STUDIES ON THE MECHANISM OF THE SELECTIVE ACTION OF CHLORTOLURON IN CEREALS AND CEREAL WEEDS

A Thesis submitted by

PATRICK JOSEPH RYAN

a candidate for the degree of

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in

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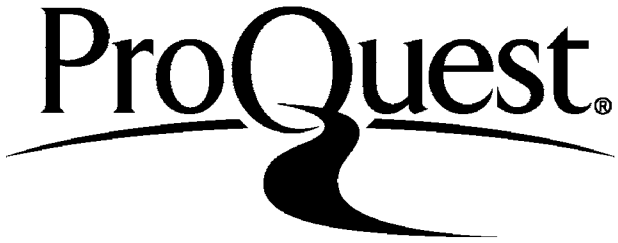
Department of Biochemistry
Royal Holloway College,
University of London,
Egham,
Surrey.



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Dedicated to Dan Ryan, my first teacher.

A B S T R A C T

The studies reported in this thesis have been primarily concerned with the mechanism of the selective action of the substituted phenylurea herbicide chlortoluron (N'-(3-chloro-4-methylphenyl)-N,N-dimethylurea) in cereals and monocotyledonous cereal weeds. The introduction reviews relevant aspects of photosynthetic electron transport, the use of the substituted phenylureas as herbicides and the mode of action of the substituted phenylureas with reference to structure/activity relationships. In addition, their selective properties are discussed particularly in relation to their metabolic fate in biological systems.

Studies on the Hill activity of chloroplasts isolated from resistant and sensitive plants and incubated in vitro with chlortoluron or the related phenylurea isoproturon (N'-(3-chloro-4-isopropylphenyl)-N,N-dimethylurea) demonstrated a similar inhibition of DCPIP photoreduction in each case. Examination of the Hill activity of chloroplasts isolated from plants treated with either chlortoluron or isoproturon, however, demonstrated a greater inhibition of DCPIP photoreduction in sensitive compared to resistant plants.

Studies on the recovery of photosynthetic activity both in whole plants and in leaf segments following inhibition by prior treatment with chlortoluron or isoproturon demonstrated a greater ability to restore oxygen evolution in the case of resistant plants, suggesting a more rapid detoxification of the herbicides in these varieties.

Metabolism studies made using (carbonyl-^{14}C)chlortoluron indicated degradation by both N-demethylation and ring-methyl oxidation pathways, followed by conjugation. The ring-methyl oxidation pathway predominated in the resistant cereal varieties to a greater extent than in the sensitive cultivars. In contrast, the N-demethylation pathway constituted the major metabolic route in the case of the cereal weeds. Studies on the inhibition of the Hill reaction by the metabolites of chlortoluron indicated that the initial product of N-demethylation retained significant phytotoxicity, whereas all products of ring-methyl oxidation were essentially non-phytotoxic. Further metabolism studies made using (carbonyl-^{14}C)diuron (N'-(3,4-dichlorophenyl)-N,N-dimethylurea) and (carbonyl-^{14}C) N'-(3-methyl-4-chlorophenyl)-N,N-dimethylurea emphasised the importance of both the presence and the positioning of the ring-methyl group in the selective action of the substituted phenylureas. Studies on the polar conjugated metabolites of chlortoluron revealed that the glycone moiety was β-D-glucose in each case. In vitro experiments indicated that chlortoluron metabolism was achieved by a microsomal-linked system requiring molecular oxygen and NADPH as co-factors.

Experiments on the absorption of chlortoluron from both soil and nutrient media indicated a greater uptake of the herbicide by the cereal weeds compared to the cereal varieties examined. A sub-cellular organelle localisation study of absorbed chlortoluron and its metabolites demonstrated the association of significantly larger amounts of phytotoxic material with the chloroplasts of sensitive compared to resistant plants. In addition,

post-mitochondrial fractions prepared from resistant plants contained correspondingly larger quantities of non-phytotoxic metabolites.

The various experimental results are discussed in terms of their significance in accounting for the observed differences in the response of cereals and cereal weeds to chlortoluron.

CONTENTS

INTRODUCTION

Herbicides

In Britain in the 19th Century each farm worker produced enough food for four people. Today that figure has risen to nearly sixty (Broadbent, 1980). A significant factor in this increase has been the introduction of herbicides. Although for many hundreds of years various industrial wastes had been used to destroy vegetation, deliberate chemical weed control can be considered to have started with the application in 1896 of Bordeaux mixture which killed yellow charlock (Sinapis arvensis L.) amongst cereals (Brian, 1976). This was rapidly followed by the use of weedkillers containing sulphates of ammonia, zinc and iron and later by arsenates, various acids, borate compounds, chlorates, and more recently ammonium sulphamate.

