites and mechanisms of herbicide action (Putnam and Penner, 1974).

Four types of antagonism have been demonstrated; biochemical due to interference with herbicide absorption, translocation, and metabolism, physiological as a result of exerting opposite biological effects;

chemical due to a chemical interaction rendering the herbicideinactive, and competitive when the two compounds share the same site of action. Also antagonism could be asymmetric, when one herbicide antagonizes the activity of the other, but not the reverse (Green, 1989).

Several examples of antagonistic interactions have been described; EPTC and 2,4-D in sorghum and maize (Beste and Shreiber, 1970), 2,4-D and 2,4,6-T in tomato (Hoffmman, 1962), EPTC and CDAA in maize (Leavitt and Penner, 1978), 2,4-D and Diclofop-methyl in maize and susceptible plants (Todd and Stobbe, 1980; Shimabukuro and Hoffer, 1991), and 2,4-D and Paraquat in maize (Wilson and Worsham, 1988).

1.5 Limitations and Shortcomings of Herbicides :

Like other successful approaches, herbicides have their own restrictions which in some cases make their use undesirable. According to Pike et al. (1991) several limitations affect the use of the herbicides; availability of equipment, the perception of need, degree of crop tolerance, spectrum of weed control, potential effect on subsequent crops, cost, convenience, and health and environmental effects. It seems that maintaining the equation of risk, benefit, and cost is a difficult task.

1.5.1 Weeds Resistance :

Weed resistance to herbicides has already reached serious levels in many agricultural areas around the world. The rate of either evolution or detection of new cases of resistance has become equal to that in insects and fungi (Holt and LeBaron ., 1990).

Resistance is defined as a decrease in the sensitivity of the weed to the herbicide, relative to a normal susceptible weed. It occurs as a consequence of the application of the herbicide (LeBaron and Gressel, 1982; Putwain, 1990).

Weed resistance to a herbicide may be due to a change in the molecular structure of the binding site, a reduction in the amount of herbicide that reaches the site, or an alteration in the pattern of uptake, translocation, and metabolism of the herbicide (LeBaron and Gressel, 1982; Putwain, 1990).

Resistance can occur under selective pressure from continuous exposure to herbicides. This could stem from either (natural) selection of pre-existing resistance in plants, or genetic changes(mutation) in plants (Benbrook and Moses, 1986), and resistance to one chemical could cause cross-resistance to others (Shaaltiel et al., 1988).

Various methods have been proposed to overcome reistance problems; using an initial high dose of the herbicidefollowed by lower ones, using synergists with the herbicide to lower its dose, rotation of herbicides and of crops, and the use of mixtures of herbicides

having different modes of action(Martin, 1987; Putwain, 1990).

1.5.2 Environmental consideration :

Herbicides should remain in the treated area for long enough to exert their biological activity, but not long enough to cause environmental problems.

Herbicides applied near shallow underground sources of drinking water could come in contact with these sources (Kearney and Kuf man, 1988; Chen, 1990). The toxicological effects of herbicides and/or their metabolites that may enter the food chains should be considered, particularly with pro-mutagenic or carcinogenic chemicals (Woo and Arcos, 1989; Isensee, 1991), while contamination of the soil and consequent inhibition of microorganisms have been demonstrated (Gunther et al., 1984; Camper, 1991),creating a carry-over problem where the succeeding crops would be injured by residues of herbicides previously applied, (Curran et al, 1991; Barns and Lavy, 1991), or when a pre-emergence herbicide synergizes injury from a post-emergence one, e.g. alteration of plant cuticles (Davis and Dusbabek, 1973).

Direct hazards from herbicides to people applying, mixing, and handling them , and the danger of these chemicals to livestock, fish, and wildlife have been reported (Gangstad, 1982; Kannak et al., 1989), while their transformation in food chains, and the subsequent

risk to the public has caused great concern with respect to the safety and contamination of food (Minyard et al., 1991). This has lead to the development of more accurate and sensitive methods for their analysis (Clower, 1991), and to restrictions on their legal use (Trichilo **et al.**, 1991).

1.5.3 Enhanced degradation :

Accelerated degradation of herbicides in soil previously treated with the same or analogou s chemicals is a recent problem reducing their biological activity (Obrigawitch et al, 1983 Harvey, 1990). This phenomonona was detected initially for thiocarbamates (Harvey et al., 1987; Muller, 1988; Menkveldand Dekker, 1988), but now includes other groups of herbicides (Avidove et al., 1990; Walker and Welch, 1991, 1992). Adaptation of soil microorganisms seems to be the cause, either due to an increase in microbial population (Skipper, 1990), or to enhancment in its activity without alteration in numbers (Mooman, 1988), or to a change in both their population and the activity of the degradators (Avidove et al., 1990).

According to Tam et al. (1987) the transfer of plasmids containing genes for the degradative enzymes via conjugation is responsible. Various methods have been explored to overcome this problem; applying extenders that act by inhibiting the activity of the degradative enzymes (Rahman and James, 1983; Harvey et al., 1986), or applying other agrochemicals with the

herbicide (Behki and Khan, 1991), while Bean et al. (1988); and Reed et al. (1989) suggested herbicide rotation and the use of encapsulated formulation as alternative methods.

1.5.4 Marginal selectivity :

A selective herbicide is one that controls weeds at rates that do not injure the crop, and leaves no residue in the soil to injure succeeding crops, and no hazardous residues within the crops. Herbicide selectivity is a relative characteristic. The herbicide is selective to a particular crop within certain limits imposed by the herbicide, the plant, and the environment. It is a term that relates to the treatment and not to the compound, depending upon the application rate and method and time of application (Hatzios and Penner, 1982; Hance and Holly, 1990).

Herbicide absorption, translocation, and metabolism determine the susceptibility or tolerance of the plant to the herbicide(Hatzios and Penner, 1982; Hassall, 1986; Schultz et al., 1990; Copping et al., 1990).

Several methods have been proposed to achieve more selective chemical weed control .

1.5.4.1 Mechanical methods :

Modification of the method and technique of application so that the herbicide comes in contact

with the weeds and not the crops is an approach that has been examined to achieve more selective control (Stephens, 1982; Hatzios, 1983; Hassall, 1986).

Applying the herbicide by hand to achieve selective control of grass in Alfalfa (Dawson, 1980), or using equipment that transfers the herbicide to the weeds by direct contact (Southcombe and Seaman, 1990) have been among the methods explored.

The requirement of time, labour, and technical training made it necessary to search for other alternatives.

1.5.4.2 Technology of formulation :

Various types of formulation were described (Hatzios, 1987). A granule formulation was used to minimize rice injury because the herbicide does not reach the growing points or the roots located under the soil, and the same effect could be achieved using control release formulations (Matsunaka and Wakabayashi, 1989).

Trimmel et al. (1982) encapsulated the herbicide in coating of starch-Borate , while Shasha et al. (1987) used a starch matrix containing the herbicide to coat maize seeds.

Recently, formulating the herbicide in such a way that its biological activity is exerted after transformation in the weeds but not in the crop (pro-herbicide) was examined (Rubin and Kirino, 1989).

1.5.4.3 Development of novel herbicides :

The use of rational synthesis has successfully resulted in developing highly selective, effective herbicides with excellent environmental safety, very low application rate, and low mammalian toxicity (e.g Sulfonylureas and Imidazolinones) (Copping et al., 1990; Leavitt, 1991; Murai et al., 1991). The cost and time requirement have imposed restrictions on this approach (Hatzios, 1983; Copping, 1990).

1.5.4.4 Genetic manipulation of crops :

Conferring crop tolerance to non-selective herbicides could be achieved through mutations that decrease the sensitivity, or cause gene overproduction of the target site(s), or cause the disruption of herbicide uptake, translocation, and metabolism. The potential of this approach became evident when herbicide target sites were studied at the molecular level, and clearly identified (Gressl et al., 1982; Schultz et al., 1990).

A number of successful cases have been reported; Atrazines acting on Qb in photosystem II, Glyphosate acting on aromatic amino acid biosynthesis, Sulfonylureas acting on biosynthesis of branch chain amino acids, and phosphinotricin acting on ammonia assimilation. On the other hand the reduction or alteration in the biological activity of the processes

concerned and the possibility of cross resistance to weeds, are limitations of this method (Schulz et al., 1990).

1.5.4.5 Chemical manipulation of crops (Safeners) :

The antagonistic type of interaction between herbicides was the basis for the development of the safeners (Hoffmman, 1962, 1978). These chemicals are applied as a seed coating or as a tank mixture with the herbicide (Hatzios, 1983). They are applied mainly with the marginally selective herbicides, those that cause damage but do not kill the crop (Gressl et al., 1982). By using these chemicals, higher doses of herbicides could be used to control a wide range of weed species including those closely related to the crops, thus increasing the period over which weeds are controlled, providing greater reliability under varying environmental conditions, permitting the use of herbicides otherwise not practicable for use in a particular crop, and allowing cheaper and/or more effective herbicides to be substituted (Blair et al., 1976; Gray and Green, 1982; Hatzios, 1989). This subject is covered in greater detail in chapter two).

1.6 Thesis objectives :

This project was concerned with exploring the effects of safeners on the herbicides concerned, with particular reference to the safener Dichlormid and

analogues against the herbicide EPTC in maize. To achieve this objective several possible interactions were proposed, appropriate methods were designed, and the relevance of the results to the safener mechanism(s) of action are discussed.

Chapter one sets out to clarify concepts related to themain objective; thiocarbamates and chloroacetanilides as examples of chemical weed control agents, herbicide mixtures and their synergistic, antagonistic, or additive interactions, limitations and shortcomings of herbicides, and the possible methods of overcoming these, with emphasis on marginal selectivity, and the exploration of techniques to acheive better selective weed control.

Chapter two is intended to give a solid and comprehensive review of herbicide safeners, from various angles; history, development, uses, chemistry, adverse effects, transformation in soil, mammals, and plants, mechanism(s) of action, and recent developments in the subject.

Chapter three comprises a study dealing with the controversial hyp othesis that the mechanism of action of Dichlormid is its interference with EPTC uptake by maize roots. To carry out that aim, an experimental system and a method of analysis have been developed. In addition the interference of Dichlormid with EPTC volatilizationfrom nutrient solution, the

relation of that to its possible absorption through maize shoots, and the overall contribution of these effects to the mechanism(s) of action of Dichlormid has been studied.

Chapter four includes a review of EPTC transformations in various media, the relation of transformation to its selectivity, the role of the enzymatic catalysis of the transforming reactions, and a review of literature dealing withthe effects of Dichlormid on EPTC metabolism from various angles. It then describes the interference of Dichlormid with EPTC metabolism in maize shoots. There were three main steps in this study; synthesis of the metabolites expected (EPTC-Glutathione, and EPTC-Cysteine conjugates) using different forms of EPTC (sulfide, sulfoxide, and sulfone), and the relation of the different forms to the enzymatic contribution to EPTC detoxification; setting up an experimental technique and analytical method for the identification and quantitative analysis of the metabolites, and studying the effects of Dichlormid on EPTC metabolism in maize shoot tissue through the Glutathione pathway, and the possible relevance of that to its mechanism of action.

In chapter five, the effectiveness of Dichlormid analogues as safeners against EPTC injury in maize, its

connection to a hypothetical structure-activity relationship, and the significance of specific functional group(s) to the safening activity are demonstrated.

Chapter six comprises a study of the photochemical fate of EPTC, Dichlormid, and EPTC+Dichlormid in aqueous and non-aqueous solutions, the separation and characterization of their photoproducts, and the possible interference of Dichlormid with the rate and nature of EPTC phototransformation.

Chapter seven draws general concluding remarks relating to the work, its contribution to the elucidation of the mechanism(s) of action of Dichlormid, the limitations and the restrictions imposed throughout the work, and suggestions and perspectives for further work .

Chapter two

Herbicide safeners : Development, Uses, Chemistry, and Mechanisms of Action

2.1 Introduction :

The termssafeners, antidotes, antagonists, protectants, modifiers, and agriregulators, all refer to chemicals that have limited phytotoxicity on their own and selectively protect crop plants but not weeds from injury by herbicides. They are applied either before or simultaneously with the herbicide. As far as their terminology is concerned, no one phrase has been fully accepted. The term safeners is the most popular one; antidotes are usually applied to reverse the action of a toxic chemical, while the chemicals concerned prevent crop injury but do not reverse it. Using the term antognists excludes chemicals that exert their activity in other ways than antagonising the action of the herbicide at the site of action, and the term protectants refers mainly to chemicals that protect crops by acting externally rather than within the plant. Even the term safeners has its limitations. It could imply safening the herbicide rather than the crop, and there is no verb to safen in the English language (Gray and Green, 1982; Hatzios, 1989a).

Safeners are applied mainly with marginally selective, soil applied, shoot absorbed herbicides such

as thiocarbamates and chloroacetanilides. They are used to protect cereal crops such as maize, sorghum, and rice (Stephenson and Ezra, 1983; Fuerst, 1987), while recent reports about using chemicals as safeners to protect broad-leaved crops, particularly against the herbicide Metribuzin in soybean have been published (Varvina, 1987; Phatak and Varvina, 1989).

Site of application of both the safener and the herbicide, time of their application, method of application i.e. seed coated or tank mixture with the herbicide, and their ratio in the mixture, all are critical factors in determination the activity of the safener (Hatzios, 1983; Jablonkai et al., 1991).

Safeners have advantages in offering selective chemical weed control, counteracting the residual activity of soil applied herbicides, extending the uses and marketability of out-of-patent herbicides, helping in the elucidation of the mechanisms of action of herbicides, allowing the replacement of expensive herbicides by cheaper ones, allowing the use of higher rates of the herbicide, and controlling weeds botanically related to the crop (Hoffmman, 1969; Blair et al., 1976; Peek et al., 1981; Gray and Greens, 1982; Stephenson and Ezra, 1985; Furest, 1987; Hatzios, 1989b).

Safeners act mainly within plants, while chemicals that act by adsorption of the herbicide in the root zone are called adsorbents.

2.2 Adsorbents :

The physical shielding of the crop from contact with the herbicide was the earliest method used to protect crops from possible herbicide injury (Blair et al, 1976; Hoagland, 1989). This approach includes the use of Activated carbon, lignin by-products, calcium polysulfides, ion exchange resins, various clays, magnesium silicate, and alumina (Hoagland, 1989).

In addition to protecting crops from herbicide injury, adsorbents are used to remove harmful constituents from drinking water, soil, and spray equipment. They are used in various ways; as a concentrated band above the germinating seeds, coated directly on the seeds, sprayed directly on the plants, applied as a root dip, as dry powder, and mixed with water to form slurry that is sprayed onto soil. They are applied at time of sowing or before to inactivate herbicides already present in the soil (Blair et al., 1976; Gray and Greens, 1982; Hoagland, 1989).

The protective efficiency of the adsorbents depends upon various factors; soil adsorptive capacity, seedling depth, rate of the wetting agent added, herbicide type, herbicide rate, type of adsorbent used, and plant species (Goffey and Warren, 1969; Bo vey and Miller, 1969; Burr et al., 1972; Lee, 1973; Hoagland, 1989).

Adsorbents have been used to protect various crops against different herbicides; maize against various herbicides (Gupta and Niranwad, 1976), barley seedlings

against Diuron (Toth and Micham, 1975), aspargus against Linuron and Chloramben (Ogg, 1978), maize against Chlorosulfron (Eleftherohorinos, 1987), tomato against Metribuzin (Toth et al., 1987), soybean against Metribuzin (Street et al., 1987), cucumber and beans against Propachlor (Bovey and Miller, 1969), maize seedlings against EPTC (Gray and Joo, 1978), and cucumber against the herbicide Chloramben (Knerr and Hoen, 1989).

The requirement for an application technique different from that used for herbicide application, the expense, the inadequate control of weeds, and the development of safeners as an effecient alternative method have caused a decline in the uses of the adsorbents.

2.3 Safeners (Historical background) :

Starting from the antagonistic interaction between 2,4-D and 2,4,6-T (Hoffmman, 1953), a long period of research by Hoffmman lead to the introduction of Nahpthalic Anhydride (NA) as the first commercial safener against thiocarbamate herbicides in maize in 1971 (Hoffmman, 1960, 1962, 1969, 1978a, 1978b).

The screening of a large number of chemicals against thiocarbamate herbicides in graminaceous crops lead to the development of the safener Dichlormid in 1972 used against EPTC and Butylate in maize (Chang et al., 1973a, 1973b; Pallos et al., 1975, 1978; Hatzios,

1983). The botanical and chemical selectivity of Dichlormid made it suitable for application as a tank mixture with the herbicide rather than as a seed coating. The advantages of this method lead to a decline in the use of Naphthalic Anhydride (Hatzios, 1989a).

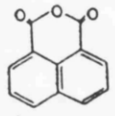
The safening activity of Oxime ether compounds has been successfully examined, and three of them were introduced as a commercial safeners(Cyometrinil, Oxabetrinil, and CGA-15281) against chloroacetanilide herbicides in sorghum (Ellis et al., 1980; Peek et al., 1981; Chang and Merkle, 1982). Later the Thiazol-carboxylic acid (Flurazole) was introduced against the herbicide Alachlor in sorghum (Sacher et al, 1983), and recently the safener Fenclorim was introduced against the herbicide Pretilachlor in rice (Hatzios, 1989a). On the other hand, various chemicals have shown different types of safening activity (Pallos et al., 1975, 1978; Gorgy et al, 1982; Wuertzer et al., 1983; Nagy and Balogh, 1985; Szell et al., 1985; Dutka and Komives, 1987; Wried et al., 1991a, 1991b), and some success has been achieved in the protection of broad-leaved crops (Varvina, 1987; Phatak and Varvina, 1989; Kneer and Hopen, 1989; Devlin and Zbiec, 1990), while the safener Naptalam has shown some success against the post-emergence application of the herbicide Paraquat in peanuts (Wehtje et al., 1991) and recently microorganisms degrading the herbicide EPTC were applied

by inoculation and used as a successful safeners in maize (Nagy et al., 1992).

2.3.1 Uses :

2.3.1.1 Naphthalic Anhydride :

Naphthalic Anhydride (NA) or according to IUPAC Naphthalene-1,8-dicarboxylic anhydride has the formula



NA

It is a light tan crystaline solid with melting point 270-274°C. It is relatively insoluble in water and in most non-polar solvents. It is stable under normal storage conditions, non-corrosive, non-hygroscopic, and relatively non-toxic to mammals.

NA was introduced as commercial safener in 1971. It is the most verstaile safener, being less specific botanically and chemically than other safeners. It protects various crops against wide range of herbicides. It has been tested successfully to protect maize against thiocarbamates, dithiocarbamates, and chloroacetanilides. It was useful in protection of rice, grain sorghum, and oats (Hoffmman, 1969, 1978; Blair et al., 1976; Stephenson and Chang, 1978; Hatzios, 1983). It was capable of providing safening activity against post-emergence application of selected herbicides (Parker, 1983). It offered safening activity to maize

against the herbicide Prefluradone (Blair and Bean, 1976). It enhanced the tolerance of wheat (Miller et al., ., 1978), and oats and barley (Chang et al., 1974; Blair, 1978) to the herbicide Barban. Also its safening activity has extended to include broad-leaved crops (e.g.bean) against the herbicide EPTC (Blair, 1979). It protects against the herbicide Chlorsulfron in maize (O'leary and Prendeville, 1985), and the herbicides Thiobencarb and Molinate in white rice (Deandrade, 1985). Codde.(1988) detected its safening activity against the herbicide Diclofop-methyl in oats, and recently Milhomme and Bastide.(1990) demonstrated that it was active against Metsulfuron in maize. Boldt and Barrett.(1991) showed that it protected maize against injury by the herbicide Imazethapyr.

2.3.1.2 Chloroacetamides :

By using the random screening method, chloroacetamide chemicals have been identified as showing safening activity particularly against thiocarbamates and chloroacetanilides (Pallos et al., 1975; Stephenson and Chang, 1978). Among the chemicals examined Dichlormid was the most effective safener when added in small amounts to EPTC and other thiocarbamates in preventing the onset of herbicide injury to maize plants (Chang et al., 1972, 1973; Pallos et al., 1978).

Dichlormid (N,N-diallyl-2,2-dichloroacetamide) is a coulerless viscous liquid, soluble in most organic

solvents. Its solubility in water is 5g/L at 20°C, with vapour pressure of 0.006mmHg at 25°C. It is relatively non-toxic to mammals (Worthing, 1979).

It was thought that Dichlormid was highly specific chemically and botanically when it was introduced in 1972 (Pallos et al., 1975; Chang et al., 1973; Stephenson et al., 1978, 1979; Dutka et al, 1979), but wider safening activity was demonstrated against other herbicides in addition to thiocarbamates; it has been used effectively with chloroacetanilides (Leavitt and Penner, 1978), with the herbicide Sethoxydim (Hatzios, 1984a), with the bleaching herbicide Clomazone (Devlin and Koszanski, 1987), and with the herbicide Chlorosulfron (Polge, 1989). This illustrates that the reactivity of Dichlormid is chemically more diverse than was previously believed, and recently Dichlormid was used as a synergist with post-emergence herbicides (Fedtke and Strang, 1990).

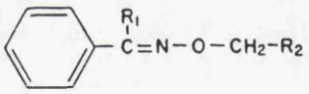
Another chloroacetamide which is mainly used as a herbicide but has safening activity when used at sub-toxic rates, is N,N-diallyl-2-chloroacetamide (CDAA).

Its safening activity has been detected against chloroacetanilides and thiocarbamates in maize (Chang et al., 1973; Pallos et al., 1978; Stephenson and Chang, 1978; Leavitt and Penner, 1978; Hatzios and Penner,

1980; Stephenson and Ezra, 1983; Ezra et al., 1985). In addition various degrees of safening activity have been detected for other members of the chloroacetamide group (Stephenson and Chang, 1978; Blair, 1979; Leavitt and Penner, 1978; Winkle et al., 1980; Gorog et al., 1982; Nagy and Balogh, 1985; Dutka and Komives, 1987; Szelle et al., 1988; Fuerst et al., 1991).

2.3.1.3 Oxime Ethers :

Oxime ethers exhibit good chemical and botanical specificity in being highly selective safeners against chloroacetanilides in grain sorghum (Peek et al., 1981; Chang and Merkle, 1982; Chang, 1983; Hatzios, 1989a). They share a common structure :



Three commercial safeners within this group have been introduced :

Cyometrinil : R_1: CN , R_2: CN

Oxabetrinil : R_1 : CN, R_2 : 

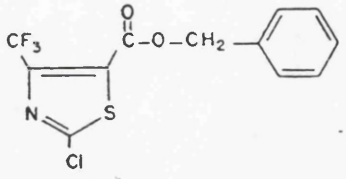
CGA-133205 : R_1 : CF_3, R_2 : 

The adverse effects of Cyometrinil on sorghum seed germination, and crop establishment lead to the introduction of its analogue Oxabetrinil (Turner et al., 1982), while the undesirable interaction of Oxabetrinil with downy mildew of grain sorghum was the reason for

the introduction of CGA-133205 (Hatzios, 1989a).

2.3.1.4 Others safeners :

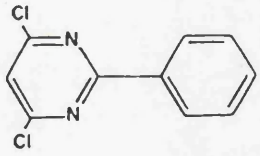
The Thiazolcarboxylate compound Flurazole was introduced as a safener against the herbicide Alachlor and Acetochlor in grain sorghum (Schafer et al., 1981)



Flurazole

Flurazole showed high degree of chemical specificity, failing to protect maize against thiocarbamate and chloroacetanilide herbicides (Hatzios, 1986).

Another safener that introduced recently on a commercial scale to protect rice against the herbicide Pretilachlor is Fenclorim :



In addition to the above commercial safeners, a wide range of chemicals have been tested and have shown various degrees of safening activity (Dutka and Komives, 1987; Hatzios, 1989a; Burckhardt et al., 1990; Ogasawara et al., 1991; Wreid et al., 1991a, 1991b; Lamoureux and Rusenss, 1992).

2.3.2 Methods of application :

The chemical and botanical specificity of the safener determine the most appropriate method for its application.

The safener Naphthalic Anhydride has limited specificity, and offers protection to the weeds when applied to the soil, so it is mainly used as a seed coating at a rate of 0.5%(w/w) of the seed (Guenyli, 1971; Hoffmman, 1969, 1978).

Dichlormid shows safening activity when applied either as a seed dressing, or as a tank mixture with a soil applied herbicide (Chang et al., 1973; Pallos et al., 1975; Blair et al., 1979; Parker, 1983), but due to its availability to plants from the soil, and simplicity in the method of application, it is sold as a pre-packaged formulation with the herbicide (Gray and Greens, 1982; Hatzios, 1983). On the other hand the possibility that the weed population will shift toward plants tolerant to Dichlormid and the herbicide formulation following their repeated application is a hazard of this method (Stephenson and Ezra, 1983).

Due to their marginal selectivity, and the protection of the weed after soil application, oxime-ether safeners are usually applied as a seed coating in grain sorghum (Ellis et al., 1980; Peek et al., 1981).

The safener Flurazole was effective when applied to the soil, but due to its protection of weeds, it is

usually applied as a seed dressing (Schafer et al., 1981), while the safener Fenclorim has been introduced as pre-packaged tank mixture with the herbicide (Hatzios, 1989a).

2.3.3 Structure-Activity relationship :

It has been demonstrated that the presence of the dicarboxylic anhydride group, and at least one aromatic ring attached directly to the anhydride is essential for the safening activity of Naphthalic Anhydride and its analogs (Hatzios and Zamma, 1986), and substantial safening activity has been detec ted in Naphthalic Acid (Codde, 1988, Sha ner, 1991).

For chloroacetamide safeners the structural similarity with the herbicides has been proposed as the basis for their activity (Stephenson and Chang, 1978; Stephenson et al., 1978, 19798; Dutka et al., 1979; Yenne, 1989; Yenne and Hatzios, 1990b), and according to Stephenson and Chang.,(1978) in a soil free system, the most effective safener is the one most similar structurally to the herbicide. On the other hand Mono and Trichloroacetamides were less effective as safeners compared with the Dichloro compounds (Stephenson et al., 1978, 1979; Dutka et al., 1979), and those with two substituents on the nitrogen are more active than those with one(Dutka et al., 1979). ; Dutka et al.(1979) , and Dutka and Komives., (1983) emphasised the significance of the dichloroacetamido moiety for the safening

activity, while later Dutka and Komives., (1987) confirmed the essentiality of the dichloroaceto group rather than dichloroacetamido to the safening activity of Dichlormid.

The presence of oxime and pyridine groups or two oxime groups and an aryl group attached to oxygen in the molecule was essential for oxime ethers to exert safening activity (Chang and Merkle, 1982; Chang, 1983).

2.3.4 Transformations:

Safeners undergo transformation in soil, plant, and mammals. The rate and nature of their transformation products depends upon the safener, the medium in which it resides, and environmental factors.

In soil, micoorganisms are mainly responsible for degradation of safeners. The safener Naphthalic Anhydride is transformed rapidly. Although its decarboxylation has been shown as the most possible pathway, its degradative routes have not been fully elucidated (Riden and Asbell, 1975).

For the safener Dichlormid, dechlorination, dealkylation, oxidation, and hydrolysis were demonstrated as pathways of degradation (Miaullis et al., 1978). No enhancement of degradation in soil previously treated with Dichlormid has been detected (Wilson and Rodebush, 1987).

In plants, Naphthalic Acid has been suggested as a metabolite of Naphthalic Anhydride (Riden and Asbell, 1975; Frear et al., 1987; Codde, 1988). Dichlormid underwent transformation through oxidation, dealkylation,dechlorination, hydrolysis, and conjugation with plant constituents (e.g Glucose) (Miaullis et al., 1978). The oxime ether safener Oxabetrinil was metabolised via conjugation with Glutathione (GSH) while the safener CGA-13305 yielded unidentified products in sorghum plants(Yenne,1989; Yenne and Hatzios, 1990a), while Brooks. (1987), and Brooks et al.(1987) reported that oxime ether safeners were metabolized by oxidation in sorghum coleoptiles.

Unidentified metabolites were detected from the safener Flurazole in sorghum (Jackson, 1984), and its conjugation with Glutathione (GSH) has been demonstrated (Breaux et al., 1989). Recently the safener Fenchlorazole ethyl was shown to be metabolized through hydrolysis and dealkylation in wheat and barley (Romano et al., 1991).