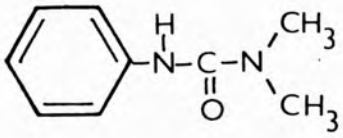
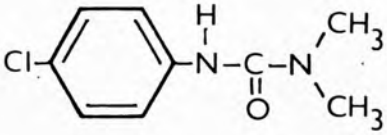
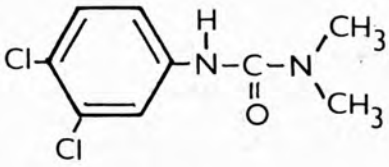
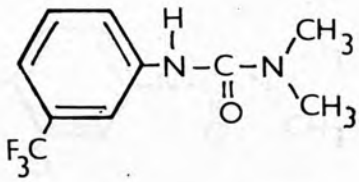
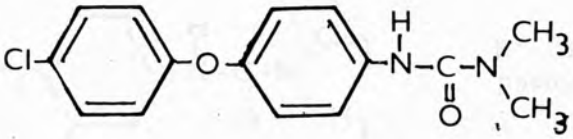
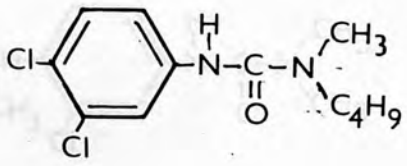
The first organic chemical to be introduced as a herbicide was the sodium salt of 2-methyl-4,6-dinitrophenol (DNOC) the forerunner of a whole family of herbicidal nitrophenols in use to the present day (Brian, 1976). The second world war delayed the publication of the discovery that synthetic plant hormones could be used for weed control. However, from 1945 onwards phenoxyalkane carboxylic acids such as 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) were introduced and resulted in a rapid expansion in the use of herbicides during the post-war period. At about this time the first benzoic and phenylacetic acids and amides were introduced, followed in the 1950's and 1960's by the substituted phenylureas, triazines, haloaliphatic acids, nitriles, thio- and dithiocarbamates, pyridines and uracils.

The importance of many of the herbicides in use at the present time is not due to their general toxicity but rather to their ability to destroy weeds without harming the crop. Herbicidal selectivity can thus be defined as the ability of a compound to either kill or to inhibit the growth of weeds whilst leaving the crop relatively unharmed. Despite twenty five years of research our understanding of the behaviour, action and fate of many such herbicides is still far from complete. In addition, the realisation that certain weed control chemicals used in the past can persist in the environment for many years emphasizes the need for more thorough understanding of the interactions of herbicides in the environment.

The Substituted Phenylureas

The first urea to be used for weed control was N,N-bis(2,2,2-trichloro-1-hydroxyethyl)urea (DCU) developed by Thompson, Swanson and Norman (1946) for use as a pre-emergent treatment for the control of grasses. This compound, however, possessed very limited selectivity and was quickly superseded by more toxic or selective herbicides. The importance of the substituted phenylureas as herbicides dates from the discovery of Bucha and Todd (1951) that N'-(4-chlorophenyl)-N,N-dimethylurea (monuron or CMU), was effective in controlling many grass species whilst at the same time exhibiting some selectivity. Since these early discoveries many urea derivatives have been screened for phytotoxicity, the structures of a selection of these compounds which have ultimately been marketed as herbicides are presented in Table 1. All effective compounds generally conform to the formula presented in Fig. 1 in which R_1 and R_2 are commonly alkoxy or alkyl groups and R_3 and R_4 alkyl, alkoxy or halogen groups.

TABLE 1: SUBSTITUTED UREA HERBICIDES

STRUCTURAL FORMULA	CHEMICAL DESIGNATION	COMMON NAME
	N'-phenyl-N,N-dimethyl urea	Fenuron
	N'-(p-chlorophenyl)-N,N-dimethylurea	Monuron
	N'-(3,4-dichlorophenyl)-N,N-dimethylurea	Diuron
	N'-(m-trifluoromethyl-phenyl)-N,N-dimethylurea	Fluometuron
	N'-(4-(p-chlorophenoxy)-phenyl)-N,N-dimethylurea	Chloroxuron
	N'-(3,4-dichlorophenyl)-N-butyl-N-methylurea	Neburon