In mammals, Dichlormid yielded similar products to those detected in soil and in plants, while its conjugation with Glutathione (GSH) was an additional route, and its terminal products were identified as Glycolamide, Glyoxamidoxamic acid, and Dichloroacetic acid (Miaullis et al., 1978).

2.3.5 Limitations and Adverse effects :

Since safeners have functional group(s) similar to those in the herbicides, they may be expected to have phytotoxic effects under some conditions. In the field both their performance and their adverse effects are influenced by environmental factors; temperature, soil moisture, soil structure, and cultivar biotype.

2.3.5.1 Naphthalic Anhydride :

When used at high rates, Naphthalic Anhydride has adverse effects (Blair et al., 1976). Stunting and chlorosis were detected as phytotoxic symptoms in maize, sorghum, rice, oats, and bean even at a commercial rate(0.5% w/w) (Thessien et al., 1980; Hatzios, 1984a, 1984b; Blair, 1979). A reduction in maize shoot development as a result of NA treatment was demonstrated (Guneyil, 1971), and variation in its safening activity with sorghum biotype has been shown (Hann, 1974). Its performance varies with soil type(Stephenson and Ezra, 1983).NA performance against EPTC was partial in sandy or silt loam soil compared with clay loam (Guenyil, 1971) while Parker and Bean., (1976) observed slight damage to rice as a result of NA treatment.

2.3.5.2 Dichlormid :

The performance of Dichlormid as a safener was affected by several factors; soil incorporation, seed placement, cultivar type and herbicide behaviour(e.g

leaching, breakdown) (Burt, 1976). Due to a difference in the degree of solubility between EPTC and Dichlormid the two chemicals leach at different rates under heavy rainfall or irrigation conditions, causing the loss of the two compounds from the treatment zone and hence plant injury(Burt and Buzio, 1980). Crook (1975) reported that

Dichlormid was ineffective against thiocarbamate herbicide in light soils. A possible shift in weed population towards species tolerant to the formulation EPTC+Dichlormid as a result of their repeated application as a tank mixture is expected (Stephenson and Ezra, 1983).

Safening activity varies according to the plant genotype. Thus some varieties are not protected by Dichlormid (Wright et al., 1974; Sagaral, 1978; Sagaral and Foy, 1982; Lay and Niland, 1985).

2.3.5.3 Oxime ethers :

As a result of the adverse effects of the safener Cyometrinil on seed germination and crop establishment in grain sorghum, its analogue Oxabetrinil was introduced (Turner et al., 1982). It in turn caused an increase in the incidence of sorghum downy mildew disease (Graig et al., 1987; Szerszan et al., 1988), which lead to the introduction of the safener CGA-133258 (Hatzios, 1989a).

Inhibition of several metabolic processes by

Cyometrinil and Oxabetrinil (Rufener et al.,1982; Zama and Hatzios, 1986), and a significant reduction in grain sorghum germination as a result of Oxabetrinil treatment have been demonstrated (Yenne and Hatzios, 1989).

2.3.5.4 Others Safeners :

The safener Flurazole has adverse effects on viability, respiration, and growth of grain sorghum (Schafer et al., 1980; Ketchersid and Merkle, 1983), and it inhibited some metabolic processes; photosynthesis, protein synthesis, nucleic acid synthesis, and lipid biosynthesis in isolated soybean leaf cells (Zama and Hatzios, 1986).

2.3.6 Mechanisms of action :

Early investigations suggested that safeners might act through a single mechanism that was assumed to be common to all crop-herbicide-safener combinations. This suggestion was disputed later. It was proposed that the action of safeners was most likely to be the result of a series of multiple interactions between the safener and the herbicide which contribute to the overall protection (Ezra et al.,1983).

The fact that the currently available safeners exhibit botanical specificity for graminaceous crops with moderate tolerance of the herbicides, and chemical selectivity toward soil applied and shoot absorbed thiocarbamate and chloroacetanilide herbicides lead to

asuggestion that their action relates to physological, biochemical, and molecular function(s) which are unique to or highly efficient in the graminaceous crops affected by thiocarbamates and chloroacetanilides and altered by the safeners, while these systems are either not present or not affected to the same extent by the herbicides and safeners in other plants (Hatzios, 1989b).

The similarity in the site(s) of uptake and action of both the safeners and the herbicides in the coleoptile region of the plant shoot is another common feature (Gray and Joo, 1978; Peek et al., 1981).

The fact that, to exert their activity, safeners should be applied simultaneously with or before the herbicide indicates that they prevent but do not reverse the damage caused by the herbicide (Hatzios, 1983; Stephenson and Ezra, 1985).

Several pathways have ben proposed explaining action of safeners; modification of the sensitivity of the target site of the herbicide, overproduction in the synthesis of target site(s), or both (Duessing, 1984), while three main hyphotheses have attracted in trest.

2.3.6.1 Interference with herbicide absorption and translocation :

For the herbicide to exert its biological activity it should be absorbed and translocated in adequate quantity to its site(s) of action. Various factors affect its pattern of uptake and/or translocation ; the

herbicide molecule, the method of application, the type of formulation, herbicide rate, and environmental factors (this subject is covered in greater detail in chapter three).

Safeners being chemicals applied with herbicides can interfere with their pattern of absorption and/or translocation. This could be through chemical interaction with the herbicide, biochemically through disruption of some biochemical processes ultimately leading to an alteration in the pattern of uptake and/or translocation, or by competition with the herbicide at the site(s) of entry (Hatzios, 1989b). The interference could result in reduction in uptake and/or translocation (Arhens and Davis, 1978; Ezra et al., 1982; Ketchersid et al., 1980 O'leary and Prendeville, 1982; Varvina, 1987; Ketchersid, 1990; Furest et al., 1991; Han and Hatzios, 1991_b; Wehtje et al., 1991; Furest and Lamoureux, 1992), or stimulation (Guneiyl, 1971; Furest and Gronwald 1986; Zama and Hatzios, 1986; Codde, 1988; Yenne and Hatzios, 1990; Milhomme and Bastide, 1990), or no significant effect(Holm and Murphy, 1972; Chang et al., 1974; Szabo, 1974; Marton et al., 1978; Winkle et al., 1980; Christ, 1981, 1985; Rubin et al., 1985; Jackson et al., 1986; Sander and Barrett, 1989a; Rowe et al, 1991; Lamoureux and Rusness, 1992).

2.3.6.1.1 Chemical antagonism :

The safener and the herbicide have functional group(s) capable of interacting chemically. This interaction could be in the tank mixture, in soil, in plant surfaces, and within the plant. The formation of products might prevent herbicide entry into the plant , or alter its pattern of translocation.

Little has been done to explore this possible mechanism , but it has been established that adsorbents act by preventing entry of the herbicide into the plant physically (Gupta, 1976; Blair et al., 1976; Hoagland, 1989).

The possiblity of complex formation between Dichlormid and EPTC in soil has not been confirmed, but the fact that Dichlormid exerts its safening activity when applied to soil as well to nutrient solution rules out the significance of soil factors in its mechanism of action (Gray and Joo, 1978; Stephenson and Chang, 1978). The formation of such complexes was the basis for applying metal ions to reduce the phytotoxicity of herbicides (e.g Glyphosate (Turner and Loader, 1978), or applying chelating agents with photosynthesic inhibitor herbicides (Jansen et al., 1990).

2.3.6.1.2 Competition at the site of entry :

The possiblity that the safener blocks herbicide uptake at the site of entry, or at some point along its

translocation pathway seems likely. Most safeners and herbicides have similar site(s) of uptake (Gray and Joo, 1978; Jackson et al, 1986; Nyffeler et al., 1980). This possiblity has been examined and different results obtained depending upon the method of study and other factors (Hatzios, 1983c; Stephenson and Ezra, 1983).

2.3.6.1.3 Biochemical alteration :

Safeners might exert their ultimate effect on herbicide uptake and translocation through alteration of physiological and biochemical processes. As an example, when safeners reversed the inhibition of epicuticular wax formation caused by the herbicides , the rate of transpiration and as a result herbicide uptake were reduced (Leavitt and Penner, 1978a; Ebert, 1982; Georog et al., 1982; Ebert and Ramsteiner, 1984).

2.3.6.2 Herbicide metabolism :

The tolerance or susceptibility of a plant to a herbicide relates to its ability to detoxify the herbicide (Hatzios and Penner, 1982; Shimabukuro, 1985; Breaux et al., 1987).i.e. its ability either to transform the active form to an inactive one or to avoid metabolizing an inactive substance into an active one (Dodge, 1990).

Safeners could have the ability to alter

herbicide metabolism directly by acting as chemical activators of particular functional groups in the herbicide, or by affecting biological system(s) (e.g enzymes) involved in the transformation of the herbicide (Lamoureux and Frear, 1987; Hatzios, 1989a). The involvement of herbicide metabolism in the mechanism(s) of action of the safener is the most popular hypothesis in this respect (Casida et al., 1974, 1975; Hubbell and Casida, 1977; Sagaral, 1980; Winkle, 1980; Jackson, 1984; Fuerst and Gronwald, 1986; Zama and Hatzios, 1986; Varvina, 1987; Gronwald, 1989; Milhomme and Bastide, 1990; Han and Hatzios, 1991b; Fuerst et al., 1991; Bussler et al., 1991; Ogasawar et al., 1991; Yaacoby et al., 1991; Lamoureux and Rusens., 1992). This hypothesis has been disputed (Leek, 1981).

Safeners are mainly applied with thicarbamates and chloroacetanilides, whose metabolism involves two established metabolic systems; mixed function oxidases and glutathione related enzymes. The possiblity that safeners interfere with one or both of the above systems has been investigated.

2.3.6.2.1 Glutathione system :

Glutathione is a tripeptide with the sequence glu-cys-gly. It exists in every cell in the living system. The glutathione status of the cell is the total cellular concentration of Glutathione and the relative distribution between its major

forms;reduced Glutathione(GSH), oxidizedGlutathione(GSSG), and mixed disulfides such as GSS-Protein (Kosower, 1976; Kosower and Kosower, 1978). Glutathione (GSH) has multifunctional properties. It is involved in enzyme mechanisms, in biosynthesis of macromolecules, in drug metabolism, in radiation, in cancer, in oxygen toxicity, and in environmental toxins (Quintiliani, 1990).

In plants, Glutathione (GSH) has a vital role; its use as a storage form of reduced sulfur in plant cells, it serves as the main long distance transport form of reduced sulfur translocated from mature leaves to roots, it protects chloroplast membranes from oxidative damage caused by hydrogen peroxide and other oxidative stresses(e.g extreme temperature, drought, air pollutants, and herbicides), it conjugates with exogenous and endogenous compounds, it is involved in the synthesis of protein and DNA, it maintains enzyme activity, and it is involved in the detoxification of pesticides (Shimabukuro, 1975; Kosower and Kosower, 1978; Lamoureux et al, 1970; Rennenberg, 1982; Meister and Anderson, 1983; Meister, 1983; Halliwell, 1984; Alscher, 1989). According to Jablonkai and Hatzios. (1991) the tolerance or sensitivity of plant to the herbicides relates to GSH content.

Environmental stresses and chemicals alter Glutathione status in plants (Meister, 1983; Polge,

1989). Exposure of beans to ozone(O_3) caused a reduction in GSH content (Gun, 1983), while exposure of maize roots to heat shock resulted in an increase in GSH content (Nietro-Soleto and Ho, 1986), and in frost-hardened spinach leaves, an increase in GSH content was detected (Dekok and Oosterhuis, 1983). Illumination of Euglena gracilis cells caused a rapid increase in total Glutathione followed by a gradual decrease (Shigeoka et al., 1987).

Safeners interfere with Glutathione status in a way that facilitates detoxification of herbicides. This could be by affecting either Glutathione(GSH) content or Glutathione-S-transferase enzymes(GST), which might take place through different mechanisms.

2.3.6.2.1.1 Glutathione(GSH) content :

A remarkable increase in Glutathione (GSH) content following safener application to graminaceous plants(maize, sorghum, and rice) has been detected (Casida et al., 1975; Hubbell and Casida, 1977; Dutka et al., 1979; Adams et al., 1983; Polge et al., 1987; Breaux et al., 1987; Gronwald et al., 1987; Gronwald, 1989; Polge, 1989; Yenne and Hatzios, 1990; Fuerst et al., 1991; Bussler et al., 1991). This increase takes place through various pathways.

2.3.6.2.1.1.1 Glutathione biosynthesis :

Glutathione synthesis in higher plants may be divided into two steps :

Cysteine + Glutamyl acid $\xrightarrow{\text{glutamylcysteine synth ase}}$ Glutamylcysteine

Glutamylcysteine + Glycine $\xrightarrow{\text{glutathione synthetase}}$ Glutathione.

An increase in the amount of precursors or in the activity of the enzymes catalyzing the synthesis leads to an increase in GSH content. An enhancement in sulfate uptake by maize roots, and in the activity of enzymes involved in its reduction and assimilation as a result of treatment with dichloroacetamide safeners leads to an increase in the content of cysteine which is ultimately incorporated in Glutathione(GSH) (Adams, 1982; Adams et al., 1983; Farago and Brunold, 1990).

Transformation of chemicals applied as safeners into cysteine has also been demonstrated (Hilton and Phillai, 1986; Polge, 1989; Hilton et al., 1990). Induction of the activity of glutathione synthase enzyme as a result of treatment with the safener Dichlormid (Carringer et al., 1978a; Rennenberg et al., 1982), and with the safener Flurazole (Breaux, 1986) has been detected.

2.3.6.2.1.1.2 Glutathione reduction :

Total Glutathione in plant exists either as the reduced form, the oxidized form, or mixed disulfides. The reduction of the oxidized form (GSSG) to the reduced one (GSH) is catalyzed by the enzyme glutathione reductase (GR). An enhancement of its activity results in an increase in the amount of the reduced form of (GSH).

The safener Dichlormid induced (GR) in maize seedlings (Komives et al., 1985a, 1985b; Komives and Dutka, 1988, 1989), while induction due to application of the safeners Oxabetrinil and CGA-133205 in sorghum seedlings has been demonstrated (Yenne and Hatzios, 1990).

2.3.6.2.1.1.3 Feedback inhibition of Glutathione biosynthesis :

Glutathione biosynthesis in mammals and plants is believed to be controlled by a feedback regulation process (Miester, 1983), and Kondo et al.(1984) reported that GS-conjugates interfered with the biosynthesis of GSH through feedback inhibition of glutathione synthesis enzymes.

The demonstration that safeners themselves could be metabolized through conjugation with Glutathione lead to a suggestion that they increase GSH content by interfering with the feedback inhibition of its

synthesis (Miallius et al., 1987; Ezra et al., 1985; Breaux et al., 1989; Yenne and Hatzios, 1990).

2.3.6.2.1.2 Glutathione-S-Transferases :

Glutathione-S-transferase (GST) is a family of isozymes exhibiting a high degree of substrate specificity (Frear and Swanson, 1970; Diesperger and Sanderman, 1979; Guddewer and Dauterman, 1979; Mozer et al., 1983; Edward and Owen, 1986; Dean et al., 1990). Their major function is to

catalyze the detoxification of hydrophobic and electrophilic compounds through conjugation with reduced Glutathione (GSH) (Mozer et al., 1983; Clark et al., 1984, 1986; Ezra et al., 1985; Mennervik and Danilson, 1988; O'connell et al., 1988; Gronwald, 1989; Schroder et al.,1990; Edwards and Dixon, 1991). Purification of GST isozymes has been carried out from various species, and their substrate specificity was confirmed (Timmerman, 1989; Grant and Matsumura, 1989; Dean et al., 1990; Hunaiti and Ali, 1991).

The tolerance and susceptibilty of various species to xenobiotics (e.g pesticides) was strongly correlated to the level and/or activity of GST enzymes that catalyze their detoxification via conjugation with Glutathione (GSH) (Shimabukuro et al., 1971; Jachetta and Rodosevich, 1981; Offea et al., 1984; Grant and Matsumura, 1989; Riedy et al., 1990).

Alteration in the activity of GST enzymes as a result of chemical treatment was reported; Shiotsuki et al.(1990) detected an inhibition in GST activity by treatment with S-benzyl Glutathione analogues, while Zama and Hatzios.(1986 demonstrated inhibition as a result of Tridiphane treatment. However, induction of the enzymes and/or increased activity as a result of chemical treatment has also been observed; with Phenobarbital (Hayaoka and Dauterman, 1983), Phenobarbital and 3-methyl chloranthrene (Ottea et al., 1984), and with essential oils and their processing by-products (Lam and Zheng, 1991). This increase in activity could be due to direct activation of the existing enzyme molecules (Rennenberg et al., 1982; Dean et al., 1990), or to an enhancement in de novo synthesis of new forms of the enzymes (Ottea et al.,1984; Hunaiti and Ali, 1990, 1991), following an increase in the steady state levels of mRNA coding for isozymes (Wiegand et al., 1980; Dean et al., 1990).

An induction of these enzymes as a result of safener application, followed by an increase in the rate of herbicide conjugation with Glutathione (GSH) has been demonstrated (Lay and Casida, 1976, 1978; Hubbell and Casida, 1977; Mozer et al., 1983; Lay and Niland, 1985; Komives et al., 1985b; Edwards and Owen, 1986b; Polge et al., 1987; Gronwald et al., 1987; Dutka and Komives, 1987; Komives and Dutka, 1988; Edwards and Owen, 1988;

Ekler and Stephenson, 1989; Yenne and Hatzios, 1990; Fuerst et al., 1991; Fuerst and Lamoureux, 1992).

2.3.6.2.2 Mono-function oxidase enzymes (Cytochrom p-450) :

Mono-function oxidase enzymes are homoproteins containing cytochrome p-450 as a central feature in their structure (Hatzios and Penner, 1982; Komives and Dutka, 1989). They are involved in xenobiotic metabolism through catalysing herbicide oxidation in plants, animals, and microbes. Cytochrome p-450 binds both xenobiotic substrate and molecular oxygen (O_2), hence catalyzing transfer of an oxygen atom to oxidise the xenobiotic, whilst the second atom of oxygen forms water. This is involved in processes like alkyl hydroxylation, aryl hydroxylation, sulfoxidation, epoxidation, and N-, and O- dealkylation (Hendry, 1986; Jones and Caseley, 1989; Komives and Dutka, 1989).

In plants, the presence of mono function oxidase enzymes in plant homogenates (shoots and roots), and their participation in herbicide metabolism has been confirmed (Taft, 1975; Casida et al., 1975; Hubbell and Casida, 1977; Leavitt, 1978; Blair et al., 1984; Cole and Owen, 1987; Komives and Dutka, 1989; Moreland et al., 1990; Fonne-Pfister and Kreuz, 1990; McFadden et al., 1990).

The inhibition or activation of MFO enzymes as a result of chemical treatment and its effect on herbicides metabolism have been studied. Inhibitory chemicals include; cysteine (Krueger, 1977),, and 1-aminobenzotriazole (McFadden et al., 1989), Tridiphane (Moreland et al., 1989), insecticide synergists (Casida et al., 1970; Fedtke and Trebst, 1987), 1-Aminobenzotriazole (Cole and Owen, 1987), carbon monoxide, piperonyl butoxide, Tetcyclasis, and Tridiphane (Moreland et al., 1990), and carbon monoxide (Fonne-Pfister and Kreuz, 1990; Zimmerlin and Durst, 1990).

Mono function oxidase enzymes are believed to be involved in thiocarbamate and chloroacetaniline metabolism in higher plants, catalysing their sulfoxidation and hydroxylation respectively (Komives and Dutka, 1989). The effect of safeners on these enzymes and hence on herbicide metabolism has been studied mainly by indirect methods by using MFO inhibitors that synergized herbicide action in the presence of the safeners (Hubbell and Casida, 1977; Ketchersid et al., 1981; Hatzios, 1981; Dutka and Komives, 1983; Hatzios, 1983b; Taylor and Loader, 1984; Krause et al., 1985; Brooks et al., 1987; Milhomme and Bestide, 1990; Fonne-Pfister and Kreuz, 1990; McFadden et al., 1990; Taft,1975; Leavitt, 1978; Zimmerlin and Drust, 1990, Mougin et al., 1991). Recently Fedtke.(1991) reported that MFO inhibitor (PBO)

pretreatment of etiolated oat roots increased the activity of MFO enzymes and hence the uptake and metabolism of the herbicide Mefenacet, while its application together with the herbicide lead to inhibitory effects. Barrett and Maxon.(1991) confirmed that the safener NA enhanced the activity of MFO enzymes and hence the metabolism of the herbicide Imazethapyr in maize, and similar results have been reported for the safener NA against the herbicides Diclofop, Chlorosulforn, and Linuron (Frear et al., 1991). On the other hand Barta and Dutka.(1990, 1991a, 1991b) indicated that safeners inhibited MFO activity rather than increasing it.

2.3.6.2.3 Other pathways of herbicides metabolism :

Conjugation of herbicides with Homoglutathione(hGSH) rather than Glutathione(GSH) in some plant species(soybean) and the possible involvement of safeners with this pathway has not been ruled out (Breaux et al., 1987, 1989; Frear et al., 1985).

2.3.6.3 Competition at the site of action :

The idea that safeners counteract herbicide action at the target site(s) has been proposed (Chang, 1983; Wilkinson, 1985; Hatzios, 1989a).

The mechanisms of action of thiocarbamates and chloroacetanilides have not been fully elucidated. The symptoms of their effects on plants indicated that more than one site of action was involved; stunting and dark-

green leathery leaves (Harvey et al., 1975), inhibition of shoot growth as a result of reduced cell division and cell elongation inhibition (Deal and Hess, 1980), failure of the young leaves to unroll (Donald, 1981), wrinkled and deformed leaves with the leaf tip trapped in the coleoptile (Wilkinson, 1983), shoot twisting (Barta et al., 1983), and severley distorted, brittle, and hard leaves (Sagaral, 1978).

Safeners compete with the herbicide at the target site(s) either through competitive antagonism when the safener acts reversibly at that site, or physiologically when the safener counteracts herbicide action by acting at a different site producing an effect opposite to the action of the herbicide (Hatzios, 1989a). Several sites of action have been proposed.

2.3.6.3.1 Lipid biosynthesis :

Lipid biosynthesis has been proposed as a potential site of action for thiocarbamates and chloroacetanilides (Wilkinson and Smith, 1975; Fedtke, 1982; Wilkinson and Oswald, 1987).The involvement of lipid biosynthesis in the mechanisms of action of safeners has been demonstrated. The safener Dichlormid reversed the inhibition of lipid synthesis caused by the herbicide EPTC in maize (Sagaral, 1978; Ezra et al., 1983), the safeners NA and Dichlormid reversed the effect of thiocarbamates on fatty acid synthesis in maize (Wilkinson and Smith, 1978), oxime ether safeners and

the herbicide Metolachlor had opposite effects on lipid metabolism and its distribution in sorghum (Yenne, 1989; Yenne and Hatzios, 1989), and recently the safener Fenclorim was shown to enhance acetate incorporation into total lipid under conditions where it was inhibited by the herbicide Pretilachlor (Han and Hatzios, 1991).

However the above hyphothesis has been disputed on the basis that no effect of either the safener Flurazole or the herbicide Alachlor could be found on lipid synthesis in sorghum seedlings (Warmund et al., 1985), and recently no significant effect of the safener Dichlormid on the inhibition of lipid synthesis by the herbicide Sethoxydim was detected (Hatzios, 1991).

2.3.6.3.2 Epicuticular wax :

Degradation of the epicuticular wax layer increases plant susceptibility to environmental stresses (e.g air pollution, microbial attack, drought, etc) and increases the rate of transpiration which causes an enhancement in the uptake of soil applied herbicides (Barta and Dutka, 1989).

Herbicides and safeners interfere with cuticle formation in opposite directions; e.g. the safener Dichlormid and thiocarbamates in maize (Leavitt, 1978; Leavitt and Penner, 1979; Gorog et al., 1982; Barta et al., 1983; Barta and Dutka, 1989), and the safener Cyometrinil and the herbicide Metolachlor in sorghum plants (Ebert, 1982; Ebert and Ramsteiner, 1984).

2.3.6.3.3 Protein synthesis :

Protein synthesis as a process inhibited by thiocarbamates and chloroacetanilides and the effect of safeners on that inhibition have been demonstrated (Jaworski, 1969; Duke et al., 1975; Deal et al., 1980; Ndahi, 1988), Sagaral.(1978) reported that the safener Dichlormid reversed the inhibition of protein synthesis caused by the herbicide EPTC in maize, while recently Han and Hatzios (1991a) observed the reversal of inhibition of valine incorporation into protein caused by the herbicide Pretilachlor in rice.

2.3.6.3.4 Others :

Inhibition of Gibberellin biosynthesis by thiocarbamates and chloroacetanilides and its reversal by safeners have been demonstrated (Wilkinson, 1981a, 1981b, 1982, 1983, 1985, 1989). An increase in peroxidase activity and lignin deposition associated with inhibition of maize growth by the herbicide EPTC and the reversal of these effects by the safener Dichlormid have been reported (Harvey et al., 1975). Alteration of membrane permeability by the herbicides EPTC and Metolachlor and its prevention by the safeners Dichlormid and NA in sugarbeet and onion roots was found by Mellis et al., (1982). The increase in ethylene

production caused by the herbicide Metolachlor was eliminated by oxime ether safeners (Paradies et al., 1981; Ndahi, 1988). The inhibition of photosynthesis as a result of CO_2 fixation inhibition caused by the herbicide EPTC was reversed by the safener Dichlormid (Sagaral, 1978), while inhibition of aryl acylamidase activity by EPTC was partially reversed by oxime ether safeners (Hoagland, 1989b), and the decrease in the total nonstructural carbohydrate content (TNC) in maize shoot and root caused by EPTC was also reversed by Dichlormid (Sagaral, 1978). On other hand Rubin and Casida.(1985), Poige et al., 1987), and Poige(1989) suggested the enzyme acetohydroxy acid synthatase as a target site for both safeners and herbicides.

2.4 Pro-safeners :

The introduction of herbicides that exert their activity after undergoing transformation process(s) within the plant (Fedtke, 1982) was the basis for developing chemicals that exert safening activity after a transformation step(s) (Rubin and Casida, 1985; Rubin and Kirino, 1989), Alternatively chemicals may be transformed into substances that are involved directly in herbicide detoxification, as in the case of OTC and TC which undergo transformation into cysteine which is incorporated into glutathione (GSH), and GSH in turn conjugates with herbicides (Hilton and Phillai, 1986; Hilton et al., 1990, Poige, 1989).

2.5 Microbial safeners :

Genes coding for enzymes that degrade herbicides may be incorporated into appropriate plasmids and transferred into bacteria colonizing roots or seeds of the susceptible plant crops (Karns, 1989), or microorganisms isolated from tolerant plants and introduced into susceptible ones, (Nagy et al., 1991; 1992) were able to provide protection to plants against phytotoxic effects of thiocarbmate herbicides.

2.6 Concluding remarks :

As appears from this review, no exclusive mechanism explaining safener action has been established. The suggestion that they act through a single mechanism seems unrealistic, while the involvement of a series of steps ultimately leading to the safening action is more likley.

Safeners act as bioregulators interfering with herbicide uptake and/or translocation, or enhance their detoxification, or possibly act as competitors at the target site(s) of the herbicides.

Research on safener development for various crops and against newly introduced effective, low residual herbicides (e.g Sulfonylureas and Imadazoloiones) is proceeding, while the introduction of selective chemicals as safeners to the crops, but as synergists

to the weeds was recently reported (Yaccoby et al., 1991).

Although the interference of safeners with the Glutathione (GSH) and related systems is the most acceptable hypothesis, the demonstration of chemicals that exert their safening activity without alteration of this system, or otherwise enhance Glutathione content without exerting safening activity leaves some doubt about that.

The study of safener action at the molecular level using computer aided molecular modelling (CAMM), or the isolation of genes encoding for enzymes involved in herbicide action are also promising routes to better elucidation of safeners' mechanisms of action.

Chapter three

Effect of the safener Dichlormid on the herbicide EPTC: (Volatility from nutrient solution and uptake by maize roots).

3.1 Introduction :

EPTC(S-Ethyl-N,N,-Dipropylthiocarbamate) is a preemergence, preplant soil applied thiocarbamate herbicide. It was introduced in 1954 for the control of several mono and dicot ylednous weeds in cereal crops. It is a colourless liquid with an aromatic odour, miscible with most organic solvents. It has a solubility in water of 365mg/l at 20°C and its vapour pressure is 0.1mmHg at 20°C.

EPTC is usually incorporated into soil to a depth of 15cm, at a rate of 3-6kg/ha in the form of an emulsifiable concentrate or a granular formulation. Due to its marginal selectivity, it is usually applied with the safener Dichlormid as a tank mixture in a ratio of 12:1 (w:w).

The mechanism of action of the safener Dichlormid against EPTC injury to cereal crops has not been fully elucidated. One of the proposed hyphothesis is that it interferes with the pattern of uptake and/or translocation of EPTC in the plant. Several reports

dealing with this hyphothesis have been published yielding controversial results.

In light of these results, coupled with a desire to understand the mechanism of action of Dichlormid, it was thought that further study was required in an attempt to clarify this issue . An appropriate method has been designed to study EPTC uptake by maize roots from nutrient solution, and the possible interference of the safener Dichlormid. Also particular interest will be given to EPTC volatilization from nutrient solution and the effect of Dichlormid on this.

The relevance of the findings to the possible uptake of EPTC by maize shoots and the mechanism of action of Dichlormid is explored.

3.2 Herbicide absorption (Fundamental) :

The physico-chemical properties of a herbicide as well as environmental factors play a significant role in its uptake by plants.

The upper part of the plant is hydrophobic by virtue of the waxy cuticle of the leaves, while the lower part is essentially hydrophilic since the major function of the roots is to take in water and various water soluble substances.

Understanding the mode of action of a herbicide is essential in determining its site of action and method

of application and hence the pattern of its absorption and translocation to the site of action.

Soil applied herbicides are taken in from the area surrounding the root and move up into the plant by the action of the transpiration process. On the other hand foliar applied herbicides are absorbed through and either remain there (contact) or transfere within the plant with the photosynthetic materials.

3.2.1 Absorption through foliage :

Interception, retention, and penetration of the herbicide through the cuticles are factors which determine the efficacy of the applied herbicide (Casseley and Walker , 1990). To crossthe cuticular barrier, the herbicide should be sorbed into the cuticle, move across the cuticular membrane, desorb into the apoplast and be taken in by the underlying cells (Fletcher and Kirkwood, 1982).

The concentration of herbicide, its chemical structure, its polarity, the cuticle thickness, the surface area of the application, the additives included in the formulation, the hairiness of the leaf surface, the surface tension of the carrier, and the possible effect of the previously herbicides applied to soil that alter cuticle formation (e.g.Thiocarbamates) are factors taken into consideration when considering foliar applied herbicides (Fletcher and Kirkwood, 1982; Barta and Dutka, 1989; Casselly and Walker, 1990).

Foliar applied herbicide come in contact with plant leaves usually in high concentration, but for a short time.

According to Cas eley and Walker.(1990), less than 1% of the applied dose reaches the site of action. The chemicals exert their biological activities either by contact (e.g.Bromoxynil, Dinoseb), or after translocation to the site of action e.g.Phenoxyalkanoic acids, Quaternary ammonium herbicides (Hassall, 1986).

3.2.2 Uptake via roots :

For soil applied herbicides to be effective, they should accumlate in weed seedlings in a toxic concentration from treated soil at an economic rate without risk of damage to the crop. The ability of the soil to supply the herbicide to the plant and the availability of the herbicide to the plant determine the amount absorbed and hence the biological activity of the herbicide.

Availability of the herbicide to the plant depends upon its behaviour in the soil- its adsorption onto soil colloids, its volatility, its leaching, and its breakdown (Kearney and Kuffman , 1976; Fletcher and Kirkwood,1982).

For herbicides to be taken in by plants, they should come in contact with the root. Herbicide chemical structure, its concentration, and soil characterstics

determine its movement in the soil . Herbicides move to the root surface via mass flow when carried passively in water which moves towards the roots in response to transpiration proceses, or by diffusion (movement along concentration gradients).

Herbicide movement to root surfaces increases as the rate of uptake of water increases, but once the herbicide is at the root surface its entry into the root is occasionally independent of water entry (Cas eley and Walker, 1990). Absorption of water and other dissolved substances e.g. Herbicides occur primarly at the root hair zone that is covered by a thin layer of lipid-like substances that are usually not a significant barrier to penetration because water ,minerals and herbicides readily penetrate through the epidermal layer, hence herbicides move into roots by simple diffusion (Hess, 1985).

Various factors that affect herbicide behaviour in the soil affect their uptake by plants and hence their biological activity. These factors are weather and soil conditions, the activity of soil microorganisms, soil moisture, soil organic content, and soil texture (Schmidt and Pestemer, 1980; Hess, 1985; Devin and Vanden Born, 1991).

3.2.3 Absorption from vapour phase :

The importance of seeds and the underground parts of the shoot as an entry routes for soil applied herbicides particularly the volatile ones has been demonstrated.

Diffusion is the main process in which volatile herbicides are moved to the vapour phase and transfer to the below ground region of the shoots and the newly germinated seedlings. During germination prior to emergence from soil, shoot tissue is exposed to the vapour of volatile herbicides which are absorbed through the cuticular layer of the underground shoot which is less well developed than the above ground parts. Thiocarbamates are relatively volatile herbicides and their entry into the underground shoot is essential for their full effectiveness (Hess, 1985; Casselly and Walker, 1990; Devine and Vanden Born, 1991).

3.3 Absorption and selectivity ;

The magnitude of uptake of a herbicide by the plant is one of the factors that determine its tolerance or susceptiblity. Variations in the amount absorbed by crop seeds that usually grow or germinate deeper than the weed seeds, and differences in the root depth of absorbing tissue protect the crop from herbicide injury (Hassal, 1986; Devin and vanden Born, 1991).

For foliar applied herbicides, the amount of herbicide that adheres to the surface of the plant depends upon the spray solution, surface tension, leaf area, leaf orientation with respect to the angle of incidence of the spray drops, spray volume, leaf waxiness and cuticle thickness, all of which determine the amount of herbicide absorbed and hence plant tolerance to the herbicide.

Variations in herbicide absorption among plant geno-types has been shown to be a cause of the difference in the tolerance to herbicides (Wright and Pieck, 1974; Chandler et al., 1974; Sargal, 1978; Dutka et al., 1978), while temperature, soil moisture, wind speed, and stage of growth of the plant are factors affecting herbicide uptake and selectivity (Fletcher and Kirkwood, 1982).

3.4 Herbicides translocation :

For the herbicide to exert biological activity, it should be translocated in sufficient amount to the site of action. Herbicides move in the plant either through non-living, cell wall materials(apoplast or xylem) , or through living cytoplasmic continuous cells(symplast or phloem) or through both. Their translocation could either be for a short or long distance.

3.4.1 Transport through xylem :

Xylem is essentially a non-living system and consists of continuous network of cells extending from the zone of differntiation(root hair zone) to all mature and developing leaves. It is an apoplastic system which acts mainly to translocate water and dissolved substances from roots into leaves by the action of the transpiration stream.

Most soil-applied herbicides are xylem mobile. Factors that affect water and dissolved substances also affect herbicide translocation. This movement could occur directly from roots to leaves or could be by redistributed to the growing points after they have been taken in by the roots (Fletcher and Kirkwood, 1982; Hess, 1985; Hassall, 1986; Cas elley and Walker, 1990).

3.4.2 Movement through phloem :

The phloem system consists of living cells(symplast). It provides a long route for the translocation of photosynthetic products from the source(leaves) to the sinks (growing points).

Foliar applied herbicides mainly translocate through this system, where their rate of translocation positively correlates with the rate of utilization and storage of photosynthetic products at various sink areas. Most herbicides that cross the cuticle membrane are translocated through the phloem (Cas eley and Walker, 1990).

Herbicides that move through the phloem enter the xylem at some point. The flow in the two systems are in opposite directions,but water flow in the xylem is more rapid so when the herbicide is able to be in the two systems. The net transport would favour the xylem, pH of the two systems is a significant factor affect herbicide ability of translocation. the PH of the phloem is 8, while the PH of the xylem is 5. It would be have in mind that most herbicides that are translocated in the phloem are weak acids (Bromilow et al., 1990).

Factors affecting herbicide movement in phloem are drought, temperature, light, rate of cuticular penetration, method of application, plant species and herbicide metabolism and phytotoxicity (Caseley and Walker, 1990; Bromilow et al., 1990).

3.4.3 Herbicide translocation (short and long distance):

Following their entry into the plant, herbicides move to their site of action. This movement may be either long distance which is neccessary to control weeds beyond the seedling stage of growth or perennial weeds that require the chemical to move within cells of organs and tissues to reach their site of action, or for short distance movement in which other neighbouring cells at the immidiate surface are to be killed. This type of movement occurs also after long distance transport. An example of short distance movement is pre

emergence growth inhibitors and post emergence photosynthetic inhibitor herbicides (Fletcher and Kirkwood, 1982; Cas eley and Walker, 1990).

3.4.4 Translocation and selectivity :

Variation in the herbicides ability to reach its site of action within the plant is a factor affecting its selectivity.

Differences in the translocation pathway of the herbicide Dicamba through the phloem in the susceptible plant and through the xylem in the tolerant plant (e.g wheat) was responsible for its selectivity (Devine and Vanden Born, 1991), while in the case of the herbicide Diclofop-methyl, the nature of its formulation was the significant factor. When applied in the ester form it entered the leaves and loaded into the phloem part in the tolerant plants, while it was transformed to the acid form in the susceptible plants (Cas eley and Walker, 1990).

3.5 EPTC absorption and Translocation (Review) :

EPTC is a soil applied herbicide. Its site of uptake has not been fully elucidated. Studies dealing with this aspect gave different results. Seeds as a main site of uptake (Chang, 1969), roots (Gray and Weierich, 1969; Yamaguchi, 1961), or shoots (Gray and

Joo, 1978). Knaak et al.(1989) demonstrated the ability of the chemical to penetrate human and rat skin.

Dutka et al.(1978) have domenstrated the high mobility of EPTC in plants compared with other thiocarbamates. It is translocated to the stems and leaves after its absorption by roots (Chang and Theisen, 1959, 1960; Yamaguchi, 1961).Its translocation and accumlation increases with time (Nalewaja, 1964).

The relation between EPTC absorption and/or translocation and its selectivity has been examined; Sorghum plants have been shown to be the most susceptible plants to EPTC due to its double uptake compared with oat,wheat, and barley (Prendevill et al., 1969), while according to Oliver et al.(1969), barley was the most tolerant plant to EPTC because its site of entry is through the roots compared with oat, wheat, and sorghum that absorbed EPTC through the shoots.However, Other studies failed to detect a significant relationship between EPTC absorption and /or translocation and plant susceptiblity (Marton et al., 1978; Ezra and Stephenson, 1985).

3.6 Effect of additives on herbicide absorption and/or translocation :

Substances applied with herbicides may alter their

pattern of absorption and /or translocation hence will have an effect on their biological activity (Hatzios and Penner, 1985).

The addition of adjuvants and surfactants to the formulation increased the solubility of foliar applied herbicides and enhanced their ability to penetrate the cuticle (Babiker and Duncan,1974, 1975a, 1975b).

For soil applied herbicides, the addition of salts($CaCl_2$, $MgCl_2$,NaCl) increased the uptake of thiocarbamates by tomato seedlings hence they synergized their action (Acosta-Nunez and Ashton, 1981b).

Among those substances that are included in herbicide formulations, particularly soil applied herbicides are safeners. The possiblity that safeners exert their safening action via interference with herbicide absorption and /or translocation has been examined, and controversial results have been reported.

3.6.1 The interfernce of safeners with herbicide absorption and/or translocation :

The site of application of both the safener and the herbicide, their method of application either as pre-plant or pre-emergence treatments, the application rate and planttype are all factors which affect safener interferance with herbicide absorption and/or

translocation behaviour (Hatzios 1983a; Fuerst el al., 1991; Jablokai et al., 1991).

Safeners present in the herbicide formulation may cause an increase, a decrease or have no significant effect on herbicide absorption and/or translocation.

3.6.1.1 Stimulation of herbicide absorption and/or translocation :

The oxime ether safener(CGA-92194) has been shown to double the uptake of Metolachlor in sorghum protoplast (Zama and Hatzios, 1986). The safeners Flurazole, Cyometrinil,and NA stimulated the uptake of Metolachlor in the sorghum plant (Furest and Gronwald, 1986). The safener NA caused an increase in the uptake of the following herbicides.Met lsulfuron methyl by maize root tissues (Milhomme and Bastide, 1990), Diclofop-methyl in oat (Codde, 1988), and EPTC in maize (Guneiyl, 1971).

A 36% increase in the absorption of Metolachlor by sorghum seeds was caused by the safener CGA-43089 (Leek, 1981), while the safener Dichlormid slightly stimulated the uptake of Acetochlor from nutrient solution by maize roots, but it retarded its translocation from roots to shoots (Jablonkai and Dutka, 1985). Recently Yenne and Hatzios.(1990) reported that the safener Oxabetrinil

increased the uptake of Metolachlor without having any effect on its translocation in sorghum seedlings.

3.6.1.2 Reduction of herbicide absorption and/or translocation :

It has been shown that oxime ether safeners had competitive effect on Metolachlor absorption by sorghumcoleoptiles (Ketchersid et al., 1982; ketchersid, 1990). The safener NA caused a reduction in the herbicide Metolachlor absorption and translocation by maize and soybean (Arhens and Davis, 1978), and in the absorption of the herbicide Chlorosulfron in maize plants (O'Leary and Prendeville, 1982). Plant growth regulators that are applied as safeners with the herbicide Metribuzin in soybean caused a decrease in the absorption of the herbicide and they interfered with its translocation (Varvina, 1987; Phatak and Varvina, 1989).

Recently, reports have been published showing that the safener Fenclorim significantly reduced the root uptake of the herbicide Pretilachlor in rice (Han and Hatzios, 1991). The safener Naptalam reduced the translocation of the herbicide Chloramben by cucumber roots and shoots (Kneer et al., 1991) and decreased the absorption of the herbicide Paraquat by peanut plants (Nehtje et al., 1991). The safener BAS-145-138 slightly reduced the herbicide Metazachlor uptake and translocation in maize (Fuerst et al., 1991a).

3.6.1.3 No interfernce with herbicides absorption and/or translocation :

Various studies failed to detect a significant effect of safeners on herbicide absorption and /or translocation. The safeners CGA-154281, Oxabetrinil, and Fenclorim had no significant effect on the absorption of Metolachlor in both tolerant and susceptible maize shoots (Kreuz et al., 1989), while Winkle et al.(1980) did not detect a significant effect of the safener CGA-43089 on the absorption of Alachlor from petri dish tests or the uptake of Metolachlor from soil by sorghum plants.

The pro-safener N-phenylmalimide had no significant effect on Alachlor uptake and /or translocation by sorghum(Rubin and Casida, 1985). Negligable effect of the safeners NA, Flurazole, and CGA-92194 on the uptake and translocation of the herbicide Imazaquin by maize roots have been detected (Jackson, 1984; Barrett, 1989a). Others studies have failed to detect significant effects on the absorption and/or translocation of herbicides in the following combinations: the safener Flurazole on Chloroacetamides absorption and/ or translocation in sorghum plants (Breaux et al., 1989), the safener MG-191 with Acetochlor by sorghum (Jablokai et al., 1991), the safener CGA-154281 with Metolachlor in sorghum (Rowe, 1989) and in maize (Rowe et al., 1991)

and the safener Flurazole with Metolachlor in sorghum (Jackson, 1986), the safener NA on the herbicide Imazethapyr in maize (Barrett, 1989b), and in combinations of safeners and chloroacetanilide herbicides in maize and sorghum (Murphy et al., 1972; Holm and Szabo, 1974; Marton et al., 1978; Christ, 1981, 1985).

6.2 Effect of Dichlormid on EPTC absorption and/or translocation :

Plant type, the medium of the study and the method of study ultimatly determine the net interferance of Dichlormid with EPTC uptake.

More than one site is involved in the uptake of EPTC and Dichlormid in maize cell cultures, in which unequal competition at different sites has been detected (Gressel et al., 1982). This competition has been proposed as a first step in a series of interactions ultimately leading to Dichlormid safening activity (Ezra et al., 1982).

However, a negligible or insignificant effect of Dichlormid on the uptake and distribution of EPTC in maize seedlings has been reported (Chang et al., 1974), while a stimulation in the uptake of EPTC and a delay in its movement in the tolerant cultivar of maize shoots has been demonstrated (Sagaral, 1978).

3.7 Herbicide volatilization (Basics) :

Herbicides dissipate through various ways; degradation, adsorption onto soil colloid s, leaching, absorption by plants, and through volatilization.

Volatilization occurs from soil, from plant surfaces and from water bodies through and after application. Knowledge of potential volatilization is useful for both estimating persistance at the target site and for assessing possible contamination of adjacent non-target sites, while balance between herbicide persistance as related to biological activity and environmental impact has to be considered.

Thiocarbamates are relatively quite volatile herbicides hence they are mainly soil incorporated (Wilkinson, 1988).

The presence of other substances in the thiocarbamate formulation may alter the pattern of volatilizing.

Safeners are examples of these substances that are added to increase selectivity. Reports dealing with their effects on thiocarbamate volatility are negligible.

3.7.1 EPTC volatilization (Review) :

EPTC is a volatile herbicide. Its vapour pressure is 0.1mm Hg at 20°C. Volatilization is the most dominant route of its dissipation from the soil surface so it is usually soil incorporated.

Soil characterstics have an obvious effect on its volatility e.g as the soil organic matter increases, its adsorption into soil increases and its volatility decreases (Ashton and Sheets, 1959). Its rate of volatilization from moist soil was higher than from dry soil (Gunther et al., 1984).

Casida et al.(1975) studied the rate of volatility of EPTC and its sulfoxidation products from glass and bean surfaces and arranged them in the following order; EPTC>EPTC-Sulfone> EPTC-Sulfoxide. Recently Knaak et al. (1989) have shown that EPTC is rapidly evaporated from the skin of workers who applied the herbicide. Around 77.80% has evaporated from the skin of the adult male rat.

The effect of the safener Dichlormid on EPTC volatility and the relevance of this to its mechanism of action has not been fully elucidated, although the presence of other non-volatile solvents and solid materials in EPTC formultion has been shown to affect its volatilization (Gunther et al., 1984). In this chapter, the effect of Dichlormid on EPTC volatilization

from nutrient solution and the relevance of this to its uptake through plant shoots will be discussed.

3.8 Methods of study (Review) :

Radiolabelled herbicides were used and their uptake and translocation was monitored in the plant (Hilton et al., 1974; Bourke and Fang, 1965).

For root absorbed herbicides, the study is carried out using three media. (1) water culture which has advantage that the herbicide is fully available to the plant roots as far as water solubility allows and avoids the possiblity of interaction between the soil and the herbicide, this method is also suitable to study the effect of other substances on herbicide absorbtion.(2)hydroponic culture(non-adsorption media) such as vermicuilite, perlite, cellulose, or agar-agar is another method, and(3) using soil as a medium of study. This method has limitations due to the effect of the physical and chemical properties of the soil, as well as others chemicals applied to the soil(e.g fertilizers, extenders, etc) (Schmidt and Pesteme, 1980).

For foliar applied herbicides, extracting the herbicide taken up by leaves or shoots and determining the amount using an appropriate method, from which the quantity that absorbed may be determined. Rinsing hands by water or organic solvent is the method usually used

to extract the herbicide from human skin, and hence determine the amount absorbed (Knaak et al., 1989).

3.9 Expermintal :

3.9.1 Chemicals :

EPTC (S-ethyl-N,N-dipropylthiocarbamate) was purchased from Greyhound Chromatography and Allied Chemicals.

Dichlormid(N,N-diallyl-2,2-dichloroacetamide) was prepared in the laboratory following the published method of Hatzios (1983a).

Solvents used are HPLC grade /Rathburn/Scotland, while the salts used in preparation of Hoagland solution were obtained from Aldrich.Chem.Co.

3.9.2 Nutrient solution :

Hoagland nutrient solution was prepared according to Epstein (1972). The solution was diluted to half strength prior to use.

3.9.3 Germination of the plants :

Maize seeds (sweet maize) obtained from FI John Innes Hybrid were germinated in petri dishes for two days prior to use, three uniform seedlings with approximate equal roots and shoots were selected for the study.

3.9.4 Method of study :

The method was set up as following; For the study of the absorption, the containers were firmly sealed to minimize volatilization losses.

EPTC and Dichlormid stock solutions were prepared using Hoagland nutrient solution in a concentration of 5mg/ml each.50 ml of EPTC and 5ml of Dichlormid stock solutions were mixed in a graduated cylinder, and the total solution was diluted to half using distilled water. The final concentrations of EPTC and Dichlormid are 2.5 and 0.25mg/ml respectively.

The samples were arranged as EPTC , EPTC+Dichlormid, and blank.

Three germinated seedlings with uniform shoots and roots were transferred to the prepared solution in such a manner that their roots were immersed into the solution in a small beaker, while their shoots grew up inside the empty plastic pot. The whole beaker was sealed using aluminum foil.

For the volatility study, the same technique was used but without plants. The study for both the absorbtion and the volatility was carried out for one to five days, with three replicates at each stage.

3.9.5 Analytical method development :

The content of EPTC remaining in solution was taken as a basis to determine the amount of EPTC that was lost either via uptake by plants or through volatilization.

3.9.5.1 Extraction :

The remaining treated solution was transferred to 500ml separatory funnel, 1g of sodium chloride was added, and the EPTC was extracted three times with 200ml of Dicloromethane. The organic layer was dried over anhydrous sodium sulphate, filtered, and the Dichloromethane removed under vacum at<40^{o}C.

The remaining residue was adjusted to 2ml using Dichloromethane, and EPTC recovery determined using GC. The extraction method was repeated using Hexane as an extractant.

3.9.5.2 Direct injection into HPLC :

The remaining solution was transfered with rinsing into a volumetric flask, and the volume was adjusted to 200ml using distilled water, a portion(10ml) of the solution was filtered using a membrane filter, and 10ul of the filtrate was injectedinto the HPLC. The recovery was determined from the EPTC standard curve, and the results expressed as a percentage of the initial applied dose.

3.9.5.3 Instrumentation :

The analysis was carried out using either GC or HPLC.

The GC used was Pye Unicam, Pu 4500 chromatograph, fitted with flame ionization detector(FID), and 2mm-4mm i.d.glass column packed with a semipolar silicon oil/3% OV17+1.95% OV202, supported on 100/200 mesh WHP. The FID signals were recorded on a chromatopac integrator, Shimadazo, C-RIB.

The HPLC consists of two M6000A dual piston reciprocating pumps with the flow rate controlled by a 660 solvent programmer. The samples were introduced into the column via a U6K external loop injection valve that was fitted with a 2ml capacity loop of capillary tubing. A stainless steel column of 25Cm-0 .5cm i.d packed with ODS(reversed phase column) was used. The eluent was monitored by a model 450 variable wavelength spectrophotometer fixed at 210nm. The output of the spectrophotometer was recorded on a chromatopac integrator, Shimadazo, C-RIB.

3.9.6 Data collection and analysis :

Plants were removed from the treated solution at different time intervals(0,1,2,3,4,5, day tests). The plants roots were rinsed with distilled water, dried, and the weight recorded. Each reading represented an average of three plants.

The remaining EPTC was determined by direct injection into HPLC. Each result represents an average of three replicates.

The effect of Dichlormid on the rates of the volatilization and the absorbtion has been evaluated. Analysis of variance was used to detect whether the effect of Dichlormid on EPTC absorption and/or volatility was significant.

3.10 Results :

3.10.1 EPTC recovery and detection limits :

Extraction and determination of EPTC residue using GC ,or via direct injection into HPLC were the two methods used.

3.10.1.1 GC :

Using Dichloromethane as an extractant, EPTC recovery was shown to be 20$\bar{+}$ 5% as determined by GC. Using nitrogen steam to remove the solvent instead of vacuum did not significantlly improves this recovery,

using Hexane as an extractant yielded a recovery of 30$\mp$ 3% as obtained using GC..

The EPTC detection limit as determined by GC was shown to be 0.2ppm(0.2mg/L) when Dichloromethane was used as solvent.

3.10.1.2 Direct injection into HPLC :

The recovery of EPTC was shown to be 72$\mp$ 3%, while the EPTC detection limit was 0.18mg/L.

3.10.2 EPTC volatility and Dichlormid effect :

As shown in Table(3.1) and Fig(3.1) the main root of EPTC dissipation was through volatilization in which 93% of the applied dose was evaporated within five days and as shown from analysis of variance, Dichlormid had no significant effect on EPTC volatilization from the nutrient solution.

3.10.3 EPTC uptake by maize roots and Dichlormid interference :

The amount of EPTC absorbed by maize roots was very low compared with what was lost through volatilization, only 7% of the applied dose was uptaken by maize roots within five days. The greatest amount was absorbed in

the first day followed by gradual decrease with complete dissipation by the fifth day as shown in Table(3.2) and Fig(3.2).

The presence of Dichlormid was shown not to have a significant effect on the absorption of EPTC by maize roots .

Table 3.1 Volatilization of EPTC from nutrient solution.

Time (day)	Concentration(mg/ml)	
	EPTC	EPTC+Dichlormid
1	1.878	1.974
2	2.026	2.139
3	2.258	2.215
4	2.297	2.273
5	2.320	2.316

Table 3.2 Absorption of EPTC by maize roots from nutrient solution .

Time (day)	Concentration(mg/ml)	
	EPTC	EPTC-Dichlormid
1	0.132	0.110
2	0.157	0.141
3	0.186	0.172
4	0.190	0.181
5	0.193	0.196

Fig 3.1 Rate of the volatilization of EPTC from nutrient solution

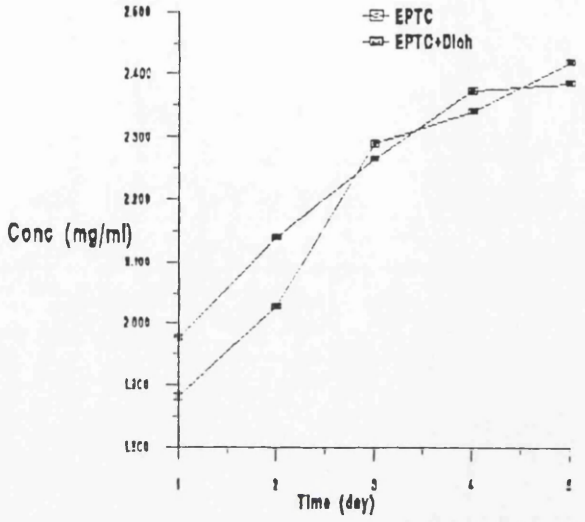
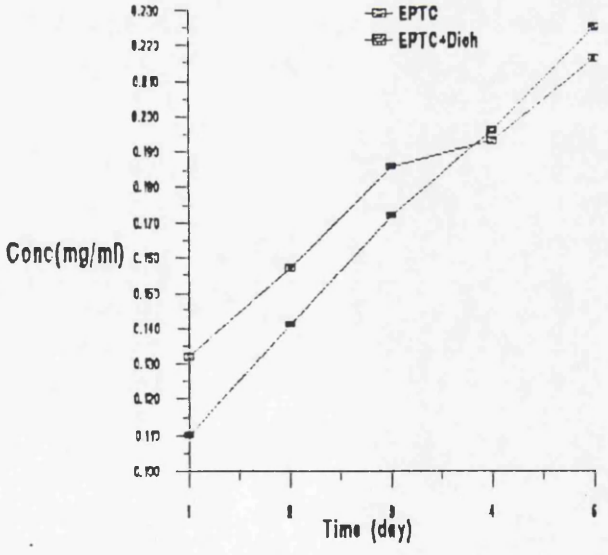


Fig 3.2 Rate of the absorption of EPTC by maize roots.



3.11 Discussion :

3.11.1 Extraction :

Using two organic solvents(Dichloromethane and Hexane) as extractants, low recoveries were obtained(20-30%).