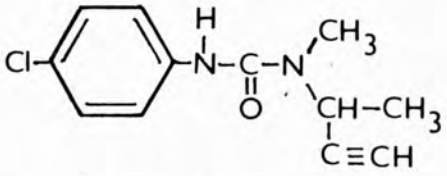
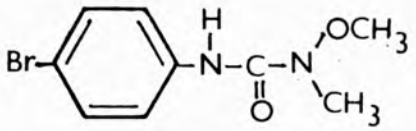
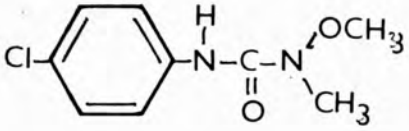
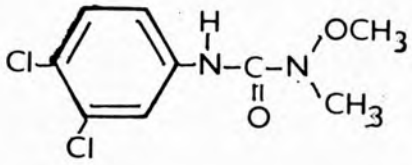
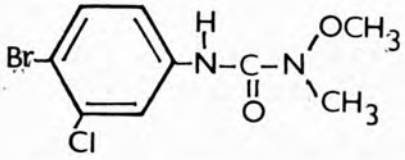
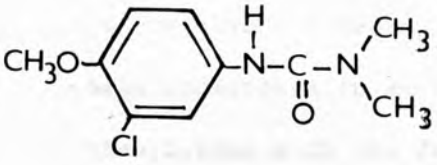
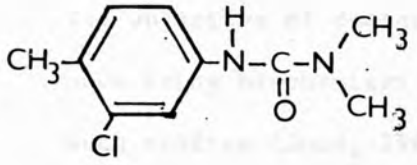
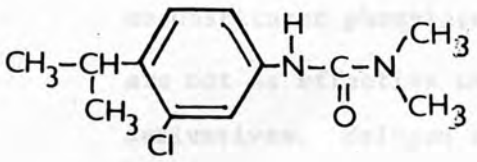
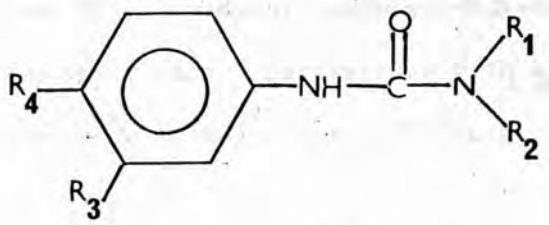
STRUCTURAL FORMULA	CHEMICAL DESIGNATION	COMMON NAME
	N'-(p-chlorophenyl)-N-methyl-N-(1-methyl-2-propynyl)urea	Buturon
	N'-(p-bromophenyl)-N-methoxy-N-methylurea	Metobromuron
	N'-(p-chlorophenyl)-N-methoxy-N-methylurea	Monolinuron
	N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea	Linuron
	N'-(3-chloro-4-bromophenyl)-N-methoxy-N-methylurea	Chlorbromuron
	N'-(3-chloro-4-methoxyphenyl)-N,N-dimethylurea	Metoxuron
	N'-(3-chloro-4-methylphenyl)-N,N-dimethylurea	Chlortoluron
	N'-(3-chloro-4-isopropylphenyl)-N,N-dimethylurea	Isoproturon

Fig. 1:

(5)



The first important substituted phenylureas possessed a high order of inherent toxicity and consequently found use mainly as total weed-killers. In the case of phenylureas developed in more recent years, however, phytotoxicity has tended to be reduced in favour of greater selectivity. Previously well documented observations that phenylureas were capable of only marginal selectivity were challenged by the introduction by Ciba-Geigy A.G of chlortoluron (N'-(3-chloro-4-methylphenyl)-N,N-dimethylurea) which displayed a high order of selectivity in cereals. Thus the relatively primitive substituted phenylureas of thirty five years ago have now largely given way to more modern analogues with enhanced selectivity though often more complex structures.

Structure-Activity Relationships of Substituted Phenylureas Inhibiting Photosynthesis

That the substituted phenylureas are potent inhibitors of photosynthesis is now beyond reasonable doubt, (Moreland, 1967). Numerous studies have been undertaken in an attempt to relate the structure of the substituted phenylureas with the degree of inhibition of the Hill reaction, often with the objective of designing herbicides by reference to a knowledge of underlying biochemical principles rather than by empirical methods. Such studies (Good, 1961; Buchel, 1972) have generally indicated that unsubstituted phenylureas such as fenuron (N'-phenyl-N,N-dimethylurea) are not as effective in inhibiting the Hill reaction as their substituted derivatives. Halogen substitution, in particular at R_3 and/or R_4 (Fig.1)

as in diuron (N'-(3,4-dichlorophenyl)-N,N-dimethylurea, or DCMU) greatly enhanced phytotoxicity. Derivatives with alkyl, alkoxy or nitro substitution were usually less inhibitory.

In order to inhibit the Hill reaction in vivo such compounds must clearly penetrate to the active site within the chloroplast, and assume the spatial configuration necessary for inhibition. Properties which may be of importance in this respect are partition characteristics (hydrophilic: lipophilic balance), steric relations, resonance and keto-enol tautomerism. A requirement for a lipophilic aromatic ring was demonstrated in a study by Hansch and Deutsch (1966) who further postulated that the substituted phenylureas act in a very lipophilic compartment within the chloroplast, with the role of the side chain considered to be related to the promotion of electron release to the carbonyl group. Although there also appears to be a correlation between the acidity of phenylureas and their toxicity to the Hill reaction, Camper and Moreland (1965) found that this relationship only holds true within groups of closely related compounds.

Moreland and Hill (1963), and Moreland (1969) have suggested that the substituted phenylureas possess the capacity for undergoing keto-enol tautomerism. In addition, resonance interaction between the amide nitrogen and the aromatic ring system would decrease the electron density at the nitrogen atom thereby reducing its attraction for the amide hydrogen. The net effect of these changes would be to lower the electronegative charge thus increasing the possibility of hydrogen bonding to the active site in the chloroplast.

The results of studies such as those discussed above can be explained in terms of a multipoint attachment of the herbicide at or near the active site involving hydrogen bonds. The requirement for a free or sterically unhindered amide or imino hydrogen has been demonstrated on several occasions (Camper and Moreland, 1965). In addition changes in molecular structure which reduce the possibilities for hydrogen bonding also appear to decrease inhibitory potency (Hansch, 1969; Moreland, 1969). The observed reversible nature of the inhibition lends further support to the belief that only weak bonds are involved in the attachment of these inhibitors to the site of action. Hansch and Deutsch (1966) concluded, however, from the results of a mathematical approach based on calculation of substituent constants and regression analysis that specific hydrophobic bonding may be responsible for inhibitor binding and that the lipophilic nature of the molecule was essential only in as much as it facilitated penetration of the chloroplast. These workers postulated that the most likely mechanism of inhibition following attachment of an inhibitor via hydrophobic and hydrogen bonds could well involve the induction of an allosteric effect, which results in a conformational change in a critical protein or lipoprotein structure involved in photosynthetic electron transport.

The Light Reactions of Photosynthesis

Since a knowledge of the mechanisms of the light reactions of photosynthesis is a prerequisite for the understanding of the mode of action of the substituted phenylurea herbicides, and data is presented in the present thesis on the effects of chlortoluron (N'-(3-chloro-4-methylphenyl)-N,N-dimethylurea and isoproturon (N'-(3-chloro-4-isopropylphenyl)-

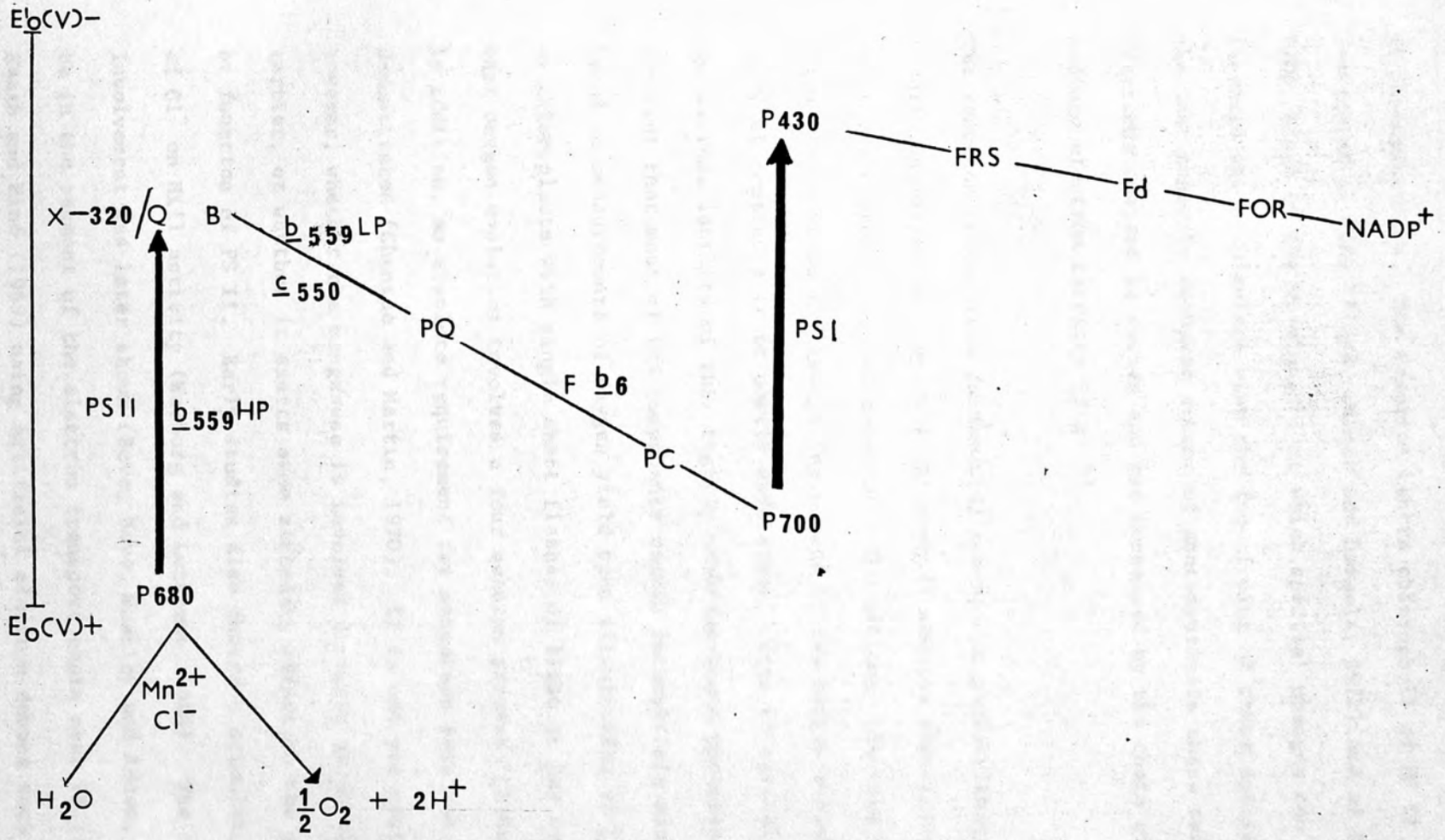
N,N-dimethylurea) on photosynthetic electron transport, a short discussion of the relevant aspects of photosynthesis would appear appropriate.

Photosynthesis involves the absorption of light energy which supports an electron transport pathway in chloroplasts coupled to ATP and $NADPH_2$ production and yielding O_2 as a by-product.