The low levels of recovery may be due to the high volatility of EPTC . It is the most volatile member of the thiocarbamates fami ly, with a vapour pressure of 0.1mm Hg at 25^{o}C. Its lossthrough the pre-analysis steps , viz extraction, handling, and through removing the solvent was undoubtly the main cause of the low recovery.

Using a stream of nitrogen to remove the solvent did not improve the recovery to any significant extent. This might give an indication that the efficiency of the extracting solvent was low, bearing in mind that EPTC is completly soluble in water under these conditions.

An alternative method that eliminated or minimized EPTC loss through the pre-analysis steps was considered essential. Direct injection into HPLC gave a recovery of EPTC of 72$\mp$ 3% .

EPTC detection limits using HPLC was 0.18mg/L, while using GC, EPTC detection limit was 0.20mg/L. It has been reported that EPTC detection limits in

Prosophia and Beer-head extracts using a TLC method were 1000ng (Ernst and Plieterse, 1977).

Although a low recovery was obtained using the method of direct injection into HPLC, this method proved reasonable taking into consideration the nature of the study in which a relative comparison between the absorbtion and the volatilization of EPTC in the absence and in the presence of the safener Dichlormid was the ultimate objective.

3.11.2 The effect of Dichlormid on EPTC volatility :

From the results reported herein, it seems that the main route of EPTC dissipation from the nutrient solution was through volatilization. This may be explained due to the high vapour pressure of EPTC(0.1mm Hg at 25^{o}C).

It has been shown that EPTC was very volatile in various media examined: from liquid, stainless steel, leaf, and soil surfaces. Its volatility from liquid surface was 57 ug/sqm/hr at 30^{o}C (Ashton and Sheets, 1959). The above authors reported that EPTC was evaporated from liquid, stainless steel, or leaf surfaces faster than from soil surfaces.

In other studies Claith et al.(1980), and Spencer and Claith. (1990) reported that 73.60% of the applied EPTC to alfalfa in irrigation water was lost as a result of volatilization within 52 hours.

Knaak et al.(1989) demonstrated that EPTC volatility was the main route of its dissipation from the skin of the adult male rat and workers skin, in which (77.80%) of the applied dose to the rat adult male has evaporated, Casida et al. (1975) showed that EPTC dissappear ed as the solvent evaporated from glass and bean leaf surfaces, and Gunther et al.(1984) indicated that significant amounts of EPTC were lost through volatilization from soil surface.

Due to its high volatility and in order to achieve an adequate biological activity, EPTC is applied by incorporation into the soil (Gyangy et al., 1988; Schreiber et al., 1978).

Others thiocarbamates have been shown to be highly volatile and their volatility decreased as their solubility increased (Draper and Crosby, 1984.

In this study, other features of EPTC were detected. Its volatilization was greatest in the first day with a gradual decrease in the following days. This is in accordance with the behaviourother thiocarbamates which initally volatiles rapidly then gradually decreased (Dutka et al., 1978).

In this study, EPTC behaviour may be explained by the fact that its molecules are saturated with water molecules so many molecules tend to leave in the vapour phase. The rate gradually decrease as the remaining molecules became more surrounded with water molecules and less available to enter the vapour phase. Clendening

et al.(1990) demonstrated that EPTC volatility from soil decreased continuosly over time.

Regarding the effect of Dichlormid on EPTC volatility, it was shown to be not significant, Dichlormid is more soluble in water than EPTC, with a solubility of 5000mg/L compared with 365mg/L for EPTC at 25°C. Its vapour pressure is also much lower than that of EPTC . Hence competition at the site(s) of entry into the vapour phase should be negligible or low.

In this study Dichlormid was used in a ratio of 1 : 10 (v:v) with EPTC which is close to the ratio in the commercial formulation (1 : 12). This indicates the amount actually present is relativley low compared with EPTC. The results do not rule out the possible effect of other non-volatile solvents and solid materials that exist in EPTC formulation on its volatility, such effects have been demonstrated (Gunther et al., 1984).

The insignificant effect of Dichlormid on EPTC volatility is in accordance with its effect on EPTC behaviour in soil, in which it did not alter significantly EPTC residual life in soil (Capper, 1975), and did not affect its enhanced degradation in soil already treated with EPTC or other thiocarbamates and Dichlormid (Wilson and Rodebush, 1987).

This is in accordance with the previous reports, that indicated Dichlormid exerted its effect and then

its safening activity by acting within the plant and not externally (Hatzios, 1989a).

3.11.3 EPTC uptake by maize roots and Dichlormid interference :

From the results obtained, various remarks could be made related to the pattern of uptake of EPTC and the effect of Dichlormid. The amount of EPTC taken in by maize roots has been determined based on the difference between EPTC in the samples containing the plants and in the samples without plants. The amount taken in was initially high then gradually decreased.This is in agreement with the pattern of absorption of thiocarbamates as reportedly (Dutka et al., 1978). Taking into consideration that most of the EPTC lost through volatilization is trapped within the sealed beaker containing the plants that directly come in contact with the plants shoots, the suggestion that significant amount of EPTC enters the plant through the shoots is reasonable, bearing in mind that reports indicate that the maize shoot is the main site of EPTC uptake (Gray and Joo, 1978; Dutka et al., 1980; Hatzios, 1989). Previous studies exposing maize shoot to EPTC evaporated from nutrient solution has been carried out (Gray and Weierich, 1969; Gray and Joo, 1978; Devin and Venden Born, 1991).

Assuming that the shoot is the main site of EPTC uptake and in comparison with EPTC pattern of

volatilization the possiblity that the same trend of absorption as via the roots(initially rapid then gradual y decline) is reasonable.

The amount of EPTC taken in by maize root in five days was (7%)of the initially applied dose. This is relatively low but seems suffecient to exert biological activity, Cas eley and Walker. (1990) reported that <1% of the herbicide dose reaches the site(s) of action. In other studies, it has been shown that the highest amount of EPTC residue found in plant tissue did not exceed 3% of the total amount absorbed (Fang and Theisen, 1959), and the amount absorbed through adult male rat skin was 14.70% when EPTC was applied in water, while for a person working as a mixer/loader/applicer of weight of 75Kg, the amount was 5.6mg/day when EPTC was applied to 120 acres per day (Knaak et al., 1989). The uptake of the thiocarbamate Propylate by plants was time and application rate dependent and continued as long as the herbicide was available (Bourke and Fang, 1965). Absorption of EPTC by alfalfa plants was almost double after five days compared with two days (Nalewaja, 1965).

From this study it appears that the method and site of application of EPTC is vital in controlling the amount absorbed, and hence its biological activity. Also for the absorption from soil, soil factors, and EPTC behaviour in soil (e.g degradation, adsorption, leaching) should be considered.

In connection with Dichlormid interferance with EPTC absorption by maize root, no significant effect was detected, and in comparison with its effect on EPTC volatility, it seems that there is no significant effect of Dichlormid on EPTC uptake through the shoots. The low level of Dichlormid applied with EPTC (1:10) and the rapid uptake of Dichlormid rule out the simultaneous competition with EPTC at the site of entry (Gray and Joo, 1978; Stephenson and Chang, 1978). The relatively non-volatile nature of Dichlormid (Worthing, 1979) ruled out the possibility of competition with EPTC at the point of entry into the maize shoot.

The method of monitoring the absorption and studying the Dichlormid effect is of particular importance to the results.

Using nutrient solution and uptake through roots, EPTC uptake was not rapid and adopting the method according to Ezra et al. (1983) by using maize cell culture to detect the competition between EPTC and Dichlormid resulted in a reduction in EPTC uptake. In relation to the effect of other safeners, competition between the Oxime ether safeners and the herbicide Metolachlor at the site of uptake has been demonstrated (Ketchersid, 1990).

In this respect, solubility of the safener in relation to its availability to the plant is another point. Dichlormid is more soluble in water than EPTC, so its availablity to the plants would be higher, thereby

creating an unequal rate of absorption. Gressel et al.(1982) reported that unequal sites of competition existed between EPTC and Dichlormid on entry into maize cell cultures.

In accordance with the results obtained here no significant effect of Dichlormid on EPTC absorption was noted, and hence the findings were of no relevance to explaining the mechanisim of action of the safener (Chang et al., 1974; Sagral, 1978; Marton et al., 1978). Similar results were reported for other safeners with thiocabamate and chloroacetanilide herbicides (Murphy, 1972; Winkle, 1980; Christ, 1981; Rubin et al., 1985; Chris, 1985; Barrett, 1989a; Jablokai et al., 1991, Lamoureux and Rusness, 1992).

3.12 Conclusion :

It appeared from this study that EPTC is quite a volatile herbicide difficult to deal with and handle. Minimum useful steps of pre-analysis were required to achieve an adequate recovery and accurate analysis.

The main route of EPTC dissipation was through volatilization in which 93% was lost through this pathway. This indicates the importance of the method of application and shoot exposure as related to its biological activity. The effect of Dichlormid was not found to be significant, and no direct relationship to its mechanis m of action could be drawn.

EPTC absorption by maize roots was initially rapid then gradually decreased. The amount absorbed was 7% of the initially applied dose. The possibility of entry through shoots should be taken into consideration.

Dichlormid did not affect significantly the uptake of EPTCby maize roots. This does not neceserly mean that similar results would be obtained using different experimental techniques and analytical methods.

In the results, the possibility of Dichlormid interferance with the absorption of EPTC does not seem to be the main cause of its safening activity, but would be make a minor contribution as a step in a multi step process leading ultimately to enhance safening action. the possibility should not be ruled out at the stage.

In the next chapter, the possibility of Dichlormid interfering with EPTC metabolism in maize tissue will be explored.

Chapter Four

EPTC metabolism in maize roots and shoots and the influence of Dichlormid on these processes

4.1 Introduction :

Transformation of herbicides proceeds through three main routes; biological, chemical and photochemical. These processes can occur in air, water, soil, plant surfaces and within the plant itself.

Following its absorption and/or translocation in the plant, the herbicide will be subject to various alterations.These may occur at the site of entry, site of action, or through the translocation pathway(s). The result of these alterations may be bioactivation, or deactivation. As a result of these alterations, the herbicide is metabolized, which according to Shimabukuro (1985) is the alteration of herbicide structure within plant cells that may or may not be catalyzed enzymatically and will ultimately lead to non-phytotoxic residues.

In higher plants, metabolism of herbicides involves three phases; bioactivation, conjugation with plant constituents particularly the reduced form of glutathione (GSH), and catabolism of the conjugate (Hatzios and Penner, 1982; Shimabukuro, 1985; Lamoureux and Russens, 1989;Dodge, 1990; Hatzios, 1991a).

Selectivity of the herbicide is related to its metabolism. This is due either to the plant's ability to metabolize the active molecule into an inactive form, or to transform the inactive form into its active molecule (Dodge, 1990).

Thiocarbamates are sulfur containing compounds which are liable to sulfoxidation processes. This increases their solubility, their ease of translocation and their reactivity as carbomylating agents. It is believed that sulfoxidation is mediated by mono-function oxidase enzymes(MFO) and considered as a pre-step for their conjugation with plant constituents e.g. Glutathione (GSH) (Hatzios and Penner, 1982; Lamoureux and Russens, 1986; Komives and Dutka, 1989; Hatzios, 1991a).

EPTC is a well known thiocarbamate herbicide. It is transformed in higher plants through sulfoxidation and conjugation with the reduced form of Glutathione (GSH) followed by catabolism of the conjugate to give various products. Others routes are also possible e.g. hydrolysis, alkyl oxidation and dealkylation) (Fang, 1969; Chen and Casida, 1978; Gronwald, 1989).

One of the limitations of EPTC is its marginal selectivity, hence it is applied with the safener Dichlormid. It is believed that Dichlormid interferes with EPTC metabolism either with its sulfoxidation, its conjugation with GSH or with both (Hubbell and Casida, 1977; Hatzios, 1989a).

The present work explores the mechanism of action of Dichlormid by studying its effect on EPTC metabolism in maize tissues through the Glutathione pathway. To carry out this work, three steps were considered. (1) Synthesis of the metabolites of EPTC (Glutathione and Cysteine conjugate) withparticular emphasis on the sulfoxidation step using different forms of EPTC (Sulfide, Sulfoxide, or Sulfone) and the possible role of MFO enzymes as a catalytic system. (2) Setting up an experimental technique and analytical method for the identification and quantitative analysis of the metabolites and (3) studying the effect of Dichlormid on the metabolism of EPTC in maize tissues.

4.2 EPTC metabolism :

Since its introduction in 1954 , several studies have been carried out examining EPTC transformations. On this basis, it seems that EPTCis subjected to chemical, biological, and photochemical alterations in soil, mammals, water and plants (Fang, 1969; Lamoureux and Russens, 1989).

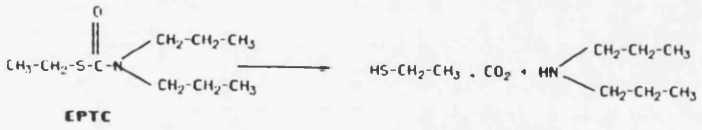
The earliest picture of EPTC transformation patterns differ from the present model (Kaufmman, 1967; Chen and Casida, 1978; Horvath and Pulay, 1980; Gronwald,1989).

4.2.1 Soil :

Microorganisms are the main cause of EPTC breakdown in soil. Its degradation was one-third as rapid in autoclaved as in nonautoclaved soil (Sheets, 1959), while Gunther et al. (1984) demonstrated its persistance for seven weeks in sterile soil compared with two weeks in non-sterile soil.

Hydrolysis with the release of CO_2 has been demonstrated. The released of CO_2 did not account for the total loss of EPTC which indicates other pathways of degradation (MacRae and Alexander, 1966; MacRae and Martin, 1965).

Fang(1969) suggested the following diagram of breakdown of EPTC in soil:



A balance between EPTC environmental impact and its biological activity is vital. Various factors affect EPTC persistance in soil; soil moisture, soil temperature, soil organic content and soil texture (Danielson et al., 1961; Burt and Akinsorotan, 1976; Obrigawitch et al., 1982). Other behaviours and features

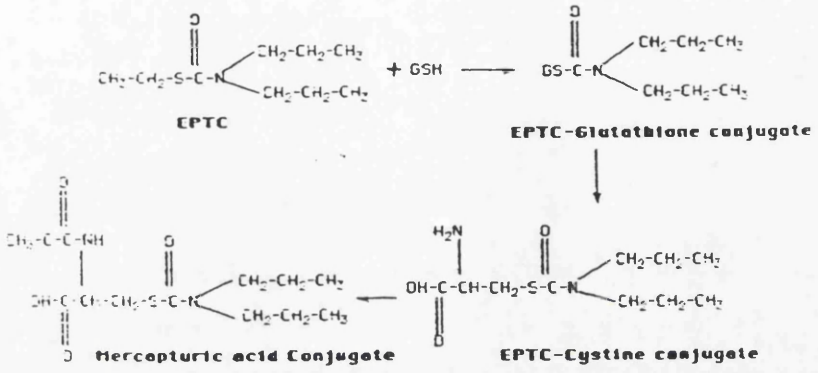
also affect EPTC persistance in soil; leaching as related to the rainfall and irrigation level (Buzio and Burt, 1980), wind velocity that decreased its persistance on the soil surface (Danielson and Gentner, 1964; Ogg, 1989) and its volatility and adsorption into soil colloids (Ekler, 1988).

The enhanced degradation of EPTC in soil already treated with EPTC,or other thiocarbamate analogues is a phenomenon which alters its persistance and its biological activity (Obrigwaitch et al., 1983; Subba-rao et al,1987; Dowler et al., 1987; Tal et al., 1989; Muller et al., 1989; Lawrence et al., 1990). Various methods have been explored to overcome this problem; employing rotations away from successive applications (Rydyanski et al., 1987; Skipper, 1990), applying EPTC as a microcapsulated formulation (Reed et al., 1989), using substances as extenders (Obrigwitch et al., 1982; Rahman and James, 1983; Miaullis et al., 1982; Harvey et al., 1986, 1987; Wilson and Rodebush, 1987; Rudyanski et al, 1987; Bean et al., 1990; Harvey, 1990; Skipper, 1990), or including other agrochemicals that inhibit its microbial degradation (Behki and Khan, 1991).

4.2.2 Mammals :

In mammals, EPTC is metabolized through sulfoxidation, conjugation with Glutathione (GSH), and catabolism of the conjugate to mercupturic acid (Casida

et al., 1974, 1975; Hubble and Casida, 1977; Shimabukro, 1976; Chen and Casida, 1978; Horvath and Pualy, 1980; Dutka et al., 1983; Lamoureux and Russens, 1989). The pathway of metabolism in rats was suggested to be :



Recently, EPTC-cysteine conjugate, EPTC-N-acetylcysteine conjugate and EPTC-N-acetylmethyl cystein conjugate were detected in the urine of a mixing/loading worker exposed to EPTC (Knaak et al., 1989).

Other pathways also have been proposed; hydrolysis with CO_2 evolution (Hubble and Casida, 1977), hydroxylation of the N- and S- moieties (Chen and Casida, 1978) and dealkylation (Casida et al., 1975b; Chen and Casida, 1978).

4.2.3 Plants :

Pathways of metabolism of EPTC in higher plants are almost similar to that in mammals with a difference in the nature of the terminal product(s) (Lamoureux and Russens, 1989). Three phases were suggested :

Phase one involves oxidation of EPTC to its sulfoxide (Casida et al., 1974, 1975b; Lay and Casida, 1975; Hubblle and Casida, 1977), and/or to its sulfone (Horvath and Pauly , 1980). It is believed that EPTC oxidation is catalyzed by (MFO) enzymes (Komives and Dutka, 1989).

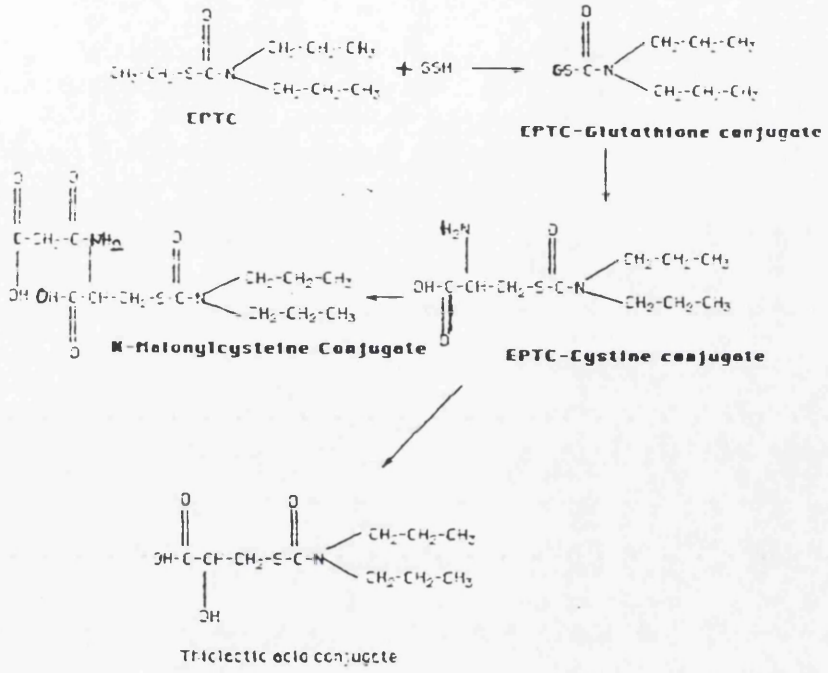
Phase two comprises conjugation of the resulting sulfoxidation product(sulfoxide or sulfone) with glutathione(GSH)and cysteine which proceeds either spontaneously (Carringer et al., 1978; Leavitt and Penner, 1979), or is catalyzed by glutathione-S-transferases (GST) enzymes (Lay and Casida, 1976; Shimabukuro, 1985; Lamoureux and Russens, 1986).

Phase three is the catabolism of the conjugate to terminal residues which varies according to the plant type. In maize, N-malonyl cysteine conjugate (Lamoureux and Russens, 1980; 1989), or Malonyl-3- thiolacetic acid conjugate (Lamoureux and Russens, 1987) have been detected.

Others pathways are possible e.g. hydrolysis with CO_2 evolution (Nalewaja et al., 1964; Chang et al.,

1974) and detection of unidentified products reported (Guneiyl, 1971; Carringer*et al*., 1978).

The proposed pathway of EPTC metabolism is :



4.3 Enzyme v. Non-enzyme :

It is believed that two enzyme systems are involved in EPTC metabolism; Mono-function oxidase(MFO) enzymes, and Glutathione-S-transferase(GST) enzymes. Indirect evidence for the involvement of MFO in EPTC oxidation into its sulfoxide and/or Sulfone form have been detected (Casida *et al*., 1976; Komives and Dutka, 1980; Hatzios, 1983b; Horvath and Pauly, 1980; Komives

and Dutka, 1989), While non-enzymes or spontaneous conjugation of GSH with EPTC-sulfoxide or sulfone has been demonstrated (Carringer et al., 1978a; Leavitt and penner, 1979; Horvath and Pauly, 1980), in the meanwhile Frear and Swanson. (1970) failed to detect enzymes activity in maize and sorghum root tissues.

However, the involvement of GST enzymes in EPTC conjugation with GSH has been clearly demonstrated (Lay and Casida, 1976; Ezra and Stephenson, 1985; Lay and Niland, 1985. Ezra et al.(1985b) gave indirect evidence by using the glutathione inhibitor Tridiphane that it synergized EPTC action.

4.4 Effect of Dichlormid on EPTC metabolism in plants :

The effects of Dichlormid on EPTC metabolism may be either in phase one (sulfoxidation), or phase two(conjugation with the reduced form of Glutathione (GSH). This may occur through various routes .

4.4.1 Mono function oxidase (MFO) :

Due to difficulties in their isolation and in their assessment,indirect methods were used to examine the effect of Dichlormid on MFO enzymes. Using MFO inhibitors e.g. antioxidants, ozone and insecticide synergists is synergized the action of EPTC in the presence of Dichlormid (Komives and Dutka, 1980; Hatzios, 1981; Dutka and Komives, 1983; Wilkinson, 1983;

Hatzios, 1983a; Ekler and Stephenson, 1987; Fedtek and Trebst, 1987; Ezra and Stephenson, 1989).

Recently, Barta and Dutka(1990; 1991) have disputed the previous reports by observing that the MFO inhibitor(1-Amino-Benzotriazole)(ABT) and its derivatives antagonized EPTC action in maize, but synergized EPTC action in wheat, barley, and oats.

In light of the controversial reports, and according to Ezraand Stephenson.(1989) more work would be required to elucidate the involvement of MFO in Dichlormid action.

4.4.2 Glutathione-S-Transferase GST :

Enhancment of GST activity increased EPTC detoxification through conjugation with the reduced form of Glutathione. The extent of the enhancment varied according to the substrate used for the assessment of the enzyme, the plant type, the plant part and the method of study and the analysis adopted.

Significant increase in GST activity as a result of Dichlormid treatment has been detected. This might be due either to an induction in constitutive activity, or an increase in the de novo synthesis of the enzymes (Lay et al., 1975; Lay and Casida, 1976; Mozer et al., 1983; Komives et al., 1985a; 1985b; Komives and Dutka, 1985b;

Ezra and Stephenson, 1985; Wiegand et al., 1986; ; Lamoureux and Russens(1989).

4.4.3 The reduced form of Glutathione (GSH) :

An increase in Glutathione GST content provides enough to conjugate with EPTC, as well as to carry out the normal functions of Glutathione (GSH) in plants.

Different degrees of increase in glutathinoe(GSH) content have been detected (Lay and Casida, 1976; Lay et al., 1975; komives and Dutka, 1980; Carringer et al., 1978; Rennenberg et al., 1982; Ezra and Gressel, 1982; Adams, 1982; Rubin and Casida, 1985; Lay and Niland, 1985; Komives and Dutka, 1985b; Ezra and Stephenson, 1985; Plo ge and Dodge(1987).

The enhancment in glutathione (GSH) content as a result of Dichlormid treatment may be due to increase in activity of Glutathione synthetase enzymes (Carringer et al., 1978; Rennenberg et al., 1982), to an induction in the activity of glutathione reductase enzyme(GR) that catalyzes the reduction of the oxidized form of glutathione(GSSG) to the reduced form (GSH) (Komives et al., 1985b; 1985c), or to an increase in sulphate assimilation and transformation into cysteine which ultimately is incorporated into glutathione(GSH) (Adams, 1982; Adams et al., 1983; Farago and Brunold, 1990).

Ezra and Gressel(1982) disputed the above reports when they noticed an increase in GSH content 12 hour

after Dichlormid treatment, while EPTC was transformed within 8 hours by maize cell suspensions.

4.4 Others effects :

Chang et al.(1974) reported that more CO_2 was released due to EPTC hydrolysis in maize seedlings treated with EPTC and Dichlormid rather than with EPTC alone.

Hydroxylation of S- and N- moeities of EPTC followed by dealkylation as a route to the metabolism of EPTC and the possible catalysis of this by MFO enzymes makes the involvement of Dichlrmid possible.

Dichlormid has other effects not related directly to the metabolism of EPTC. It causes a dramatic decrease in the formation of sulpholipid in greening maize seedlings (Blee, 1986), an induction in Acetohydroxy acid synthetases activity in maize roots and shoots (Rubin and Casida, 1985), and an increase in the enzyme(Adenosine 5-phosphosulphate sulphotransferase) activity which plays a role in sulphate assimilation and reduction (Farago and Brunold, 1990).

4.5 EPTC metabolism (Methods of study) :

The technique of study and the method of analysis depends upon the particular objectives, for residue determination, to determine the rate of EPTC

transformation, or identification or quanification of the metabolites.

EPTC residues in the plants has been determined colermatically which involve hydrolysis of EPTC with sulfuric acid to dipropylamine followed by the formation of a complex with cupric dithiocarbamate in which absorbance is measured at 440nm (Batchelder and Patcher, 1960).

Chromatographic methods using GC have also been used; either via direct injection into GC (Yip, 1975), or through hydrolysis to dipropylamine, derivativization with nitrophenyl, prior to injection into GC (Crosby and Bowers, 1968), also TLC was used to separate and identify EPTC and its sulfoxidation products (Komives et al., 1979).

Studying EPTC metabolism was carried out using radioassay methods; which involve extraction with 80% methanol, partitioning between organic and aqueous phases, in which the level of radioactivity in the aqueous phase represents the metabolites (Carringer et al., 1978), or through separation, and characterization of the metabolites using radiochemical detectors (Hubble and Casida, 1977; Casida et al., 1975; Horvath and Pualy, 1980).

Recently Knaak et al., (1989) used GCMS to separate and identify EPTC metabolites in rat and human urine,

while Gee et al., (1990) used an immunoassay method to determine EPTC residue levels.

4.6 Experimental :

4.6.1 Chemicals :

EPTC(S-ethyl-N,N-dipropylthiocarbamte) was purchased from Greyhound Chromatography and Allied Chemicals.

The reduced forms of Glutathione(GSH) and Cysteine were purchased from Sigma Chemical Co.

Diallylamine obtained from Aldrich Chemical Ltd, Dichloroacetylchloride from BDH Chemicals Ltd, Triethylamine from Prolabo, and m-Chloroperoxybenzoic acid from Sigma Chemical Ltd.

Dichlormid was prepared as previously described (Hatzios, 1983a), (see Section 4.6.3) EPTC-sulfoxide, and EPTC-Sulfone were prepared according to Casida et al.(1975).

The solvents used were either HPLC or A.R grade from Rathburn/Scotland.

4.5.2 Materials :

Maize seeds(sweet corn) were obtained from .John.Ins.

Soil compost from Leving ton, while Hoagland nutrient solution prepared according to Epstein (1972).

HPLC conditions were as previously described in Chapter three, while TLC plates prepared in the laboratory using Silica Gel 60 F-254 of 2mm thickness as the coating layer.