In view of the enormous literature which exists in the area of photosynthesis research it would seem reasonable to state without detailed documentation, a number of generally accepted premises, and to reserve a more in depth consideration to those aspects which bear directly on investigations described in this thesis. Many excellent review articles have appeared in the literature, in particular those by Boardman (1968), Owen (1971), Cramer and Whitmarsh (1977), Trebst and Avron (1977) and Emami-Saravi (1979). The path of photosynthetic electron transport generally accepted by many workers in this field is shown in Fig. 2.

Photosynthesis represents a transfer of electrons against a thermodynamic gradient from a donor to an acceptor (water and $NADPH^+$ respectively in the case of higher plant and algal photosynthesis). A variety of electron carriers participate as intermediates in this process, e.g., plastoquinones, cytochromes, plastocyanin, non-haem iron proteins and flavoproteins. Light energy enters the system at two points in green plants. One of these is located on the water oxidation side (PS II) of photosynthesis, and the other on the pyridine nucleotide reduction side (PS I)

Fig. 2: Diagrammatic representation of photosynthetic electron transport



FRS: Ferredoxin Reducing Substance. Fd: Ferridoxin. FOR: Ferridoxin-$NADP^+$ Oxidoreductase
All other notations have their conventional meanings.

of photosynthesis. The reaction centre chlorophyll of PS II is usually designated as P680 (Floyd, Chance and Devault, 1971) and of PS I as P700, based on the wavelengths at which spectral changes can be observed which correlate with the functioning of these species. In the most commonly accepted scheme of photosynthesis these two light reactions operate in series and are connected by the chain of intermediate electron carriers (Fig. 2).

The reaction centre is a fundamental concept in photosynthesis and is usually envisaged as a special chlorophyll complex associated with a primary electron donor and acceptor. The ultimate electron donor in green plants is water, though the nature of the oxygen evolving complex of PS II continues to be poorly understood. This is partially due to the extreme lability of this tightly membrane-bound photosystem and the fact that most of the components remain incompletely characterised. Based on measurements of oxygen yield upon illumination of Chlorella or chloroplasts with single short flashes of light it has been deduced that oxygen evolution involves a four quantum process (Cheniae, 1970). In addition, an absolute requirement for manganese ions has also been demonstrated (Cheniae and Martin, 1970). It is not yet certain, however, whether the manganese is involved directly as an electron carrier, or whether it exerts some secondary effect on the structure or function of PS II. Early studies also showed a stimulatory effect of Cl^- on Hill activity (Warburg and Luttgens, 1946). The site of Cl^- involvement was later shown (Bove, Bove, Whatley and Arnon, 1963) to be in the segment of the electron transport chain near PS II. Izawa, Heath and Hind (1969) using artificial electron donors such as

hydroxylamine and ascorbate, subsequently located the site of involvement of Cl^- as being close to the site of water oxidation.

The primary electron acceptor for PS II is thought to be a special plastoquinone molecule designated Q (which may ultimately prove to be identical to or at least intimately associated with component X-320. It would appear that Q is a one electron carrier forming a semiquinone in the reduced state. The second electron carrier on the reducing side of PS II, component B is also thought to be a quinone molecule which appears to be polypeptide bound. This is a two electron carrier which delivers an electron pair into the plastoquinone (PQ) pool which links PS II to PS I. It has been suggested that the substituted phenylureas interrupt electron flow by acting at B.

The experiments of Böhme and Cramer (1971, 1972) suggested that a low potential form of the b-type cytochrome ($b_{559\ LP}$) is located before the main pool of plastoquinone on the basis of an observed inhibition of PS I-dependent oxidation by the plastoquinone antagonist dibromothymoquinone (DBMIB). A role for cytochrome b_{559} on the acceptor side of PS II is also suggested by the observation that it functions as a secondary quencher of fluorescence in the presence of DCMU (Ke, Vernon and Chaney, 1972) when electron flow between the primary and secondary acceptors is inhibited. An additional component in this region of the photosynthetic electron transport chain was identified by Knaff and Arnon (1969) as a light induced absorbance change near 550 nm, which has subsequently been designated C550. Butler (1973) has suggested that this component possibly represents an absorbance change in a sensing

pigment for the redox state of the quencher. The involvement of cytochromes in the intermediate electron transport chain, had been discovered as early as 1951 (Hill, 1951; Davenport and Hill, 1952; Hill, 1954). The first to be discovered was cytochrome *f*, which is bound to the chloroplast structure though it can be released by alkaline non-polar solvents. The α-band of its reduced form is at 555 nm, thus this component is alternatively known as cytochrome c_{555}. Cytochrome b_6, or b_{563}, is found in the PS I-enriched digitonin fraction as well as in a complex with cytochrome *f* (Nelson and Neumann, 1972). Chloroplasts also contain a blue copper-protein called plastocyanin, two quinones, namely vitamin K_1, and plastoquinone, and in all probability other electron carriers which remain to be identified. Elucidation of the sequence of electron carriers in the photosynthetic electron transport chain has been attempted by many of the same methods used in analysis of mitochondrial electron transport. By collating information on the standard redox potentials of the carriers, their behaviour as observed spectroscopically, and the action of inhibitors and of artificial electron carriers, it has been possible to construct the sequence, basically as shown in Fig. 2.