4.6.3 Synthesis of Dichlormid :

Dichlormid(N,N-diallyl-2,2-dichloroacetamide) was prepared according to the published method of Hatzios, (1983a) with slight modification :

0.05 mole(7.34gm) of Dichloroacetylchloride was dissolved in 20ml Dichloromethane in a 100ml round bottomed flask on an ice bath, to this solution 0.05mole(3.64gm) of Diallylamine was added dropwise with continuos stirring, and the whole mixture was stirred for 5 hours at room temperature. The resulting solution was washed twice with 20ml of distilled water, the aqueous phase discarded, while the organic layer was dried over magnesium sulphate (anhydrous), filterd, and the solvent removed under vacuum employing a stream of nitrogen gas. Dichlormid was recovered as a colourless liquid by vacuum distillation at 120^{o}C at2mm Hg, confirmation of the identity of Dichlormid was carried out using HPLC by matching its retention time with that extracted from the commercial formulation, and by submitting the material to mass spectrometric identification..

4.6.4 Preparation of EPTC -Sulfoxide :

A modified procedure to that of Casida et al.(1975) was used:

1mmole of EPTC(189mg) was dissolved in 10ml Chloroform. To this solution 1mmole of m-chloroperoxybenzoic acid was added, the mixture was stirred for two hours at room temperature, then cooled, and the solvent removed under a stream of nitrogen gas. The by-product(m-Chlorobenzoic acid) was removed by filtration.

Confirmation of the identity of EPTC-sulfoxide was carried out according to the method of Komives et al.(1979): a drop of the resulting solution was spotted on a TLC plate, which was developed in a mixture of Cyclohexane : Acetone :Acetonitrile (16 :3 :1) (v:v:v). After a 10min run the plate was removed from the chamber, dried at room temperrature, and sprayed with a 0.5% solution of 2,6-dichloroquizone-4-chlorimide in acetic acid (Gibbis reagent), and heated for 10min in the oven at 105-110^{o}C.

Detection was based on colour of the spots, and the Rf values for each spot.

4.6.5 Synthesis of EPTC-Sulfone :

EPTC-sulfone was prepared as mentioned in the previous section with some modifications :

5mmole of the oxidizing agent(m-chloroperoxybenzoic acid) instead of 1mmole was used, and the reaction mixture was stirred for five hours instead of two hours.

Identification of EPTC-Sulfone was carried out using the previous method mentioned in section 4.6.4.

4.6.6 Preparation of the EPTC -Cysteine conjugate :

The free base of cysteine was used in combination with different forms of EPTC .

4.6.6.1 EPTC and Cysteine :

100mg of cysteine was dissolved in 5ml of methanol containing 0.2ml of Triethyamine (Hubble and Casida, 1977). The solution was held under nitrogen with continous stirring, then 100mg of EPTC was added, and the whole solution stirred for 24 hours at 30°C, methanol was removed under vacuum at<40°C and the remaining residue washed twice with diethylether. The ether layer was removed by decantation, and the remaining residue was subjected to identification by Mass-Spectrometry.

4.6.6.2 EPTC-Sulfoxide and Cysteine :

The above procedure was repeated using EPTC-Sulfoxide instead of EPTC. The resulting residue was recrystallized from methanol, and its structure confirmed by mass-spectrometry.

4.6.7 Synthesis of EPTC-Glutathione conjugate :

4.6.7.1 EPTC and Glutathione (GSH) :

The method of Hubble and Casida(1977) was followed as described in section 4.6.6.1 . The resulting oily product was subjected to mass-spectrometric analysis.

4.6.7.2 EPTC-Sulfoxide and Glutathione (GSH) :

Repeating the above procedure (section 4.6.6.1), an oily product was also obtained.

4.6.7.3 EPTC-sulfone and Glutathione (GSH) :

100mg of EPTC-sulfone was used with glutathione(GSH) following the method of Hubble and Casida(1977). The resulting white precipitate was recrystallized from methanol, dried, and its mass - spectra recorded.

4.6.8 Method of study :

An appropriate method selected here comprise plant germination, chemicals application, plant moinitoring, harvesting, extraction, purification, separation and identification of metabolites.

4.6.8.1 Soil :

Maize seedlings that previously germinated for three days in petri dishes were transferred into

soil(compost), different treatments were used- Control sample, EPTC alone, and EPTC+Dichlormmid in a ratio of 12:1 (w\w)the plants were monitored, the injury symptoms caused by EPTC observed. The plants were harvested at the first, second, third, and seventh day after planting then separated into leaves and shoots. The shoots were cut into small pieces, and homogonized in 100ml of 80% aqueous methanol solution for 10min. The homogenate was transferred into a 500ml separatory funnel and extracted three times with 100ml of petroleum ether. The aqueous layer was lypholized then dissolved in 5ml methanol for further analysis.

4.6.8.2 Nutrient solution :

Half-strength Hoagland nutrient solution (Epstein, 1972), was used as a medium for germination, and the same procedure used above for the soil study was used here.

4.6.9 Methods of analysis :

4.6.9.1 HPLC :

HPLC conditions as described in Chapter three were used, standard solutions of EPTC, Dichlormid, EPTC-Cysteine conjugate, and EPTC-Glutathione conjugate were injected into the HPLC instrument, and an adequate separation was acheived using mobile phase of water: Acetonitrile in a ratio of 50:50 (v:v). The eluents were

measured using a UV detector at a wavelength of 200nm, with flow rate of 1.5ml/min. 10ul of maize shoot extract are injected under the same conditions as that used for the standards.

4.6.9.2 TLC :

Drops of both plant extract and standard solution were spotted on TLC plates and developed as described by Casida et al.(1975). The identification of the eluents was carried out under UV light, and their Rf values calculated.

4.7 Results :

4.7.1 Dichlormid synthesis :

Confirmation of the identity of Dichlormid was acheived based on three criteria :

1- Boiling point similar to that reported in the literature, 95°C at 3mm Hg.

2-HPLC retention time matched that extracted from the formulation (Fig 4.1).

3-By mass-spectrometric analysis (Fig 4.1).

The purity of Dichlormid was 98% as judged by HPLC.

4.7.2 Preparation of EPTC-Sulfoxide and Sulfone :

The formation of EPTC-Sulfoxide and EPTC-Sulfone was characterized using the method of Komives et al.(1979) which involves using Gibbis Reagent as an

indicator: yellow and brown spots indicate the presence of the Sulfoxide and the Sulfone respectivley, a comparison was also made between the calculated Rf values and those reported in the litreture .

4.7.3 Synthesis of EPTC-Cysteine conjugate :

As shown from the mass-spectral fragments, no conjugate was formed from the reaction between EPTC and Cysteine, while the formation of the conjugate confirmed when EPTC-Sulfoxide was used as a reactant with Cystein (Fig 4.3)

Fig 4.1 HPLC chromatogram of Dichlormid

a) Extracted from the commercial formulation

b) Locally synthesized

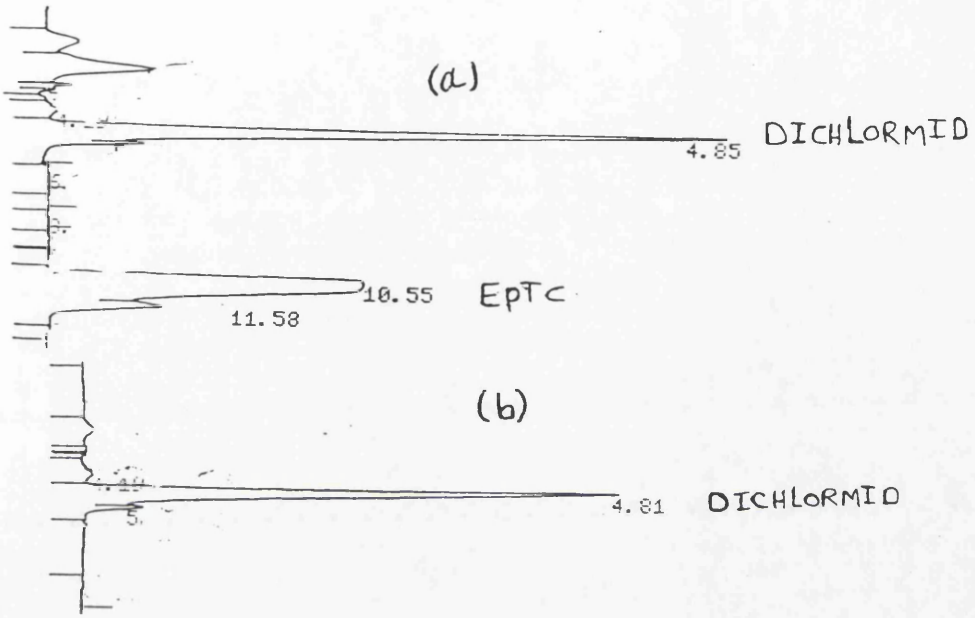
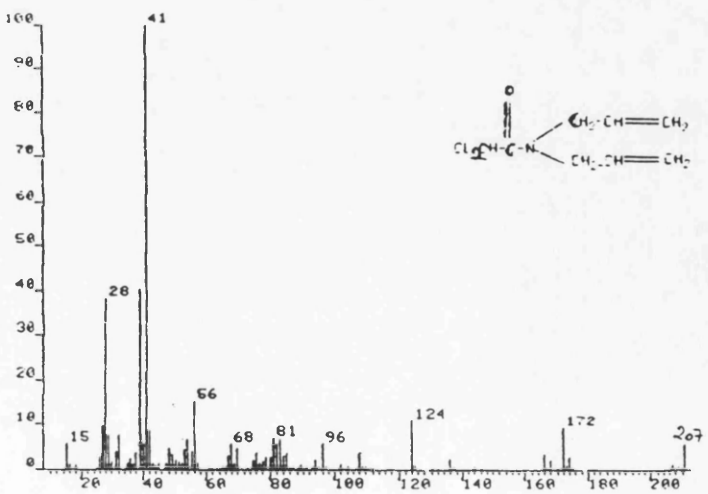


Fig 4.2 Mass-spectra of Dichlormid



4.7.4 Synthesis of EPTC-Glutathione conjugate :

Using EPTC,and EPTC-Sulfoxide as a reactant with the reduced Glutathione(GSH) did not yield the required conjugate. The formation of the conjugate did however take place when EPTC-Sulfone was used as a reactant, (Fig 4.4).

4.7.5 Methods of analysis :

4.7.5.1 HPLC :

The separation of EPTC, Dichlormid, EPTC-Cysteine conjugate and EPTC-Glutathione conjugate was achieved using the following conditions :
Mobile phase : water : Acetonitrile (50:50) +1% Acetic acid. Flow rate 1.5ml/min. Wavelength 200nm.

A search for metabolites in the in maize shoot extract was not successful.

4.7.5.2 TLC :

TLC plates spotted with standard mixture and maize shoot extract are developed in the following solvent systems :
Water : Methanol(50:50) , (25:75) (v:v)
Butanol:Acetic acid :water(6 : 2 : 1) (v:v)
Hexane:methanol(50:50), (75:25), (90:10) (v:v)

Using UV light to search for the expected metabolites revealed nothing when compared with the standard mixture.

Fig 4.3 Mass -spectra of EPTC-cysteine conjugate

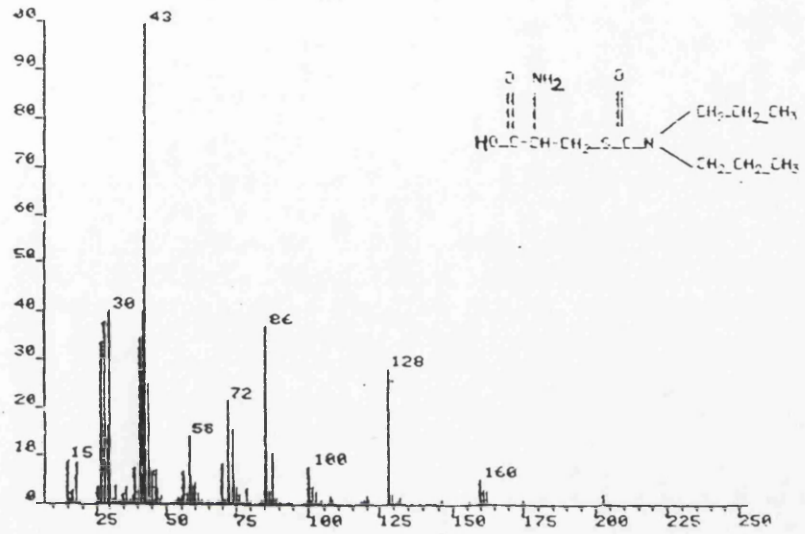
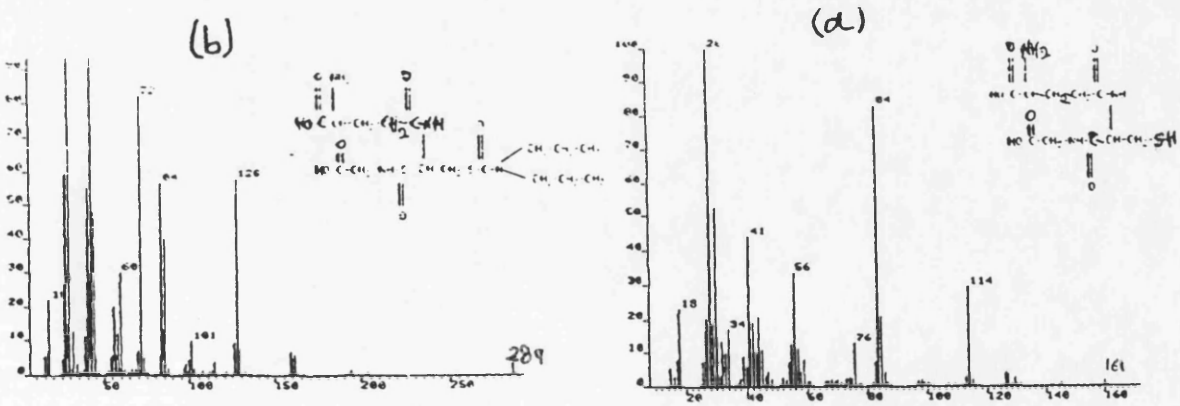


Fig 4.4 Mass spectra of : a) Glutathione (GSH) strandard.b) EPTC-Glutathione conjugate.



4.8 Discussion :

4.8.1 Synthesis of the metabolites :

4.8.1.1 EPTC sulfoxidation :

Two factors were considered in the preparation of EPTC-Sulfoxide and /or Sulfone; (1) time of the reaction, and (2) concentration of the oxidizing agent(m-chloroperoxybenzoicacid) which was previously used for this purpose for EPTC and other thiocarbamates (Bellet and Casida, 1974; Hubbell and Casida, 1977; Schuphan et al., 1981).

The significance of the above factors indicates concerning the metabolism study as to the period of study, and the activity of the catalyzing enzymes(e.g MFO) in plants tissue.

By-product (m-chlorobenzoic acid) interference with the oxidation products was minimized by careful removal through filtration, while the thermal instability of the sulfoxide and sulfone (Casida et al., 1975; Komives et al., 1979); Schuphan and Casida, 1979; Draper and Crosby, 1984; Chen, 1990; Liu et al., 1991) necesiated careful handling and purification.

4.8.1.2 EPTC-Cysteine conjugate :

For the reaction to take place between EPTC and Cysteine, electrophilic site of EPTC C-S- should come in contact with the nucleophilic site (thiol) of cysteine, they facilitate the departure of the leaving group from EPTC (mercaptan).

Under the experiment conditions employed, the low polarization of the carbon of the carbonyl group of EPTC made it difficult for the mercaptan group to leave and the conjugate to form between EPTC and Cysteine.

Oxidation of EPTC to its Sulfoxide increased its reactivity by polarizing the carbonyl group which increased its electrophilicity, and the basicity of the leaving group decreased hence its rate of departure enhanced, as a result the reaction between the thiol site in cysteine and the carbonyl group in EPTC was encouraged and the conjugate formed.This was confirmed from its mass spectrum.

Previously, it was reported that thiocarbamte sulfoxidation increased reactivity in reactions with ester fission, or as carbamylating agents (Chen and Casida, 1978; Dutka et al., 1978; Hutson, 1981; Blair et al., 1984; Cashman and Olsen, 1990).

The significance of EPTC sulfoxidation as a pre-step to its conjugation with plant thiols, indicates the importance of MFO enzymes that catalyze this oxidation

in plant tissue. The possiblity exists that Dichlormid is involved in these processes by affecting the activities of these enzymes.

4.8.1.3 EPTC-Glutathione conjugate :

Neither EPTC,nor EPTC-sulfoxide appeared to conjugate with Glutathione(GSH) under the conditions used, while it seems that EPTC-Sulfone is the only (true) form to conjugate with GSH as shown from its mass-spectra as compared with Glutathione(GSH) mass-spect.

The difference between cysteine and Glutathione as thiol reactant is in the bulk of the glutathione compared with cysteine. The ability of glutathione molecules to come in contact with the electrophilic site in EPTC in order for the reaction to take place is much restricted compared with cysteine .

Further oxidation of EPTC-Sulfoxide to the Sulfone increased the polarization of the carbonyl group. It decreased the basicity of the leaving group(sulfonite) hence facilitated the rate of its departure, and they increased the rate of the formation of the conjugate of Glutathione, previously the sulfone form of a range of thiocarbamates was used to synthesize their corresponding conjugates with Glutathione (Hubbell and Casida, 1977), also Horvath and Pualy(1980) detected the

formation of a Glutathione conjugate of EPTC when the sulfone form was used as a reactant, but little or no non-enzymatic conjugation took place between Glutathione and EPTC or EPTC-Sulfoxide within 10 days of incubation. However, under different conditions, conjugation of glutathione(GSH) with EPTC-Sulfoxide and not EPTC has been detected (Carringer et al., 1978a; Leavitt and Penner, 1979).

The formation of an EPTC-Glutathione conjugate in plants in term of the involvment of the enzyme (GST) in the catalysis of the conjugation is a contraversial issue; Non-enzymatic conjugation is the true way according to Carringer et al. (1978a) and Leavitt and Penner.(1979), while Lay and Casida (1976), Ezra et al.(1985b), and Lay and Niland(1985) confirmed the essential role of GST enzymes for the conjugation to take place. The same contraversy exists for other herbicides conjugated with Glutathione,e.g.; For the herbicidesDiallate (Chen et al., 1979), Metolachlor (Gronwald et al., 1987), and Pretilachlor(Han and Hatzios, 1991).

From this study, it appeares that oxidation of EPTC to its sulfone is vital for its detoxification via conjugation with Glutathione, and the role of Dichlormid in that via its effect on MFO enzymes amount should not be ruled out. The formation of a conjugate between EPTC or EPTC-Sulfoxide in the presence of GST enzymes is

possible , as Dichlormid has enhanced their activity as oreviously reported (Lay and Casida, 1976). It increases the rate of the conjugation, hence the detoxification. However, the catalysis of EPTC sulfoxidation by peroxygenase and lipoxygenase enzymes and the effect of Dichlormid on these reactions has been recently demonstrated (Blee, 1991).

4.8.2 EPTC metabolism :

Attempts to extract and characteize EPTC metabolites in maize shoots either using HPLC, or TLC were not sucessful, hence a study of the effect of Dichlormid on EPTC metabolism was not possible. The failure in the detection of any of these metabolites could due to different reasons, viz. Assuming their formation in the plant, their levels would be below the detection limits of HPLC, and TLC under the conditions used.

The transformation of the EPTC-Glutathione conjugate to other products other than what have been looked for in this study could have occured.

The possibility that EPTC metabolized through other pathways(e.g hydrolysis, alkyl-oxidation, dealkylation) rather than conjugation with Glutathione could take place.

Losing the metabolites through the pre-analysis steps e.g extraction, purification, handling,

separation), and through decomposition or other ways could also be a possibility.

The senstivity and the nature of the analytical method used here as compared to the nature and/orsenstivity of the previous described methods employing radiolabelled compounds for studying EPTC metabolism in plants (Lay and Casida, 1976; Casida et al., 1975; Hubbell and Casida, 1977; Carriger et al, 1978a; Lay and Niland, 1985; Knaak et al., 1989) is also relevant.

4.9 Conclusion :

From the results reported in this chapter, the following remarks can be drawn :

The significance of EPTC oxidation as a pre step for its detoxification through its conjugation with Glutathione and the possible involvement of Dichlomid in this process via its effect on MFO enzymes that catalyze the process.

The presence of GST enzymes determines EPTC form that is able to cojugate with glutathione. In this study and under the conditions used, it was obvious that EPTC-sulfone was the true form of EPTC to conjugate with Glutathione.

The analytical method used, as its detection limits, and suitability is vital in facilitating the study of the effect of Dichlormid on EPTC metabolism.

In this study no clear picture of the effect of Dichlormid on EPTC metablism through conjugation with Glutathione and the relation of that to its mechanism of action would be drawn. In the next chapter the significance of specific functional group(s) to the safening activity of Dichlormid will be examined.

Chapter Five

Dichlormid analogues as safeners for maize against EPTC injury.

5.1 Introduction :

The development of the present commercial safeners came as a result of a random screening exercise that involved the herbicide, the propsed safener and the plant (Parker, 1983).

Structure-activity relationship studies is a promising area of future work. If the functional groups required for herbicidal activity are clearly defined, structurally similar molecules lacking these functional groups may have a chance of being effective safeners for these herbicides, or non-phytotoxic analogues of herbicides may become effective safeners.

Earlier, it has been suggested that a similarity in the chemical structure between the safener and the herbicideis the basis for safener action (Stephenson et al.1978; 1979; Stephenson and Chang, 1978; Dutka et al. , 1979), however in addition the essentiality of specific group(s) for safening activity has been studied (Chang and Merkle, 1982; Dutka and Komives, 1983; Chang, 1983; Hatzios and Zama, 1986; Dutka and Komives, 1987; Codde, 1988).

Recently, the structural similarity between the safener and the herbicide at the molecular level has been examined using Computor Aided Molecular Modeling (CAMM) (Yenne, 1989; Yenne and Hatzios, 1990).

Dichlormid is a safener used mainly to protect maize against thiocarbamate phytotoxicity. Contraversial results dealing with the significance of specific group(s) responsible for the safening activity have been published (Dutka et al., 1979; Pallos et al., 1978; Dutka and Komives, 1983, 1987).

The present work set out to examine the effectivness of the safening activity of the Dichlormid analogues; Diallylamine, Dichloroacetylchloride and CDAA against EPTC injury in maize, by which the significant of specific group(s) to the safening activity was examined.

5.2 Safeners (Chemical Classification) :

Different chemicals have been developed as safeners. They all fit into the following chemical catagories :

(1) Dicarboxylic Anhydrides ; include NaphthalicAnhydride and other analogues (Hoffmman, 1969, 1978; Hatzios and Zama, 1986; Codde, 1988). Their structures are shown inFig 5.1.

(2) Chloroacetamides viz: Dichlormid, CDAA, and other analogues (Chang et al., 1973; Pallos et al., 1975, 1978; Dutka and 1978; Dutka and Komives, 1983, 1987; Hatzios, 1989b; Szell et al., 1985, 1988, (Fig 5.2).
(3) Oxime ethers viz : Cyometrinil, Oxabetrinil, CGA-133205, and others (Ellis et al., 1980; Chang and Merkle, 1982; Peek et al., 1981; Hatzios, 1989a) (Fig 5.3).

(4) 2,4-Disubstituted-5-thiazole carboxylates viz : Flurazole (Sacher et al., 198; Breaux et al., 1989) (Fig 5.4).
(5) More recently introduced safener Fenclorim against the herbicide Pretilachlor in rice (Ebert and Geber, 1989) (Fig 5.5).

The above safeners are used mainly to protect cereal crops such as maize, sorghum and rice against soil applied, shoot absorbed thiocarbamate and chloroaceanilide herbicides (Hatzios, 1989a).

Fig 5.1 Chemical structures of Dicarboxylic Anhydride safeners.

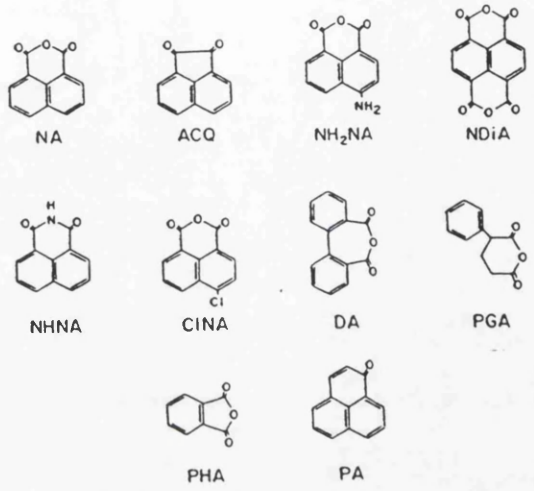
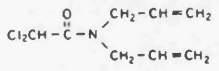
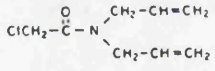
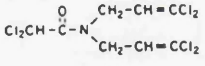


Fig 5.2 Chemical structures of Chloroacetamide safeners.


Dichlormid; R-25788; DDCA


CDAA; Allidochlor (RANDOX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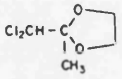
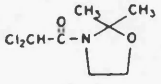
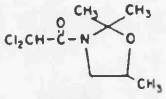
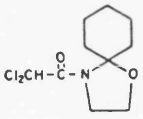
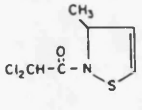
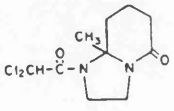

MG-191


R-28725


R-29148


AD-67


M-32988


LAB-147886

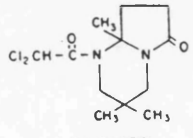
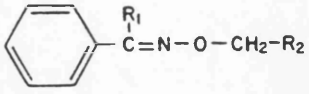
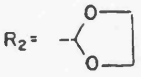

LAB-145138

Fig 5.3 Chemical structures of Oxime ether safeners



Cyometrinil $R_1 = C\equiv N$, $R_2 = C\equiv N$

CGA-92194 $R_1 = C\equiv N$, $R_2 =$ 

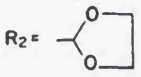
CGA-133205 $R_1 = CF_3$, $R_2 =$ 

Fig 5.4 Chemical structure of Flurazole

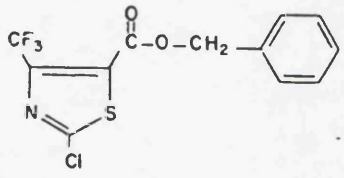
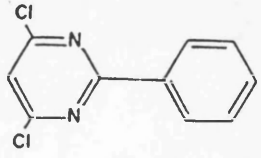


Fig 5.5 Chemical structure of Fenclorim



5.3 Safeners (Basis of Development) :

5.3.1 Parameters of safening activity :

Continous research is underway in academic and industrial research centres for the development and improvement of safener action . As a result of this and in addition to the present safeners, safeners for broad leaved crops have been examined (Varvina, 1987). Safeners that exert their safening action after transformation step(s) within the plants (pro-safeners) have also been developed (Robin and Kirino, 1989). Microorganisms isolated from tolerant plants then introduced into susceptible plants were able to provide protection to plants against the phytotoxic effects of thiocarbamate herbicides (Nagy et al., 1991; 1992).

Various parameters were consideredin the determination of the safening activity viz: the visible injury symptoms due to the herbicide, plant height, plant fresh and dry weight and plant yield. Some characteristics were taken to consideration in determining whether the compound could be developed as a practical safener; its selectivity, its optimum rate for safening activity, safener-herbicide dose ratio, its suitability for formulation and its reliability under field conditions.

In judging the extent of the safening activity , two terms are used; viz : excellent, sufficient,

significant or effective is one term used to refer to complete protection, while moderate, marginal or limited is the other term used to describe partial protection.

5.3.2 Factors affect safening activity :

The degree of safening activity is varied according to the style of study e.g. laboratory, greenhouse or field.

Several factors play a role in the determination of the safening activity; viz : the availability of the safener to the plant from the soil was the basis behind the selection of Dichlormid as the most effective analogue for maize against the phytotoxic effects of thiocarbamates (Pallos et al., 1975; Stephenson and Chang, 1978; Stephenson et al., 1979).

The amount of the herbicide applied, the herbicide : safener dose ratio, the timing of application of the herbicide either as pre plant or as a pre or post emergence treatment, the technique of the application of the safener either as a tank mixture with the herbicide or as a seed dressing and the timing of safener application as related to the time of herbicide application, all have a significant effect on safening activity (Pallos et al., 1975; Hoffmman, 1978; Miller et al., 1978; Blair, 1978; Leavitt and Penner, 1978b; Taft, 1975; Burt, 1976; Winkle et al., 1980; Simkins et al., 1980; Malefyt and Duke, 1981; Dawson, 1983;

Hatzios, 1984; Rubin and Casida, 1985; Christ, 1985; Catizone and Lovato, 1987; Barrett, 1989; Knerr and Hopen, 1989).

Plant type, soil characterstics e.g.moisture, temperature, and soil organic content and weather conditions e.g. rainfall all can influence safening activity of the examined chemicals (Burt, 1976; Myfferler et al., 1978; Okii et al., 1979; Theissen et al., 1980; Ketchersid et al., 1981; Jhonson and Wax, 1981; Sagral and Fog, 1982; Simkins et al., 1980; Hatzios, 1984; Rubin and Casida, 1985; Lay and Niland, 1985; Catizone and Lovato, 1987; Barett, 1989; Sander and Barrett, 1989). Stage of plant growth has been shown to be a factor affecting Dichlormid safening action for maize against EPTC (Buret and Buzio, 1979).

The present work examines the effect of Dichlormid and analogues on the phytotoxicity of EPTC to maize, The injury symptoms of EPTC to maize plants, EPTC inhibition of plant height and plant fresh weigt are considered as parameters in judging the safening action of the applied chemicals.

5.4 Experimental :

5.4.1 Chemicals ;

EPTC(S-ethyl-N,N-dipropylthiocarbamate) was purchased from Greyhound Chromatography and Allied Chemicals.

Dichlormid was prepared as previously described(Section 4.6.3).

Diallylamine was obtained from Aldrich Chemical Ltd and Dichloroacetylchloride from BDH Chemical Ltd and CDAA (2-chloro-N,N-diallylacetamide) was obtained from Greyhound and Allied Chemicals.

The solvents used were either of HPLC or AR grades.

5.4.2 Plants and soil :

Soil Compost(Levington) was used for plant germination.

Maize seeds(sweet corn) were obtained from FI.John.Innes.Hybride. Seedlings have germinated in Petri dish and having uniform shoots and roots were selected for the study.

5.4.3 Treatment :

Plastic pots were filled with the soil to 2cm below the surface. The remaining 2cm of the soil were mixed in a plastic bag with the chemical(s) concerned prior to planting. The chemicals were applied either in methanol or in acetone (Diallylamine and Dichloroacetylchloride

were applied in acetone). They were applied in weight by weight ratio in the following manner :

Control (9ml of either methanol or acetone),

EPTC(12.6kg/ha) , EPTC+Dichlormid in ratio of (10:1)

EPTC+CDAA in ratio(10:1), EPTC+Diallylamine in ratio (10:1)

EPTC+Dichloroacetylchloride in ratio(10:1)

EPTC+Diallylamine+Dic hloroacetylchloride for each in ratio of (10:1), Dichormid(1.26kg/ha), Diallyamine(1.26Kg/ha), Dichloroacetylchloride (1.26kg/ha). The development of the injury symptoms was mointored. The plants were harvested after five weeks and plant height and plant fresh weight were measured. Each reading represents three samples with three plants in each.

The analysis of variance method was used to predict if the safening action of the applied chemicals was significant.

Results

The injury symptoms :

The injury symptoms were recorded based on a comparison with the control sample.

Table 5.1 Injury symptoms of maize plants treated with the herbicide EPTC, the safener Dichlormid and its analogues.

Sample	Injury symptoms
Control	None
EPTC	shoot growth inhibited deformed, wrinkled, unrolled, twisted, and dark-green leathery leaves.
EPTC + Dichlormid	None
EPTC + CDAA	None
EPTC + Diallylamine	slightly twisted, wrinkled, and loopy leaves, no green- dark leathery leaves.
EPTC + Dichloroacetyl-chloride	slightly twisted, wrinkled, and loopy leaves but not shoot, slightly gren leaves.
EPTC + Diallylamine + Dichloroacetylchloride	slightly loopy leaves, no twisted leaves or shoots, green-dark leathery leaves
Dichlormid	None
CDAA	None
Diallylamine	None
Dichloroacetylchloride	None

Plate 5.1 Maize plants treated with EPTC, Dichlormid and Dichlormid analogues under greenhouse conditions.

Plant height :

Plant height was measured from the soil surface to the tip of the highest leaf, the values represent an average of three samples, with three plants in each.

Table 5.2 The height of maize plants treated with the herbicide EPTC, the safener Dichlormid and its analogues.

Sample	Plant height (cm)
Control	47.50
EPTC	22.33
EPTC+Dichlormid	43.76
EPTC+CDAA	28.63
EPTC+Diallylamine	27.35
EPTC+Dichloroacetyl-chloride	23.92
EPTC+Diallylamine+ Dichloroacetylchloride	26.42
Dichlormid	52.18
CDAA	44.33
Diallylamine	46.80
Dichloroacetylchloride	51.50

Plant fresh weight :

Plant fresh weight was measured immediatly after harvesting in which a combination of plant shoot and leaves comprise the weight. Each reading is an average of three replicates, each containing three plants :

Table 5.3 The fresh weight of maize plants treated with the herbicide EPTC, the safener Dichlormid and its analogues.

Sample	Plant fresh weight (gm)
Control	9.13
EPTC	5.50
EPTC+Dichlormid	6.62
EPTC+CDAA	1.96
EPTC+Diallylamine	3.42
EPTC+Dichloroacetylchloride	3.99
EPTC+Diallylamine +dichloroacetylchloride	4.45
Dichlormid	12.54
CDAA	9.35
Diallylamine	9.61
Dichloroacetylchloride	12.60

Discussion :

Three parameters were considered when examining the safening activity of the applied chemicals; viz:(1) counteraction the injury symptoms of EPTC,(2) plant height and (3) plant fresh weight.

The injury symptoms noted for EPTC were typically those of thiocarbamates; shoot growth inhibition, stunting, twisting of shoots and leaves, wrinking, looping, brittle, hard and dark-green leathery leaves (Plate 5.1). Similar symptoms have been demonstrated by others (Harvey et al., 1975; Pallos et al., 1978; Sagral, 1978; Wilkinson, 1978; Donald, 1981; Wilkinson, 1983; Barta et al., 1983).

Application of Dichlormid or CDAA with EPTC resulted in a complete elimination of these symptoms, while various degrees of recovery were achieved by addition of either Diallylamine or Dichloroacetylchloride or both. Diallylamine significantly reduced leaf and shoot twisting, leaves wrinkled and loopy leaves and eliminated the green-dark leathery character of the leaves caused by EPTC.

Dichloroacetylchloride reduced leaf and shoot twisting, and wrinkle and loopy leaves, but the green-dark colour remained. While application of diallylamine

and dichloroacetylchloride with EPTC resulted in the prevention of twisting of the leaves and shoots only.

From these results, it appears that an essential functional group(s) not present in Diallylamine or in Dichloroacetylchloride is required for the full safening activity that Dichlormid as one unit has. It has been reported that the structural similarity between EPTC and Dichlormid was the basis for its safening activity (Pallos et al., 1975; Stephenson and Chang, 1978; Stephenson et al., 1978; 1979; Dutka et al., 1979; Yenne and Hatzios, 1990). However, researchers have disputed this hypothesis and indicated that particular group in the Dichlormid structure is essential for its safening activity. This group being either the Dichloroacetamido moiety (Dutka and Komives, 1983), or the Dichloroaceto group (Dutka and Komives, 1987). This hypothesis contradicts to results obtained here which emphasise the importance of Dichlormid as a whole unit for complete safening action.

Regarding the partial safening activity exerted by Diallylamine and Dichloroacetylchloride, it seems that the functional groups that they contain prevent some physiological and/or biochemical effect(s) caused by the herbicide EPTC.

Previously, it has been reported that application of exogenous gibberellin(GA) has prevented the stunting effect caused by EPTC but it did not prevent leaf

deformation (Wilkinson, 1978), while the addition of ethylene generator inhibitors decreased ethylene enhanced release caused by the herbicide Metolachlor, but did not prevent the accompained injury symptom (Paradis et al., 1981; Ndahi, 1988). In this respect, the essential roles of specific group(s) for the safening activity in other safeners has been detected; oxime and/or pyridine group for oxime ethers (Chang and Merkle, 1982; Chang, 1983) or dicarboxylic group attached to the aromatic ring for Naphthalic Anhydride (Hatzios and Zama, 1986). Recently Palogh et al.,(1992) reported that addition of Methyl Dichloroacetate to the mixture of EPTC and Dichlormid in maize increased the safening activity from 90 to 100%. With reference to plant height, it seems it is the most acceptable parameter for predicting safening activity, EPTC caused 55% reduction in plant growth while

Dichlormid decreased it to 10%, and CDAA decreased it to 40%. Diallylamine and Dichloroacetylchloride caused a reduction to 43 and 49% respectively, while their application together reduced the inhibition by 10%, However, no adverse effect on plant height inhibition was observed as a result of the application of the chemicals without the herbicide.

These results also confirmed the necessity of Dichlormid as a whole unit for the safening activity. The reduction in shoot growth inhibition by 50% as a

result of safener application was considered as the basis for the safening activity (Chang et al., 1973).

Plant fresh weight did not provide an adequate tool to judge the safening action. This is in accordance with Ezra et al., (1985) who indicated that the significant differences between treatments in term of plant weight does not make it a basis for predicting the safening activity, while they considerd plant height as a basis for predicting the safening action.

In this study, Dichlormid eliminated 80% of plant height inhibition caused by EPTC. This is in agreement with several previous studies (Taft, 1975; Sagral, 1978), while a reduction of 27% as a result of CDAA application does indicate that Dichloroacetamides are more effective as safeners than the mono or trichloro analogues. This has also been obtained previously (Stephenson and Chang, 1978), also due to the adverse effects of CDAA where it is mainly used as a herbicide (Ezra et al., , 1985). Diallylamine and Dichloroacetylchloride partial safening action indicates that no specific functional group in the examined chemicals is responsible for the full safening activity, while their failuer to act more effectivlly when applied togother with EPTC seems to be due either to their unavailibility to plants, or due to their interaction to form an inactive product, or to their interaction with soil constituents.

5.7 Conclusion :

One point in particular could be drawn from these results, viz: it is that Dichlormid as whole unit is required for safener action, while the partial effect noted for Diallylamine and Dichloroacetylchloride indicates that EPTC interferes with more than one physiological and/or biochemical process in maize, some, but not all of which are affected by Dichlormid analogues.

Chapter six

Phototransformation of EPTC, Dichlormid, and EPTC+Dichlormid in aqueous and non-aqueous solutions.

6.1 Introduction :

In the environment, herbicides are dissipated through various processes, viz: volatilization, leaching, adsorption into soil colloids and through transformation. Their transformations proceeded chemically, biologically and photochemi cally yielding almost similar products on occasion (Bellet and Casida, 1974; Benson, 1974). For phototransformation to occur, the herbicide should absorb light, either directly or through sensitizing substances. The absorption might occur in the air, during spraying in water droplets and on the plant and soil surfaces (Hulpke et al., 1983).

The chemical structure of the herbicide, the formulation of the herbicide, the characterstics of the soil and the climate are all factors affecting herbicide liability to phototransformation.

Photolysis of the herbicide may (1) increase its biological activity towards weed control, (2) decrease its activity hence facilitate its removal as harmful residues, or (3) yeild compounds with different

biological activities or/and different significant mammalian toxicity (Watkins, 1979).

EPTC is a highly volatile soil applied herbicide, which might undergo phototransformation in the soil, at plant surfaces, and in the air. Neither its photochemical fate, nor that of the safener Dichlormid have been fully explored. This work in effect is to shed some light on their phototransformation both as indiviuals and in combination under different conditions.

6.2 Photochemistry (Fundementals) :

Photochemistry is defined as the study of the chemical processes that occur after electronic excitation of molecules with electromagnetic radiation (Davidson, 1979), while environmental photochemistry is a study of these processes relevant to environmental conditions (Roof, 1980).

Light is electromagnetic in nature. It consists of photons, each photon only activates one molecule. Photon energy is related to its wavelength and its intensity. The shorter the wavelength, the greater the energy of radiation. Energy absorbed by the molecule may increase its transitional, rotational, vibrational, or electronic energy.

If the wavelength of the radiation imparts sufficient energy to interact with the valence

electrons, an electronically excited molecule is produced. This energy is defined as:

$$E = hv = hc/\lambda$$

v:light freguency , h :Planck constant ; c :light speed and λ : wavelength of the light.

The amount of radiation absorbed by the system is proporationl to the number of molecules involved :

$$E = E^{\backslash}CI$$

E : absorbance ,C :concentration , $E^{\backslash}$ extinction coeffecient, and I cell distance(Path).

As a result of the excitation, two excited states may be produced, singlet and triplet. Inter-crossing from excited singlet to excited triplet is possible. The excitation energy might dissipate in a number of ways; 1) emission as fluoresence, 2) as phosphorsence, 3) as heat, or 4) through undergoing chemical reactions (Plimmer, 1970).

The efficiency of the excitation process is defined as the number of moles of the resultant products divided by the number of Einsteins of light absorbed. This process may be direct by which molecules absorb light and undergo transformation, or indirect by which the receptor molecules contact the excited donor species whererby it transfers the excitation energy to the receptor that undergoes transformation just as if it had acquired the energy directly. These donors are called

sensitizers; an efficient sensetizer functions in the following sequence :

$Sens(s_0)$ + hv ---------- $Sens(s_1)$, s_1 : singlet excited state.

$Sens(s_1)$ _____ISC $Sens(t_1)$, t_1 : triplet excited state , ISC :Inter System Crossing.

$Sens(t_1)$ + $A(s_0)$ ____ Energy transfer $Sens(s_0)$ + $A(t_1)$, s_0 : singlet ground state .

The sensitizer should have 1) efficiency of Inter-System Crossing (ISC), 2) the ability to transfer energy and 3) it should absorb at higher wavelengths than the acceptor will absorb. Its effeciency will decrease if it undergoes transformation. For sensitizers in solution, transfer of triplet excitation energy is generally more effecient than transfer of the singlet unit. The opposite to sensitizers are quenchers that are mainly used to suppress the photochemical process (Roof, 1980).

6.3 Phototransformation of the herbicides (Review) :

Different processes were identified as routes of phototransformation of herbicides; viz: reduction, dechlorination with the replacement of the chlorine either with a hydrogen or a hydroxyl group, hydroxylation of the alkyl chains and the aromatic ring, dealkylation, sulfoxidation, hydrolysis, and coupling of the radicals from which a variety of dimers are formed

(Pilmmer, 1970; Crosby, 1976; Zepp, 1980; Marchettee et al., 1988; Cessna and Muir, 1991).

The intensity of the light, the distribution of the wavelengths of sunlight, concentration of the herbicide and the physical state of the environment in which the herbicide resides are all factors which have an effect on the herbicide pattern of phototransformation (Miller and Zepp, 1983).

Herbicides that absorb light in the UV-spectrum undergo direct transformation, while those exhibiting no UV spectrum above 290nm are photochemically stable when irradiated with sunlight. However, if the system contains other components, indirect phototransformation might take place. These components that act as photosensitizers can be present either naturally in the field or within the formulation of the herbicide (Watkins, 1979; Cessna and Muir, 1991). Examples are Riboflavin (Rejto et al., 1984), H_2O_2 (Draper and Crosby, 1984b), Benzophenone and Anthraquinone (Casida et al., 1975), Methylene blue and Tryptophan (Draper, 1979; Draper and Crosby, 1981), Tyrosine (Ross and Crosby, 1985), and Plant pigments (Dodge and Knox, 1986).

6.3.1 Thiocarbamates :

Their phototransformation varies according to the chemical structure of the herbicide; viz: the herbicide Thiobencarb has an absorption maximum at 290nm, thus it weakly absorbs wavelengths greater than 290nm. As a consequence, under sunlight, phototransformation of Thiobencarb is very slow (Casida et al., 1975; Ross and Crosby , 1984; Cessna and Muir, 1991), on the other hand Ishikawa et al.(1977) reported that Thiobencarb undergoes sulfoxidation, ring hydroxylation, hydrolysis and dealkylation via exposure to UV light.

The thiocarbamate Molinate that exhibits no absorption at wavelengths greater than 290nm was recovered unchanged after 16 hour of exposure to sunlight on filter paper (Casida et al., 1975), or after seven days irradiation of simulated sunlight in aqueous solution (Soderquist et al., 1977). Common photochemical processes were demonstrated for thiocarbamates; viz: sulfoxidation, dealkylation, hydrolysis, ring hydroxylation and dimerization (Draper, 1979; Draper and Crosby, 1984a; Demarco and Hayes, 1979; Draper and Crosby, 1984b).

The effects of other substances e.g. natural water components and sensitizers on the photolysis of thiocarbamates have been demonstrated (Ivie and Bull, 1976; Gohre and Miller, 1986).

6.3.1.1 EPTC :

EPTC has been shown to be transformed to its sulfoxide form via exposure to sunlight for 16 hours on filter paper (Casida et al., 1975), while irradiation with UV light at 254nm in hexane yielded Dipropylformamide, Dipropylamine, Propylamine, Mercaptane, and Disulphide (Demarco and Hayes, 1979).

6.3.2 Choroacetamides :

Exposure of the herbicide CDAA to UV-light on filter paper produced various unidentified products (Mitchell, 1961), while in the presence of Riboflavin, the herbicide Propachlor underwent hydroxylation via exposure to sunlight, and cyclization via irradiation with UV light (Rejto et al., 1984).

Pilmmer.(1970) reported that dechlorination is a common photochemical process for the phototransformation of chloroacetamides.

6.4 Methods and Apparatus :

Monitoring phototransformation of herbicides in the environment is a difficult task because molecules might interact with several environmental components. It is

necessary to resort to laboratory models, but even these models do not provide a total guide to environmental behaviour although valuable information may be obtained using relatively limited ranges of conditions (Cavell, 1979).

Herbicide phototransformation may be studied in thin solid films, in organic and aqueous solutions, in adsorbed state on particles, and in the vapour phase (Crosby, 1976; Dilling and Goersch, 1980). Hulpke et al.(1983) used silica gel as a pure and standardized method for studying the phototransformation of the herbicides. Various UV-light sources were used in the above (Crosby, 1976; ,Marcheterre et al., 1988).

6.5 Experimental

6.5.1 Chemicals :

EPTC(S-ethyl-N,N,-dipropylthiocarbamate)(99.00%) was purchased from Greyhound Chromatography and Allied Chemicals.

Dichlormid was prepared as previously described (section 4.3.6)

2,6-dichloroquinone-4-chlorimide(98.0%) was purchased from Aldrich. Chem.Ltd

Solvents used were HPLC grade obtained from Rathburn/ Scotland.

Dipropylamine and Diallylamine were obtained from Aldrich. Chem.Ltd, while propylamine was obtained from Koch-Light Lab Ltd.

TLC plates were prepared using silica gel 60F-254- Dimensions 20 x 20cm and 5mm thickness.

6.5.2 Photochemical apparatus :

The photochemical appartus consisted of a three-necked vessel of 1050cm^3 capicity. It was equipped with magnetic stirring bar and a water cooled internal quartz immersion well. The light source was a 125 watt Hanovia high pressure mercury lamp that produced an irradiation peak at 253.7nm.To eliminate wavelength below 290nm, a pyrex tube was used as a filter.

6.5.3 Irradiation :

EPTC (100mg), Dichlormid(250mg), and a mixture of EPTC (100mg) and Dichlormid(10mg) were dissolved in one litre of water or methanol in the photolysis vessel. The solution was stirred for 5min, then it was irradiated at 254nm.

Samples were withdrawn at zero time and at 5 min intervals for one hour.

6.5.4 Analysis :

6.5.4.1 Equipment :

The analysis was carried out using a GC-Pye Unicam PU-4500 chromatograph, fitted with a flame ionization detector(FID) and 2mX4mm id glass column packed with a semipolar silicon oil(3.5% OV 17), supported on 100/200 mesh WHP. FID signals were recorded on a Chromatopac Integrator(SPU 290). Column temperature was 145^{o}C, while the detector and the injector temperatures were set at 220^{o}C and 250^{o}C respectively. Nitrogen was used as a carrier gas with a flow rate of 30ml/min.

6.5.4.2 Irradiation in methanol :

Samples were withdrawn at zero time and at 5min intervals for one hour. The samples were injected directly into the GC and the rate of the phototransformation of EPTC was calculated either as mg/l of solution or as a percentage of the remaining EPTC.

6.5.4.3 Irradiation in water :

Samples(25ml) of either EPTC, or Dichlormid were withdrawn at the same time intervals as above. They were transferred into a 100ml separating funnel and extracted three times with 25ml Dichloromethane. The

organic layer was combined, dried over anhydrous sodium sulphate. The Dichloromethane was removed under vacuum at<40°C and the remaining residue was adjusted to 1ml with Dichloromethane prior to injection into the GC

6.5.5 Separation and Identification of photoproducts :

6.5.5.1 Methanol :

The remaining residue after one hour of irradiation was concentrated under vacum at<40°C prior to separation and identification.

6.5.5.2 Water :

The remaining solution was extracted three times with one litre of Dichloromethane, the extracts combined, dried over anhydrous sodium sulphate , filterd and the Dichloromethane removed under vacum at <40°C prior to further analysis.

6.5.5.3 Identification :

Three identification techniques were used :

6.5.5.3.1 TLC/MS :

The concentrated photolysates from both the methanol and water media were chromatographed over thin layer

plates. The plates were developed in different solvent systems, viz : Water : Methanol (50 : 50) , (70 : 30) , (90 : 10) (v : v). Butanol : Acetic Acid : Water (6 : 1 : 1). (v : v). Hexane : Diethylether (70 : 30) , (30 : 70) (v : v).

After developing for 10-30min, the plates were removed, dried, and examined and visualized either by eye or under UV light. The localized bands were scraped off, extracted and eluted with methanol, then concentrated and submitted to more spectrometric analysis.

6.5.5.3.2 TLC/Gibbs reagent :

This method was used mainly to detect the thermally unstable products viz: the Sulfoxide and the Sulfone form of EPTC. Spots of the photolysates were chromatographed on TLC plates. The plates were then developed in a mixture of Cyclohexane : Acetone : Acetonitrile , (16 : 3 : 1) (v : v : v). After the solvent front had travelled 10cm the plates were removed from the chamber, dried at room temperature, then sprayed with 0.5% solution of 2,6-Dichloroquin-4-chlorimide in acetic acid(Gibbs reagent), and heated in an oven for 10min at 105-110^{o}C. Identification was based on the loc ation of the coloured bands and by their Rf values, as compared with authentic compounds .

6.5.5.3.3 Cas Chromatography :

The analysis was based on a comparison between the retention time of the standard compounds Dipropylamine, Diallylamine and Propylamine, and of the photolysate products. GC was used as previously described (section 6.5.4.1). Column temperature was kept at 145°C, and the detector and injector temperature were 220°C and 250°C respectively. The carrier gas was nitrogen with a flow rate of 30ml/min and the FID signals were recorded on Chromatpac Integrator(SP 4290). Both the authentic and the photolysate products were injected under the same conditions, then the photolysate solution was spiked with the predicted standards and injected into the GC.

6.6 Results :

6.6.1 Recovery :

Using Dichloromethane as a solvent for extraction, EPTC and Dichlormid recoveries were as follows :
EPTC 87$\bar{+}$ 2% EPTC in the presence of Dichlormid 93$\bar{+}$ 2.3%
Dichlormid 95$\bar{+}$ 1.72% The results were corrected according to the above percentages.

6.6.2 The rate of phototransformation of EPTC in methanol and water :

Irradiation of EPTC with UV-light at 254nm caused a rapid phototransformation in both media (Fig 6.1). EPTC half-life of phototransformation in water and methanol is shown in Table 6.3. A remarkable change in the colour of the EPTC solution from clear to yellow then to orange, and an unpleasant smelling odour were two phenomena accompanying EPTC phototransformation at 254nm.

Under artificial sunlight conditions (>290nm), negligable or very slow degradation of EPTC was detected in both the aqueous and the organic solutions(Fig 6.3). A significant difference between the rates of photolysis of EPTC in the two media (water and methanol) was detected using the analysis of variance method.

6.6.3 Dichlormid rate of phototransformation in aqueous and non-aqueous solutions :

Dichlormid underwent rapid degradation via irradiation with UV-light at 254nm in both water and methanol(Fig 6.2) with a relatively short half-life (Table 6.4). A slight change in the colour of the solution toward yellowish, with no change in the odour were noticed.

In both media, negligible degradation at>290nm was observed (Fig 6.4).

A significant difference between the rate of degradation of Dichlormid in water compared with methanol was detected.

6.6.4 Effect of Dichlormid on EPTC phototransformation :

At 254nm and at>290nm, Dichlormid had no significant effect on the rate of phototransformation of EPTC in both media, while there was a significant difference in the rate of phototransformation of EPTC in the presence of Dichlormid in water compared with methanol at 254nm.

Table 6.1 Phototransformation of EPTC at 254nm.

Time (min)	% of the initial EPTC Concentration			
	water		methanol	
	EPTC	EPTC + Dich	EPTC	EPTC +Dich
0	100	100	100	100
5	79.68	91.08	95.67	94.72
10	66.26	78.53	88.71	85.49
15	50.89	62.35	78.00	76.01
20	44.20	53.61	69.38	67.79
25	30.85	40.17	64.65	58.68
30	19.85	29.58	57.14	53.41
45	4.96	14.27	43.00	36.02
60	1.92	5.43	30.38	25.60

Table 6.2 Phototransformation of Dichlormid at 254nm.

Time (min)	% of initial Dichlormid concentration	
	water	methanol
0	100	100
5	92.71	40.83
10	58.46	33.29
15	31.73	15.73
20	19.25	6.90
25	8.08	n.d
30	n.d	n.d

Table 6.3 Half-life of the phototransformation of EPTC at 254nm.

Sample	Medium	
	water (min)	methanol (min)
EPTC	14.03 ∓ 0.093	37.22 ∓ 5.16
EPTC +Dich	18.50 ∓ 1.20	32.19 ∓ 3.37

Table 6.4 Half-life of the phototransformation of Dichlormid at 254nm.

Sample	water (min)	methanol (min)
Dichlormid	10.22 ∓ 0.79	5.20 ∓ 0.72

Table 6.5 photolysis of EPTC at wavelength >290nm.

Time(min)	% initial EPTC concentration					
	water			methanol		
	dark	EPTC	EPTC+Dich	EPTC	EPTC+Dich	Dark
0	100	100	100	100	100	100
5	98.76	97.52	95.50	96.66	98.18	98.76
10	97.90	97.94	98.18	95.98	96.54	97.54
15	96.54	95.50	96.56	94.26	97.06	96.34
20	97.31	96.37	97.06	95.31	94.85	97.85
25	96.40	94.45	94.85	94.27	95.78	98.30
30	98.21	97.62	95.32	94.16	94.45	98.57
45	96.30	96.33	93.58	93.57	93.21	96.90
60	96.87	95.89	94.13	95.16	93.45	97.12

Table 6.6 Phototransformation of Dichlormid at wavelength > 290nm.

Time (min)	% of initial Dichlormid concentration		
	Dark	water	methanol
0	100	100	100
5	99.10	97.52	95.65
10	98.76	97.94	97.81
15	95.43	95.50	96.53
20	96.49	94.45	98.01
25	97.41	95.98	96.43
30	98.40	93.89	94.56
45	96.63	94.71	94.39
60	97.03	92.10	93.13

Fig 6.1 Rate of phototransformation of EPTC at 254nm.

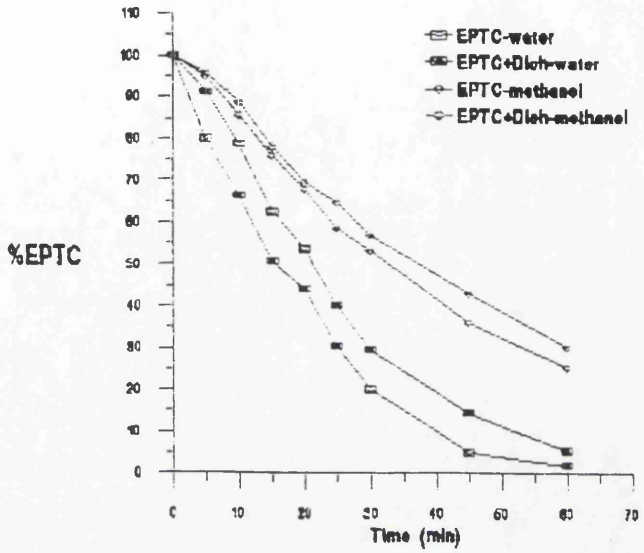


Fig 6.2 Rate of phototransformation of Dichlormid at 254nm.