Following the pool of plastoquinone the only known participants in the electron transport chain are cytochrome b_{559}, cytochrome *f* and plastocyanin. The proposed function of plastoquinone is supported by the observations that reduced plastoquinone is oxidised by light absorbed by PS I, oxidised plastoquinone is reduced by light absorbed by PS II. The evidence obtained by Böhme, Reimer and Trebst (1971) and Böhme and Cramer (1971) using 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone

(DBMIB), suggests that plastoquinone may be positioned between cytochrome $\underline{b}_{559}$ and cytochrome $\underline{f}$ in the electron transport sequence.

One of the most powerful experimental approaches to the identification of the sequence of carriers in the intermediate electron transport chain is the use of algal mutants, such as Chlamydomonas and Scenedesmus which are deficient in one or other of the electron carriers. Work reviewed by Levine and Smillie (1962) and Boardman (1970) propose plastocyanin as the primary photooxidant of PS I. An electron transport role for cytochrome $\underline{b}_{559}$ was proposed from studies made using chloroplast fragments of wild-type and mutant strains of Chlamydomonas reinhardii (Levine and Gorman, 1966; Levine, 1969). These studies indicated that a cytochrome component with a reduced α-band at 559 nm was auto-oxidisable, reduced by red light and by ascorbate and was oxidised by far-red light in the wild-type algae but not in mutants deficient in cytochrome $\underline{f}$ or plastocyanin. The precise location of cytochrome $\underline{b}_{559}$ is a matter for continuing debate, having been reported to function at a number of locations depending on whether it is in the high-potential or low-potential form.

P-430 has been assigned as the primary electron acceptor of PS I, and appears to be a bound iron-sulphur protein. Substantial evidence comes from the discovery that the quantum yield and action spectra for P-430 photoreduction mimic those for P-700 photoreduction and that subsequent reduction of ferredoxin coincides with the rate of disappearance of the 430 nm signal. The reducing side of PS I is the best understood segment of the electron transport system. It is generally accepted

that ferredoxin participates in the reduction of $NADP^+$, and functions as a one electron carrier. It was later realised that a second component, ferredoxin-$NADP^+$ oxidoreductase was necessary for the transfer of electrons from ferredoxin to $NADP^+$ (Golbeck, Lien and San Pietro, 1977). In addition a compound known as ferredoxin reducing substance has been claimed to mediate electron flow between P-430 and ferredoxin though the relatively low mid-point potential quoted for the compound casts some doubt on this postulated role (Junge, 1977).

Mode of Action

Symptoms induced by the toxic action of the substituted phenylureas on plants are generally expressed via the leaves. Chlorosis is observed as one of the first visible effects of phenylurea treatment this being followed by more extensive leaf injury (Bucha and Todd, 1951; Muzik, Cruzado and Loustalot, 1954; Minshall, 1957). Symptoms may take up to a week to appear after treatment, initial chlorosis and the appearance of waterlogged areas being followed by wilting, stem collapse, yellowing and ultimately abscission of plant parts (Brian, 1976). Symptoms can, however, vary with both species and the dosage of herbicide applied.

The initial publication of the discovery of herbicidal activity amongst the substituted phenylureas (Bucha and Todd, 1951) was followed by the demonstration (Wessels and Van Der Veen, 1956) that 2×10^{-7}M diuron and 4×10^{-6}M monuron caused a 50% inhibition of photosynthetic electron transport measured as a Hill reaction with 2,6-dichlorophenolindophenol (DCPIP) as the electron acceptor. This was one of the earliest reports to indicate that a herbicide could act by inhibiting photosynthetic electron transport. Bishop (1958) demonstrated that diuron inhibited

the photosynthetic production of oxygen from water by a Scenedesmus mutant. CO_2 fixation, however, proceeded unimpaired when hydrogen was supplied, this alga being capable of by-passing PS II by using hydrogen for generation of reducing power. Vernon and Zaugg (1960) found that whereas 1 x 10^{-5}M diuron caused more than 90% inhibition of normal photosynthetic reduction of $NADP^+$ by isolated chloroplasts, the addition of DCPIP and ascorbate which allow the by-passing of PS II restored photosynthetic activity to 94% of the control values.

In agreement with the above result is the data of Good (1961) who reported that diuron did not have any significant effect on PS 1-mediated activities such as phenazine methosulphate-catalysed cyclic photophosphorylation. The observed lack of effect of diuron on photosynthetic reactions activated by far-red light only, (Moreland, 1967) similarly implies the absence of a phenylurea inhibitory site associated with PS I. Many studies have implicated PS II as being the location of the phenylurea sensitive site.