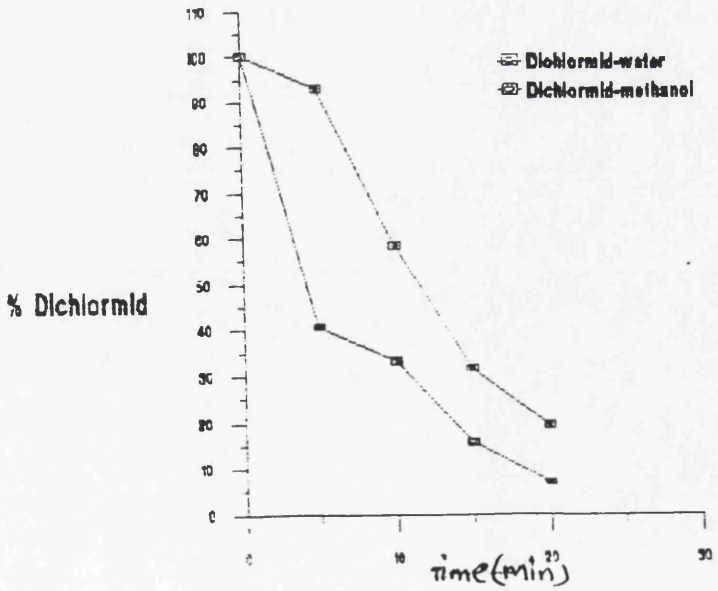


Fig 6.3 Rate of phototransformation of EPTC at >290nm.

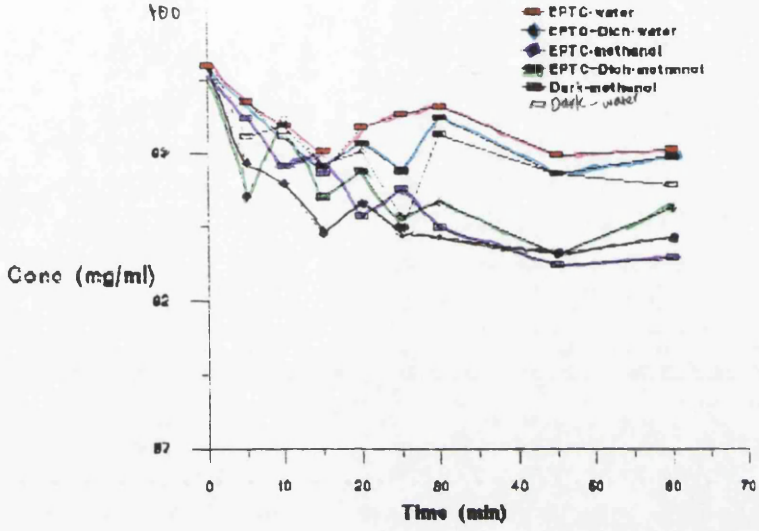
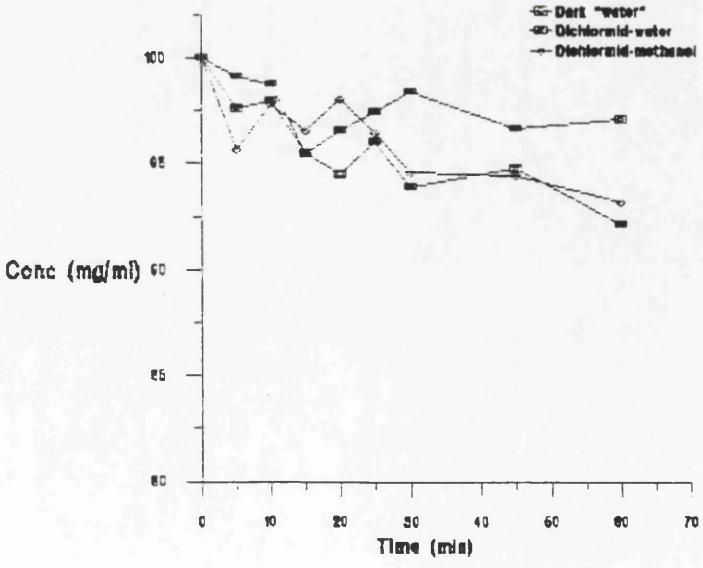


Fig 6.4 Rate of phototransformation of Dichlormid at >290nm.



6.6.5 Separation and identification of photoproducts :

Separation and identification of the photoproducts that formed at 254nm were carried out. At >290nm, although negligible degradation had taken place (2%), several peaks were detected as shown from the GC chromatograms, However, due to the low levels present, no further separation and identification steps by other methods were carried out.

6.6.5.1 EPTC phototransformation :

6.6.5.1.1 TLC/MS method :

From the identity of photoproducts separated using different solvent systems, it appeared that these products had a high molecular weight resulting possibl y from coupling of radicals which formed through EPTC degradation in both methanol and water. The cleavage of C-S, and C-N bonds will account for the formation of these radicals.

Gradual dealkylation of the acid chains of EPTC has also occurred, but the coupling of the dealkylated radicals made it difficult to bring about their separation and identification. Possible pathways of EPTC degradation are shown in Fig 6.5.

6.6.5.1.2 TlC/Gibbs reagent :

Two yellow spots representing EPTC and EPTC-Sulfoxide were detected. Confirmation of their identity was based on a comparison of their Rf values with those of EPTC and EPTC-Sulfoxide standards and with those values reported previously (Komives et al., 1979).

6.6.5.1.3 Gas chromatography :

Propylamine and Dipropylamine (Fig 6.11) were detected as photoproducts of EPTC at 254nm. Their identity was confirmed based on matching of their retention times with those of the standards.

Dipropylformamide (Fig 6.13) was predicted as the peak appearing at 4.30 min and then gradually disappeared . The absence of Dipropylformamide standard made definite confirmation difficult.

At> 290nm, various peaks (six) were detected (Fig 6.9). They were completely different from those which appeared at 254nm. Their low levels made it difficult to carry out further separation and identification steps.

6.6.5.2 Dichlormid photoproducts :

Dichlormid routes of phototransformation at 254nm were characterized as dechlorination, dealkylation, and hydrolysis both in water and in methanol as demonstrated by GC-MS, GC, and TLC-MS.

At >290nm, several products were formed as shown by injection of the photolysate into the GC (Fig 6.10).

6.6.5.3 Interferance of Dichlormid with EPTC photoproducts:

At 254nm, it appeared that products detected in EPTC photolysate solution were almost similar to those detected in EPTC+Dichlormid solution. An extra product was formed as a result of the coupling of EPTC and Dichlormid radicals. This compound was separated by TLC and was identified using a mass spectrometer.

Dichlormid did not interfere with EPTC photoproducts at >290nm .

Fig 6.5 Pathways of EPTC phototransformation in water and in methanol at 254nm.

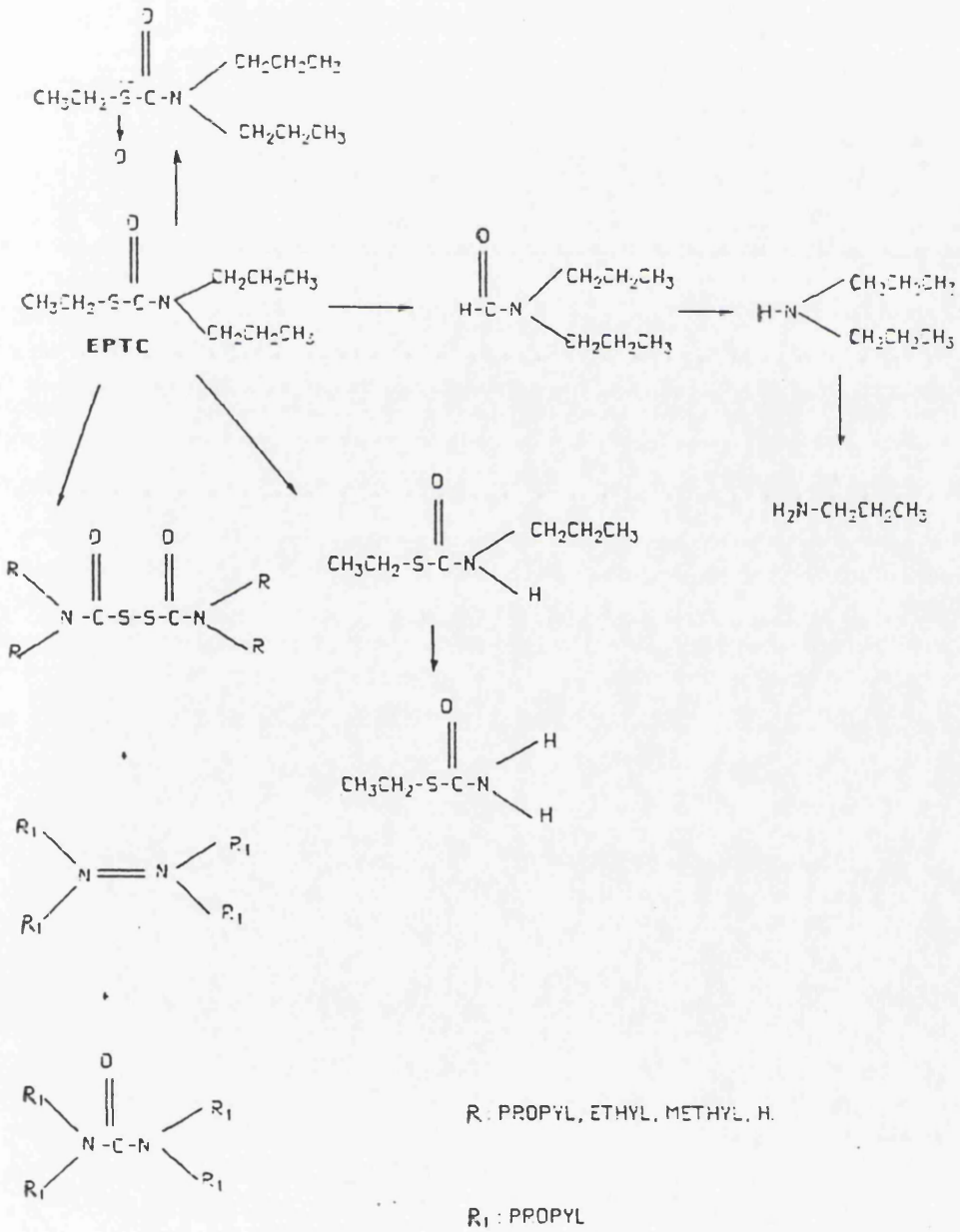


Fig 6.6 Routes of the phototransformation of Dichlormid in water and in methanol at 254nm.

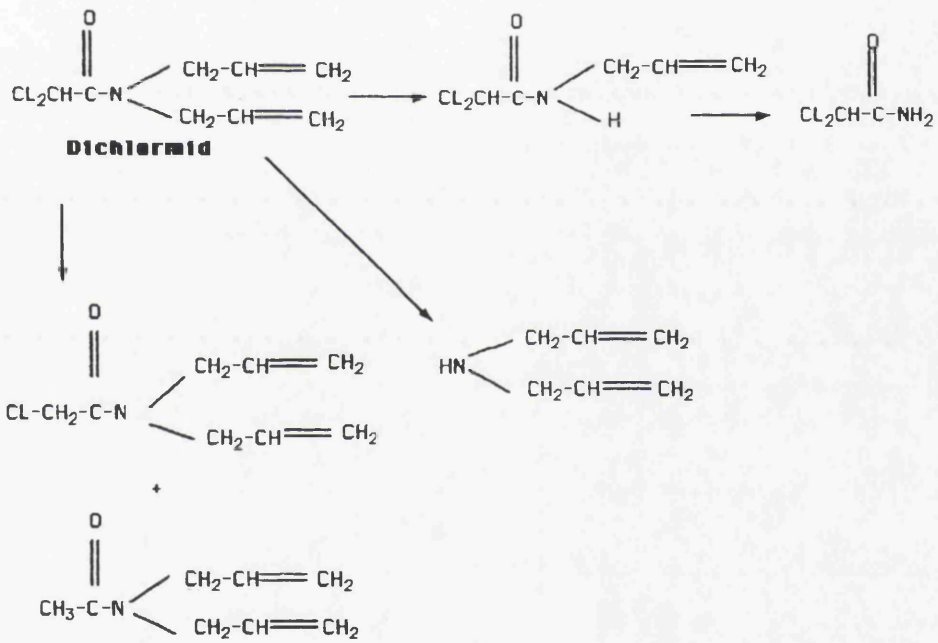
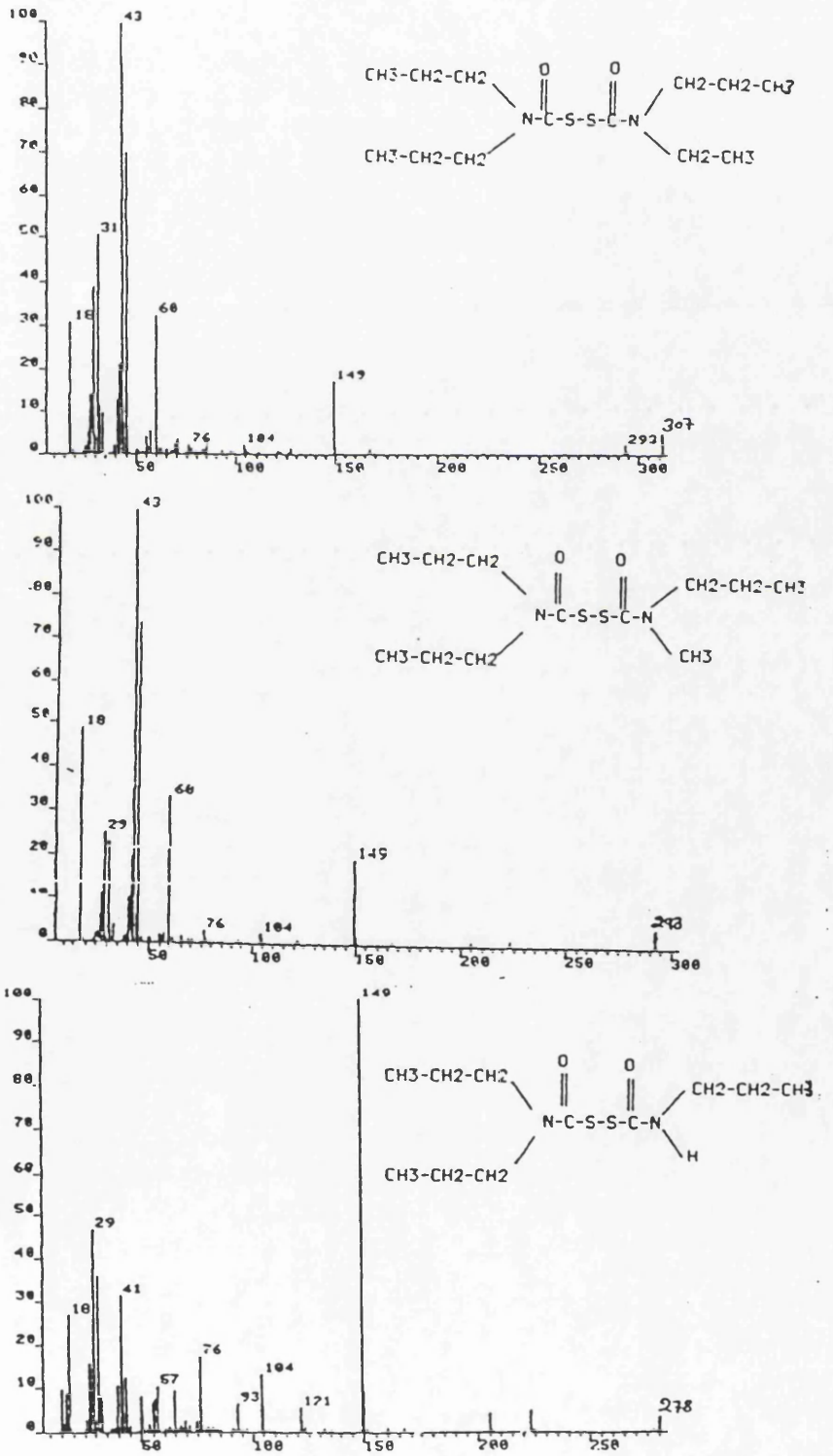
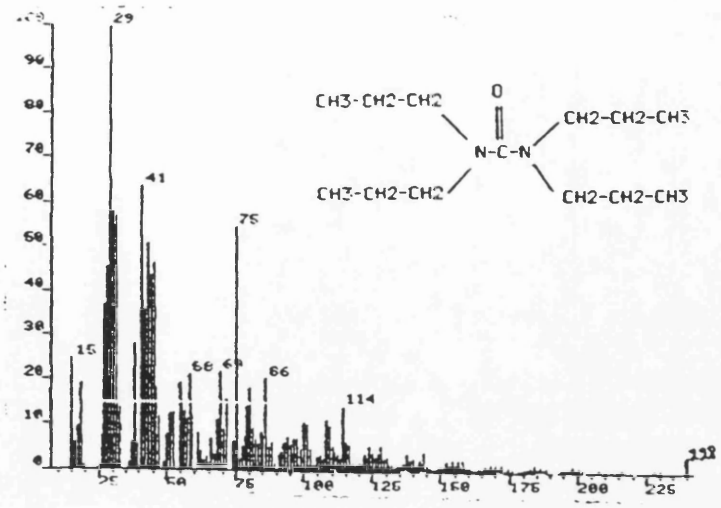


Fig 6.7 Representative mass spectra of EPTC photoproducts in water and in methanol at 254nm.




29
41
75
15
66
114
CH3-CH2-CH2
O
CH2-CH2-CH3
N-C-N
CH3-CH2-CH2
CH2-CH2-CH3

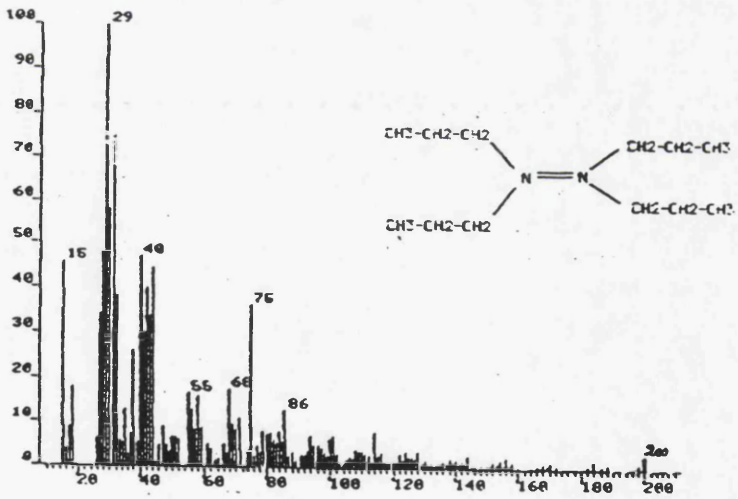
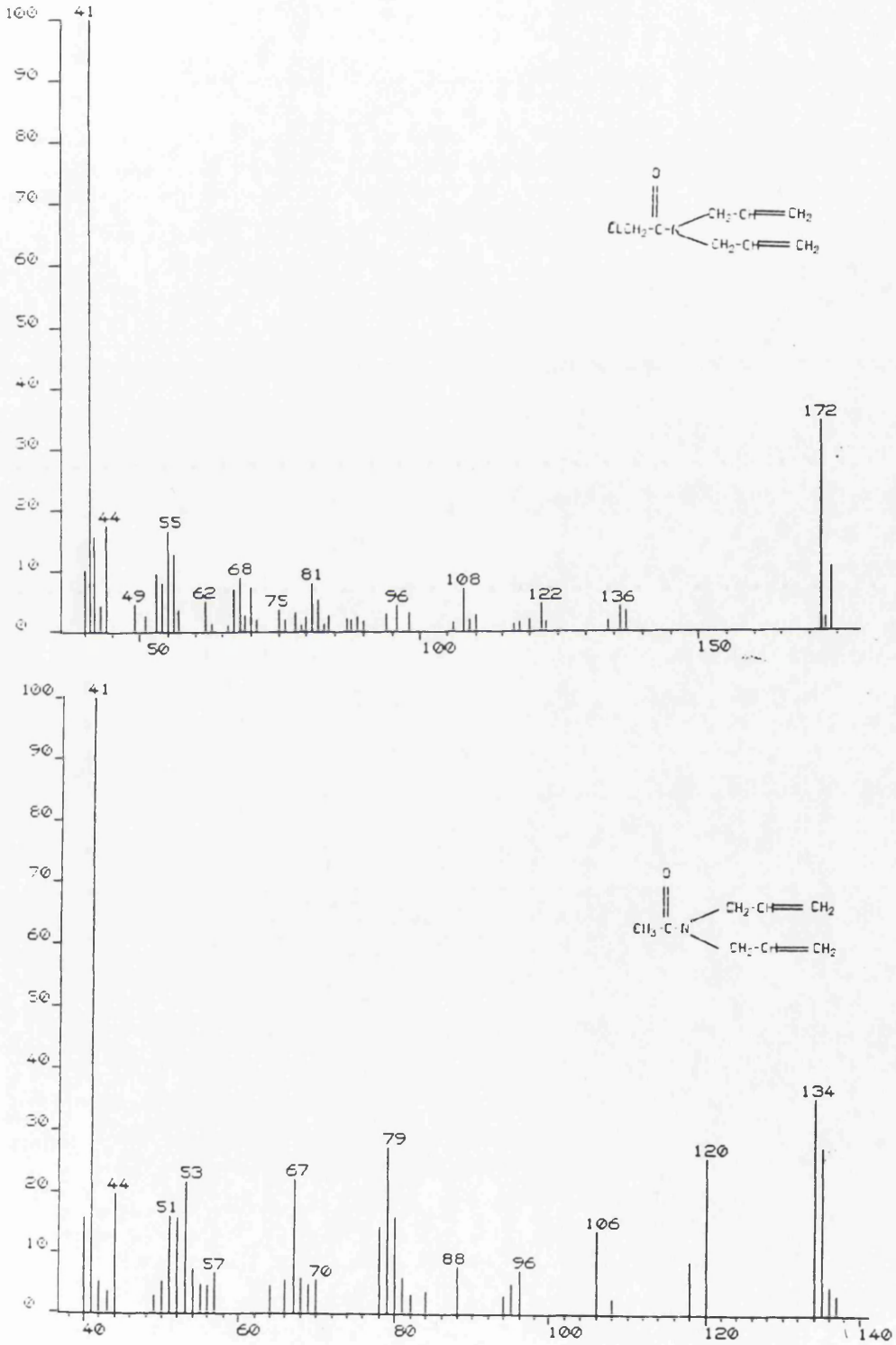
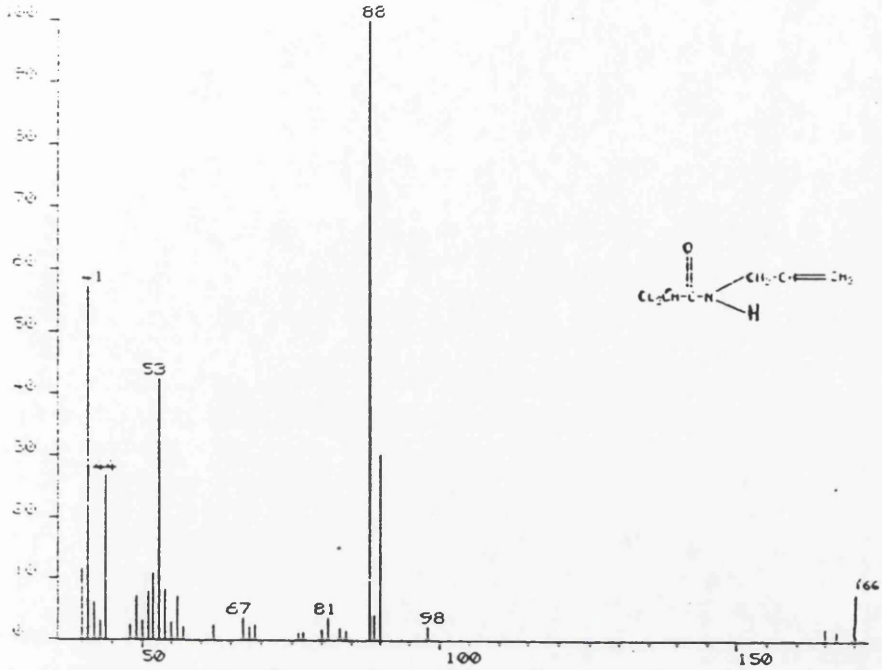

29
15
40
75
55
66
86
CH3-CH2-CH2
N
N
CH2-CH2-CH3
CH3-CH2-CH2
CH2-CH2-CH3

Fig 6.8 Representative mass spectra of Dichlormid photoproducts in water and methanol at 254nm.

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98
166
50
100
150

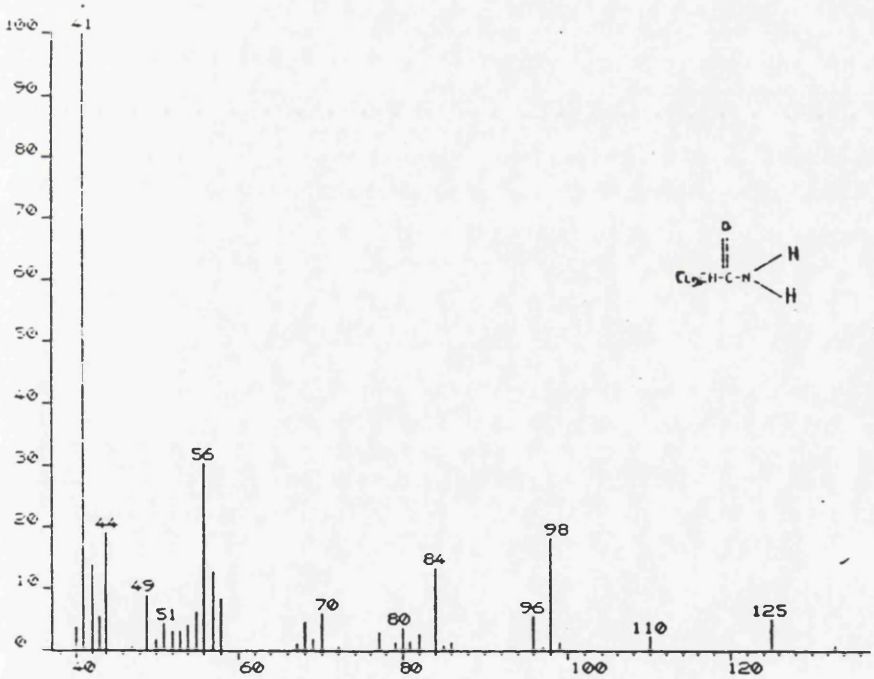

41
56
44
98
84
49
51
70
80
96
110
125
40
60
80
100
120

Fig 6.9 Chromatogram of EPTC photoproducts in water and in methanol at >290nm.

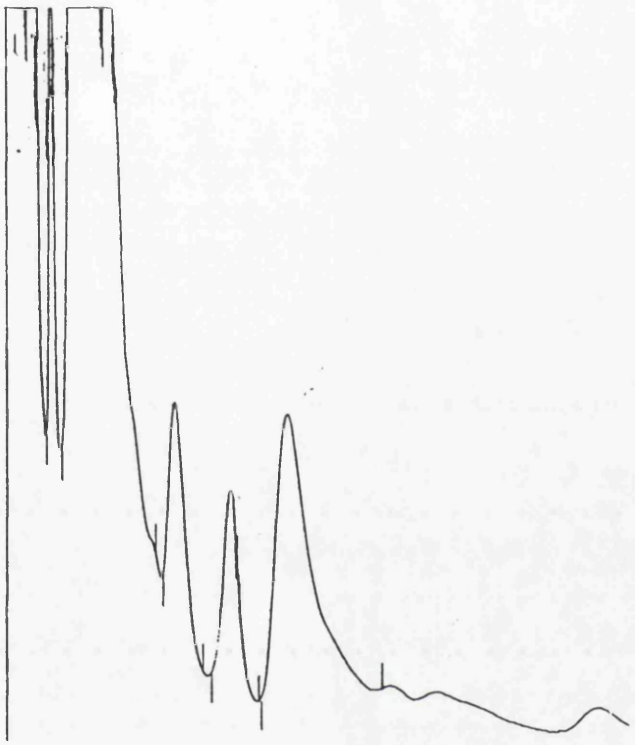


Fig 6.10 Chromatogram of Dichlormid photoproducts in water and in methanol at >290nm.