In a review of the effects of diuron on the redox states of photosynthetically important cytochromes, Moreland (1967) concluded that the site of action of diuron was at or near the beginning of the intermediate electron transport system linking PS I and PS II. Thus Levine (1969) observed that in the presence of diuron cytochromes f, b_6 and b_{559} could still be oxidised by PS I but that they no longer became reduced in light absorbed by PS II, suggesting that inhibition by diuron was at a site between PS II and the cytochrome components of the electron transport system. There are several reports in the

literature which suggest that diuron acts either at the level of the primary photochemical events of PS II or on one of the electron transport steps immediately following. Thus Izawa and Good (1965) determined that the inhibitory efficacy of diuron was dependent on light intensity, 50% inhibition of electron transport being achieved by a diuron concentration of 5.5×10^{-8}M at low light intensities, whereas a concentration of 7.3×10^{-8}M was required to achieve the same percentage inhibition under higher light regimes. This indicated that diuron affected a reaction regulated by the amount of light available.

In a study of the effect of tris buffer (tris(hydroxymethyl)amino-methane) on the Hill reaction of isolated chloroplasts Yamashita and Butler (1968) noted that washing chloroplasts with tris buffer reduced the rate of electron donation from water, however, a low concentration of the artificial electron donor p-phenylene diamine restored photo-reducing capacity. Electron flow, however, from p-phenylene diamine to $NADP^+$ was found to be sensitive to diuron. At higher concentrations of the electron donor other sites beyond the diuron block seemed available for receipt of electrons, so that inhibition by diuron was no longer apparent. Yamashita and Butler concluded from these studies that diuron inhibited electron flow at the site of the primary electron acceptor of PS II, any diuron-sensitive electron flow implying donation of electrons prior to PS II. Izawa, Connelly, Winget and Good (1966) studied the effect of diuron on photosynthetic electron transport in the presence of an autoxidizable electron acceptor such as methyl viologen. They noted also that diuron inhibited electron flow from water to an artificial electron acceptor, but that electron flow could

be restored by the addition of an artificial electron donor such as the ascorbate/DCPIP couple. From their results Izawa et al (1966) placed the diuron inhibition site near PS II and C550. A degree of caution, however, is necessary in arriving at any firm conclusions concerning the site of action of the substituted phenylureas from studies involving the use of artificial electron acceptors and donors, as their precise sites of action are not absolutely defined.

The most conclusive evidence for the location of the site of diuron inhibition on the reducing side of PS II has accumulated from numerous studies of chlorophyll fluorescence. The studies of Duysens and Sweers (1963), Duysens (1964) and Lavorel (1964) have led to the conclusion that monuron prevents the oxidation by PS I of the primary electron acceptor, Q, of PS II.

A study (Izawa and Good, 1965) on the binding of monuron and diuron within chloroplasts showed that the behaviour of the inhibitors was analogous to the binding of substrate to an enzyme, implying the existence of discrete binding sites within the chloroplast.

It is now generally accepted that the redox components which act as the primary acceptors from PS II do not react specifically with the substituted phenylureas (Moreland, 1980). Recent experiments on the effect of trypsin on isolated chloroplasts have led to the conclusion that the redox components X-320 and B are embedded in a proteinaceous region of the thylakoid membrane which can be considered to be a regulator of electron flow from the primary acceptors of PS II

into the plastoquinone pool. In addition this proteinaceous component would prevent the interaction of exogenous redox reagents with the PS II primary electron acceptors. Evidence that many Hill reaction inhibitors bind at the same site has been obtained by Tischer and Strottman (1977) who demonstrated that several groups of herbicides which inhibit the Hill reaction compete directly for a very similar binding site. A further study by the same authors (Tischer and Strottman, 1979) indicated that a quantitative relationship existed between the degree of binding within the chloroplast and the inhibitory efficacy of these compounds on photosynthetic electron transport. The suggestion was made, therefore, that the herbicide receptor was either an electron carrier or a membrane component present in a stoichiometric amount with the redox components responsible for electron transport.

Mild treatment of chloroplasts with trypsin (Renger, 1979) has been found to relieve the inhibitory effect of diuron provided that a suitable electron acceptor such as ferricyanide was present. Such results have led to the suggestion (Renger, 1979; Tischer and Strottman, 1979) that the diuron acceptor molecule may be situated either on or within a membrane protein which is digested by treatment with a peptidase such as trypsin. The lipid phase of the membrane, however, may also contribute in some way to the binding of PS II inhibitors. Recent results presented by Böger and Kunert (1979) indicate that the response of trypsin treated chloroplasts is different to certain groups of Hill reaction inhibitors, implying the presence of several binding sites on the protein shell. In agreement with this

view on the basis of studies on the effect of trypsin on diuron inhibition of electron flow and of the kinetics of the release from inhibition, Renger (1979) has proposed the existence of a binding area with different receptor and sub-receptor sites. This concept has been endorsed by Trebst and Draber (1979) and by Draber and Fedtke (1979) who postulated that, in view of the structural diversity of PS II inhibitors, the binding area may consist of a grouping of obligate receptor and facultative sub-receptor sites, all of which need not necessarily be occupied by one inhibitor.