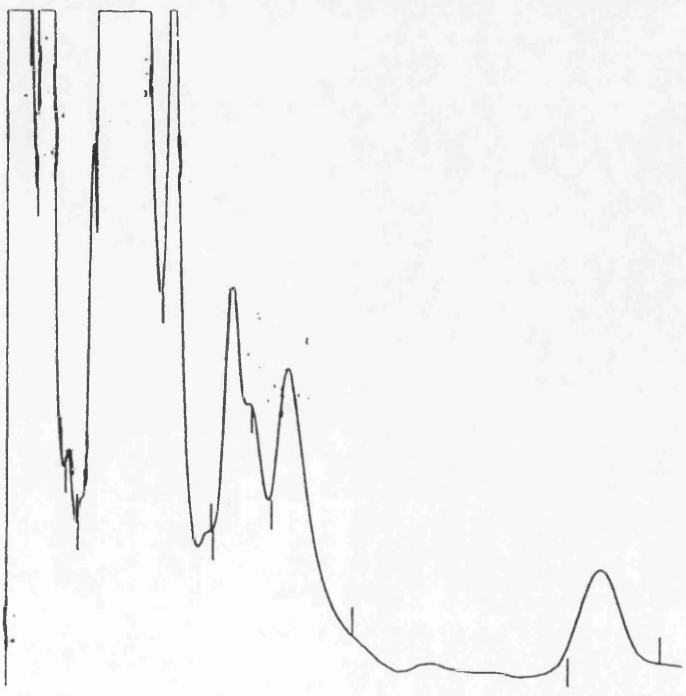


Fig 6.11 Chromatogram of EPTC photoproducts in water and in methanol at 254nm.

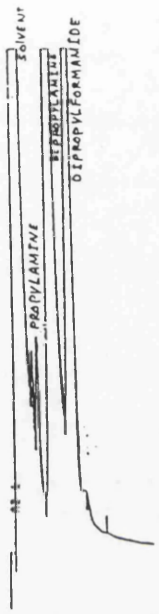


Fig 6.12 Chromatogram of Dichlormid photoproducts in water and in methanol at 254nm.

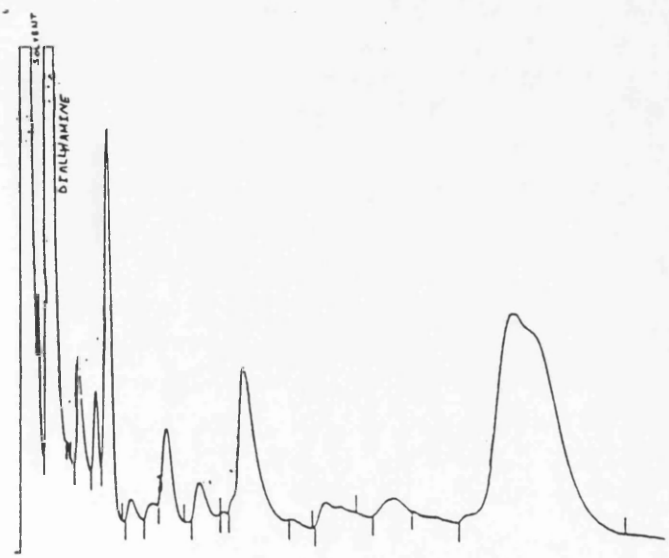
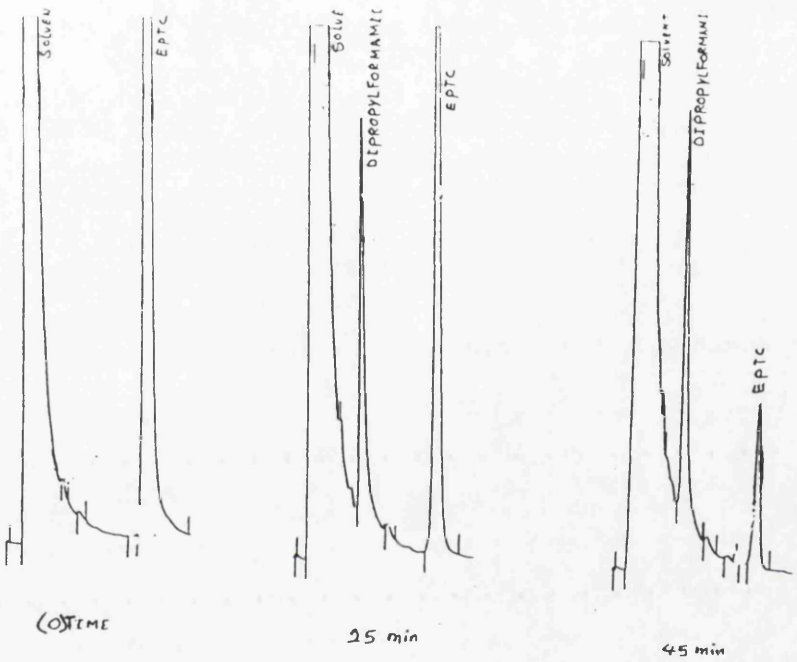


Fig 6.13 Rate of formation and disappearance of Dipropylformamide at 254nm.



6.7 Discussion :

6.7.1 EPTC rate of phototransformation :

The rapid photodegradation of EPTC at 254nm reflects its chemical structure. The energy available at 245nm is equivalent to 313 kcal/mole which is sufficient to overcome C-S, C-N and C-C bonds and hence the disruption of EPTC.

The significant difference in the rate of EPTC phototransformation between water and methanol is due to the nature of the media. Draper and Crosby (1981) indicated a difference in the mechanism of photooxidation between organic and aqueous solutions, in which singlet oxygen is involved in a low dielectric medium (organic medium), while in water a free radical mechanism due to formation of peroxide became important. Draper and Crosby(1984) reported the involvement of OH radicals in the photodegradation of thiocarbamates.

At >290nm, almost negligable degradation has been shown to take place. This is due to the relative low energy available at this range of wavelength which is not enough to break down the normal bonds in EPTC molecules. This situation is similar to sunlight conditions in which the presence of the ozone layer in the upper atomsphere absorbs sunlight spectrum below 290nm (Woodrow et al., 1983).

6.7.2 Rate of Dichlormid phototransformation :

Dichlormid underwent transformation more rapidly than EPTC when irradiated at 254nm. This is due to the high lability of the C-Cl bond to the breakdown. While at >290nm the low level of energy available was not sufficient to breakdown Dichlormid bonds hence it remained unchanged.

6.7.3 Effect of Dichlormid on EPTC rate of phototransformation :

As shown by the analysis of variance method, no significant effect of Dichlormid on the rate of phototransformation of EPTC was noted. This was due to the fact that 1) Dichlormid is degraded more rapidly than EPTC at 254nm, and 2) to its presence at low concentration compared with EPTC (1:10) (w:w) which is close to the ratio in the commercial formulation, and 3) to the fact that Dichlormid does not act as sensitizer or quencher.

6.7.4 EPTC pathways of phototransformation :

As shown in Fig 6.5, EPTC underwent transformation in a number of different ways; viz: 1) sulfoxidation with the formation of sulfoxide, 2) hydrolysis yielding Dipropylamine, Propylamine, and Dipropylformamide, 3) gradual dealkylation, and coupling of various radicals

from which a variety of dimers formed. This is in accordance with the vulnerability of the bonds involved in the EPTC structure to breakdown at 254nm. Previously EPTC sulfoxidation under sunlight was detected (Casida et al., 1975), as was its hydrolysis via irradiation with UV light in hexane (Demarco and Hayes, 1979). Other thiocarbamates were phototransformed through similar pathways (Draper and Crosby, 1981; Ruzo and Casida, 1985). The failure to detect EPTC-Sulfone may be due to its instability and its transformation into other products which has been reported for EPTC biological transformations in soil and water (Chen, 1991).

EPTC dealkylation was confirmed from the identity of Propylamine and from the identification of dimers, but whether dealkylation occurred before or after coupling the radicals needs further clarification EPTC dealkylation in mammals has been demonstrated (Chen and Casida, 1979).

At >290nm, although negligible degradation has taken place, some products were formed as shown from injection of the photolysate into the GC. These compounds were different from those formed at 254nm. The amount of energy available at each wavelength determines the nature of the phototransformation processes that take place. The actual identification of the nature of these products was difficult due to their low levels (as stated above).

6.7.5 Routes of phototransformation of Dichlormid :

Dichlormid underwent rapid transformation via irradiation at 254nm in both water and methanol. The separation and the identification of products formed revealed that routes of photolysis of Dichlormid were : dechlorination, dealkylation and hydrolysis. The lability of the C-Cl bond to photocleavage explains the rapid dissipation of Dichlormid .This is a common pathway of phototransformation of Chloroacetamides according to Pilmmer.(1970). Dechlorination was demonstrated as a route of transformation of Dichlormid in soil, water and mammals (Miallis et al., 1978).

The identity of Diallylamine was confirmed from a comparison with the standard using GC. Dichlormid hydrolysis is reasonable while this amount of energy available, and has been reported in other media (soil, plants, and mammals) (Miallis et al., 1978)

Dealkylation of Dichlormid was confirmed from identification using GC-MS spectra. This pathway is common for herbicide photolysis (Marchetere, 1988), and has been identified as a route of Dichlormid transformation in soil, plants, and mammals (Miallis et al., 1978).

At >290nm, several products were formed although only negligible transformation had occurred. The identity of these products are different from those

formed at 254nm. The difference in the amount of energy available at each wavelength accounted for this.

Effect of Dichlormid on EPTC Photoproducts :

An additional compound was formed from the phototransformation of EPTC and the Dichlormid mixture. This product resulted from coupling of radicals through S-N bond. The low level of Dichlormid and its rapid transformation compared with EPTC made its interference with EPTC photoproducts negligible. The biological/toxicological properites of this product needs further examination.

Conclusion :

EPTC, Dichlormid and EPTC + Dichlomid underwent rapid transformation via irradiation at 254nm. This is due to the lability of the bonds involved in their chemical structure to cleavage by the amount of energy available at this wavelength. Dichlormid underwent rapid transformation compared with EPTC. This is due to the susceptibility of the C-Cl bond to rapid cleavage. Dichlormid had no significant effect on the rate of phototransformation of EPTC. This may be due to its rapid degradation compared with that of EPTC hence it could not act as a sensitizer or as a quencher. This was also supported by the low level of Dichlormid present in the solution.

At >290nm which reflects artificial sunlight conditions, negligible transformation has taken place.

This does not reflect the actual field situation in which soil characterstics, such as H and individual components present might act as sensitizers, as well the effects of additives present in the formulation of the herbicide . The recent report about the significant inhibition extenders of the phototransformation of the thiocarbamate Butylate hence retaining its biological activity is an example (Zsolt et al., 1990). In this respect, another two contradictory factors exist. The method of application of EPTC and Dichlormid-soil incorporation would reduce the effect of the sunlight, while their presence in the field for a longer period compared with this study might increase the effect.

The biological/toxicological properties of the photoproducts needs further study particularly the dimeric compounds. Reports about the mutagenic/carcinogenic properties of photoproducts of thiocarbamates have been published(Schuphan et al., 1981; Woo and Acros, 1989) .

In connection with the mechanism of action of Dichlormid, no direct such relation could be drawn from this study, however most safeners exert their safening action by acting within the plants. This study would not suggest otherwise.

Chapter Seven

Concluding Remarks and Research Prospects

The project was carried out to contribute to studies on the mechanism(s) of action of herbicide safeners and their impact on the development of more effective and selective safeners. The effect of the safener Dichlormid and analogues on the herbicide EPTC in maize has been taken as a particular reference.

Several possible effects were proposed and experimental techniques and analytical methods were designed to carry out the study. Various restrictions and limitations were identified and suggestions made for minimizing their effects and for further work.

The hypothesis that safeners interfere with the pattern of uptake and/or translocation of chemicals is still a controversial issue (Ekler and Stephenson, 1991; Yuyama and Shirakura, 1991; Viger et al., 1991; Wehtje et al., 1991; Fuerst and Lamoureux, 1992).

The technique employed for the study, the analytical method, plant type, part of the plant used in the study and the environmental conditions all have a significant effect on the results.

In this study, nutrient solution was used as a general medium of the study, the advantage of using this method being the availablity of both the safener and the herbicide to the plant since both are fully soluble in

water under these conditions and avoid interaction with soil constituents.

HPLC was used as an analytical method to minimize the pre analysis steps that cause a loss of the highly volatile herbicide (EPTC). This method is not the most appropriatetechnique, but the absence of radiolabled chemicals made it a reasonable alternative.

The results indicated no significant effect of Dichlormid on EPTC uptake via maize roots, this is reflected in the general pattern of the results previously reported. The lack of radiolabled chemicals was an obstacle to monitoring EPTC translocation within the maize plant and the possible effect of Dichlormid.

A tudy of the Dichlormid effect on EPTC volatility from nutrient solution was considered to give an indication of the possible interference of Dichlormid with EPTC uptake through the maize shoot which is thought to be the main site of uptake and action of both EPTC and Dichlormid. The results indicated no significant effect, but the lack of a direct method to carry out such a study makes the results less convincing. This study is of particular importance, as EPTC is a highly volatile herbicide and readly enters the vapour phase in which chemicals present in its formulation could alter its pattern of movement into the vapour phase and hence the degree of injury to the plants and hence its biological activiy.

The capacity of plants to detoxify certain herbicides by specific biochemical reactions has been recognized as an important process contributing to the selectivity of herbicides, thus it is reasonable to expect that the protective action of safeners may be related closely to the physiological or biochemical processes contributing to the moderate tolerance of the protected cereal crops to the antagonized herbicides. However the botanical specificity of the commercial safeners (only cereal crops are protected) is still a puzzling issue that remains to be solved. Safener induced enhancement of herbicide detoxification in protected plants seems to be the major mechanism involved in the protective action of the currently developed safeners. Their effects are on specific systems involved in herbicide detoxification (e.g Glutathione and Mono-function oxidase systems). The current safeners apparently affect glutathione systems either by elevating GSH content and/ or by enhancement of the activity of GST enzymes.

In the study, an attempt was carried out to examine the effect of the safener Dichlormid on EPTC metabolism in maize tissues through the determination of the levels of the metabolites (Glutathione and Cysteine Conjugates). Particular attention was given to the sulfoxidation process of EPTC as a pre step for its detoxification. Synthesis of the metabolites using different forms of EPTC (Sulfide,

Sulfoxide, or Sulfone) gave indications of the significance of this process.

The results have revealed that the true form of EPTC able to form conjugates was the sulfoxide form with cysteine and the sulfone form with glutathione (GSH) under the conditions used. This is of particular importance in indicating the significant of EPTC sulfoxidation to its detoxification and its selectivity and to the role of MFO enzymes in catalyzing the sulfoxidation and the possible interaction with Dichlormid. This does not mean under different conditions similar results would be obtained. HPLC was used as an analytical method. This was adequate for the separation of EPTC, Dichlormid, EPTC-cysteine conjugate and EPTC-glutathione conjugate standards. However, the detection of the metabolites or the parent herbicide in maize tissue was not successful. This blocked the main aim of the study which was to examine the Dichlormid effect on EPTC metabolism. This may have been due to several reasons most notably the lack of accuracy due to the absence of a specific detection system resulting from the unavailability of radiolable chemicals. Studies carried out in this field used radiolable chemicals with radioanalytical methods. Further studies in this field are required with particular emphasize on the role of EPTC sulfoxidation to its selectivity and to the mechanism of action of Dichlormid. Using a direct assay in vivo with EPTC as a substrate would be the ideal

method to examine closely the role of MFO in its catalysis and the possible enhancement by the safener Dichlormid. Previously, indirect methods were used by applying MFO inhibitors in conjunction with the safener and the herbicide.

Structure-activity relationship studies help to optimize chemical properties of both the herbicides and the safeners and to understand their biological mode of action. Several examples indicated close similarity between chemical structures possessing both herbicidal and safening properties. In some cases this differentiation was marginal as shown in crops pretreated with low herbicide doses leading to safening effects. In other examples, however, structural optima for safening and herbicidal efficacy can be clearly differentiated.

The knowledge of the effects of chemical structural modifications on the biological activity of herbicide safeners has expanded greatly in recent years. The importance of structural similarity between a herbicide and its safener has been shown to be advantageous but not absolutely necessary for efficient protective action. Recent studies demonstrated the requirement for at least one electrophilic site in molecules intended for herbicide safeners which will be available for possible nucleophilic displacement reactions. Others studies indicated the essentiality of specific

functional group(s) for unknown biological reactions required for safener action, while others laid emphasis on the whole structure of the safener as a biologically active chemical, One of the major questions that still needs further clarification is the possible role of safener conjugates with glutathione and /or other endogenous sulfhydryl containig molecules in safener action and the elucidation of structural requirements for optimum activity of such conjugates.

Although detailed examinations have been published on structure-activity relationships of herbicide safeners, only a few data are available on chemical reactivity-safener activity relationships. Chemical reactivity of acetamide type compounds as well as their safener activity against thiocarbamate herbicides change with the number of chlorine substituents in the order : non-chlorinated< monochloro< dichloro. Also acetals and ketals involving a dichloromethyl group on their central carbon atom were found to be active or highly active as safeners for maize against thiocarbamte and chloroacetanilide herbicide injury. A mechanism for the biotransformation of these compounds as prosafeners to the actual dichloroacetic ester safeners has been proposed

Structure-activity relationship studies on Dichlormid and analogues yielded contraversial results; some indicated the essentiality of specific group (s)

(e.g. Dichloroacetamido or Dichloroaceto moities), while others emphasized the structural similarity between Dichlormid and EPTC. In light of these results coupled with the need for more understanding of Dichlormid mechanism of action, an attempt was carried out to examine the safening activity of Dichlormid analogues(Diallylamine, Dichloroacetylchloride and N,N-diallyl-2-chloroacetamide) against EPTC injury to maize. Injury symptoms, plant height and plant fresh weight were taken as parameters in judging the safening effect.

The results revealed that various degrees of safening activity were exerted and elimination of some of the injury symptoms of thiocarbamates was achieved: Diallylamine mainly prevented the development of green-dark leathery leaves, reduced leaf and shoot twisting and looping and wrinkleand caused a 22.5% reduction in the inhibition of maize shoot growth caused by EPTC. Dichloroacetylchloride eliminated leaf and shoot twisting and looping and reduced maize shoot inhibition by 7.1%, while CDAA completely eliminated EPTC injury symptoms and caused a reduction in plant height inhibition only by 28.2%, while Dichlormid caused 95.96% reduction. The combination of Diallylamine and dichloroacetylchloride prevented leaf and shoot twisting, and caused a 18.3% reduction of EPTC inhibition of maize height.

These results indicated that more than one bichemical and/or physiological process was affected by

EPTC and Dichlormid. No specific functional group(s) responsible for the safening activity was identified, and Dichlormid as one unit was required for complete safening activity.

In this respect, more work to examine other possible functional group(s) and combinations is required. This type of work must be carried out under field conditions. In this respect Jablonkai and Dutka (1989) referred to the relation between molecular structure and N-alkylating ability of chloroacetmides where their phytotoxicity is charcterized primarily by their reduced capability to conjugate with GSH, while transacylation reaction according to Dutka.(1991) is involved in Chloroacetamide biological activity hence the Dichloroacetyl moeity may be responsible for the safening activity.

The phototransformation of herbicides by sunlight has rapidly become an integral part of studies concerning the environmental transformation of pollutants present in the environment and has become an essential element in the registration of new agrochemicals. Based on this, more accurate and quantitative analytical methods were developed.

The presence of additives in the herbicide formulation may significantly alter the pattern of phototransformation. Studies on the phototransformation of the safener Dichlormid usually applied in EPTC formulation, and the effects on EPTC phototransformation

and the relation to mechanism of action and environmental considerations has not been carried out. Based on this, the phototransformation of EPTC, Dichlormid, and the mixture of both in aqueous and non-aqueous solutions under UV light at 254nm and >290nm (artificial sunlight) were examined.

The results revealed that both EPTC and Dichlormid underwent rapid phototransformation when irradiated at 254nm in water and in methanol and Dichlormid had no significant effect on EPTC rate of photolysis. Dichlormid underwent photolysis more rapidly than EPTC in both media. At >290nm which reflects the sunlight conditions, negligible degradation of both EPTC and Dichlormid took place.

Separation and identification of a complex mixture of products formed via irradiation at 254nm revealed the presence of Azo compounds which have toxicological properties. Further work is required exploring the toxicological and biological propreties of EPTC and Dichlormid photoproducts. Dichlormid did not interfere significantly with the nature of the photoproducts of EPTC. Although negligible degradation has occured at >290nm, various products were formed which were completely different from those formed at 254nm.

In assessing the results, the field factors(e.g. sensitizing materials, soil characterstics) and other components in the herbicide formualtion should be considered, also a study of EPTC phototransformation in

the vapour phase would be of particular importance as it is a highly volatile herbicide and readily transferred into the vapour form.

The mechanisms of action of safeners has not been fully elucidated. To carry out further work, several facts should be considered. Safeners are most effective when applied prior to or simultaneously with the herbicides whose injury they prevent. Safeners exhibit a high degree of botanical and chemical specificity in protecting only certain cereal crops against injury caused from specific classes of herbicides, and the protected cereal crops are moderately tolerant to the antagonized herbicides.

The chemical and the botanical specificity of the currently marketed safeners indicate that safeners do not act by a single mechanism. A series of multilevel interactions between safeners and antagonized herbicides appear more likely as an explanation for the protective action of herbicide safeners. Some safeners are also likely to act by different mechanisms depending on the nature and specificity of particular crop-herbicide-safener combinations.

In general, safeners are believed to act either as bioregulators influencing the amount of a given herbicide that reaches its target site in an active form or as an antagonist of herbicidal effects at a common site of action. Although safeners can compete with

herbicides at common target sites, such a mechanism seems unlikely. The ratio of safener to herbicide doses in prepackaged formulated mixtures of herbicides and safeners range from 1 : 6 - 1 : 30. Such ratios do not favour the antagonist theory of safener action since very little safener will be available at the site of action to compete with the herbicide, which would be present at considerably higher concentration. A better understanding of the mechanisms of action of current safeners and herbicides will allow more positive attempts towards increasing the number of situations in which crop safeners for herbicides could be used. Advancement in the molecular biology techniques and the potential involvement of "gene activation" and gene manipulations in the molecular action of herbicide safeners undoubtedly would provide a key for the elucidation of the exact mechanisms of the protective action of safeners.

In this field, the work unravelling the mechanisms of action of herbicide safeners will continue to challenge the ingenuity of investigators in the future. Selected areas that need to be investigated further include :

Further investigations on the mechanisms of safener action should be extended to the molecular level, we must determine whether and how safeners affect gene expression in plants and whether these regulatory effects of safeners are related to the biochemical and

physiological effects exerted by the antagonized herbicides.

Further studies on the physiological, biochemical and molecular interactions between herbicides and safeners on plants should establish which effects of the safeners and herbicides are of primary or secondary importance and whether the secondary effects are totally unrelated to the safening function.

What is the physiological or biochemical significance of hormonal interactions with selected safeners? Do safeners mimic the effects of endogenous plant hormones in protecting cereal crops from herbicide injury?.

What is the role of glutathione reductase (GR) enzyme in the action of safeners? Is this enzyme activated or induced by these safeners?.

Do safeners conjugate with glutathione (GSH)? Is this conjugation catalyzed by specific glutathione -S-transferase enzymes (GST) or by the same GST enzymes that catalyze the conjugation of the herbicides?.
Do safeners interfere with the feedback inhibition of glutathione biosynthesis and what is the relation of that to the increase in glutathione (GSH) content in plant?.

How stable are the safener-GS conjugates? Are they further catabolized in the same way as GS-conjugates of herbicides?.

Do safeners affect sulphate uptake and assimilation by plants and then transformation into cysteine which is ultimately incorporated into glutathione (GSH)?.

What is the role of Mixed-function oxidases in the metabolism of herbicides whose effects are counteracted by safeners? Are they induced by safeners? Using direct methods with herbicides as substrates will be the ideal method.

Are other oxidative enzymes such as peroxygenases, peroxidases, lipooxygenases, etc... involved in the metabolism or action of selected herbicides, or herbicide safeners? Recent reports confirmed this involvment (Blee, 1991).

If safeners act by more than one mechanism, what determines the sequence of steps or events involved in the safener action and what triggers the function of a particular mechanism?.

What is the impact of herbicide safeners on herbicide metabolism by soil microorganisms, especially in soils where high herbicide residues may exist? What is the effect of that on the amount of the herbicide available to the plant , as well as the influence on the carry over problems, their effect on herbicide enhanced degradation and to environmental considerations?.

What is the effect of safeners with mycoherbicides and other naturally occuring phytotoxins? Do safeners protect crops against plant pathogen attack?.

How is safener action affected by surfactants, adjuvants, extenders and other additives that are commonly found in herbicide formulations?.

New aspects of safener structure-activity relationship studies are required to be explored, particularly by examining the safening action of specific functional group(s).

Further work is required on the effect of safeners on herbicide phototransformation, particularly under field conditions using the commercial formulation. Recent reports about the significant inhibition of the thiocarbamate Butylate photolysis by extenders hence retaining its biological activity is an encourging feature (Zsolt et al., 1990).

Effect of the safeners on herbicide volatility, and hence its uptake from the vapour phase requires further work, particularly with the volatile herbicides where the shoot is the main site of uptake.

Interference of safeners with herbicides adsorption-desorption phenomena and the effect of that on the amount of herbicide available to the plant need to be studied.

Studies on the safener and the herbicide at the molecular levels using CAMM would provide clues to more understanding of the mechanism of action of the safener.

Further research is definitely needed to provide answers to these and other questions that are currently unanswered or, at best, poorly understood.

As related to the development of herbicide safeners, prosafeners have a promsing future. They are of great importance because they provide an alternative way for increasing the selectivity of safeners. Differences in the ability of crop and weed plants in biotransforming prosafeners to actual safeners could be exploited to advantage and enhance the practical usefulness of this concept. In addition, the bioavailability to plants and the transport mobility in the biophase of prosafeners may be prefered to that of safeners for improving the protective activity. On the other hand, highly effective safeners which are very reactive chemically are not usable in practice since they are consumed partially or completely before reaching the sensitive active site(s) of action. However, these chemicals can be transported at critical concentrations to their biological target in the form of prosafeners.

Microbial safeners exploiting the herbicide degradative potential of soil bacteria and fungi which colonize the roots of desirable crops may be developed in the near future. Alternatively, microbial safeners could result from the manipulation of endophytic bacteria colonizing the transport system of the plant. Such microbes could be manipulated genetically to synthesize appropriate substrates or enzymes known to serve as target sites for specific herbicides.

A further challenge in this field, is the development of safeners for dicotyledonous crops. Some studies have shown promise. There is not yet a safener that is very close to commercial use on dicotyledonous crops and despite extensive research on the mode of action of safeners, few investigators have even offered hypothetical explanations as to why it seems so easy to protect monocots as compared to dicots from various herbicides.

The other challenge is the development of safeners for postemergence herbicides, with the expected trend toward greater use of postemergence herbicides. Increased efforts to develop postemergence safeners would certainly seem to be warranted, particualary for Dicotyledonous crops.

The growing environmental and toxicological concern about the Dichloroacetamide type herbicide safeners recently promoted a search for alternative safener types. Hopefully these types should fulfil the recent environmental regulations. The safening effect of Dithiocarbamate derivatives is an example (Matolcsy et al., 1991).

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