Two mechanisms have been proposed concerning the basis of the inhibition of photosynthetic electron transport following the binding of a phenylurea herbicide within the chloroplast. Firstly that structural modification of the proteinaceous shell altered the mutual orientation and separation of X-320 and B so as to interfere with the kinetics of electron flow between these components. Renger (1979), however, considered it to be more likely that electron flow between X-320 and B was thermodynamically regulated via the respective redox potentials of X-320 and B, which are dependent on the state of the proteinaceous shell. Modification of the protein shell, such as that caused by herbicide binding, is therefore considered to effect the relationship of the redox potentials of the B^-/B and $X\text{-}320^-/X\text{-}320$ couples. Support for this view comes from studies of luminescence and fluorescence changes induced by DCMU (Lavorel, 1975; Etienne and Lavorel, 1975) where a shift of the equilibrium of the reaction $X\text{-}320^- + B^+ \rightleftharpoons X\text{-}320 + B^-$ towards $X\text{-}320^- + B$ has been demonstrated. In addition Renger and Tiemann (1979) concluded from studies of the proton transport pattern, that a

modification of the proteinaceous component by mild trypsination changed the thermodynamic properties of X-320.

Data obtained from the effect of inhibitors on fluorescence after activation with weak light, indicated that Q remained functional as an electron carrier but that B did not. It appeared that diuron could cause a reverse electron donation from B^- to Q, producing Q^- thus decreasing the redox potential of B with respect to Q (Velthuys and Amesz, 1974; Velthuys, 1976; Pfister and Arntzen, 1979) which would result in the inhibition of electron flow.

The latest information on the structure of the proteinaceous shell comes from the results of studies on the mode of action of PS II inhibitors in herbicide resistant weeds (Arntzen, Ditto and Brewer, 1979; Pfister, Radosevich and Arntzen, 1979), which generally support the concept of receptor and sub-receptor sites on or within the protein shell. Laval-Martin, Dubertret and Calvagrac (1977) have described a strain of Euglena which was highly resistant to diuron but less so to O-phenathroline, resistance being an inherent property of the chloroplast, rather than extra-chloroplast as is normally the case. Of particular interest are the studies of Pfister and Arntzen (1979) with triazine-resistant biotypes, which in addition to displaying extreme resistance to the symmetrical triazines, were also strongly resistant to the asymmetrical triazines, partially resistant to the pyridazones and uracils but only slightly resistant to the phenylureas. The observed differences in resistance could not be attributed to differences in herbicide absorption, distribution or metabolism. These studies with

preparations of chloroplast membranes suggested that alterations to the triazine-binding site was directly related to tolerance to the herbicide. As atrazine binding to resistant chloroplasts could not be detected, and as atrazine could not replace diuron from such chloroplasts, it was concluded that the atrazine binding site was modified in resistant chloroplasts resulting in a decreased binding affinity. Studies on the polypeptide composition of sensitive and resistant chloroplast membranes indicated changes in a 18-20,000 molecular weight polypeptide. This would suggest that a surface-exposed polypeptide is essential for the binding of PS II inhibiting herbicides.

As there are no reports in the literature indicating any differences in the mode of action of monuron and diuron compared to other substituted phenylureas, the evidence accumulated for the mode of action of these two compounds, described in this section, will be taken to equally apply to other substituted phenylureas.

Mechanism of Toxicity

A number of hypotheses have been proposed concerning the mechanism by which plants treated with substituted phenylurea herbicides are killed. In an early study of the effect of monuron on carbon assimilation, Ashton, Uribe and Zweig (1961) found that when the herbicide was applied via the roots of plants, carbon dioxide fixation by leaves in the light was greatly reduced. The subsequent distribution amongst carbon fixation products following exposure of phenylurea-treated red kidney bean (Phaseolus vulgaris L.) to $^{14}CO_2$ both in the light and in the dark

served to demonstrate that these herbicides almost completely blocked the pathway of photosynthetic carbon fixation, but that this effect did not result from an interference with any of the reactions of the carbon reduction cycle. Thus the earliest hypothesis proposed, simply stated that plants killed by treatment with a substituted phenylurea starve to death. There is little evidence that this is the case, indeed, the mechanisms which lead to phytotoxicity appear to be considerably more complex than would be anticipated solely from a limitation of carbohydrate supply. Hoffman, Sweetser and Todd (1960) suggested that the toxicity of monuron to Euglena species in the presence of succinate in the light, but not in the dark, was due to the formation in the light of a phytotoxic compound. Along similar lines is the proposal (Sweetser and Todd, 1961) that the toxicity of monuron in the light to a culture of Chlorella pyrenoidosa supplemented with carbohydrate, was due to the accumulation of a toxic photosynthetic intermediate. In agreement with this view, Davis (1966) suggested that leaf injury in shrub wild oak (Quercus turbinella - Greene) seedlings following treatment with fenuron was due to the accumulation of toxic components in the inhibited photosynthetic unit.

A somewhat different explanation for the mechanism of toxicity of phenylurea herbicides was presented by Stranger and Appleby (1972) based on their observations that in diuron-treated chloroplasts, degradation of carotenoid pigments preceeded the breakdown of chlorophyll. These results were interpreted as providing support to a hypothesis that diuron induced toxicity by catalysing a lethal photo-sensitised oxidation in the chloroplast, possibly caused by an inhibition of the

NADPH formation, necessary to maintain a functional carotenoid protective mechanism. More recently Pallet and Dodge (1976; 1979) have suggested that phenylurea herbicides may induce toxicity via the redirection of the pigment absorbed excitation energy which would initiate cellular deteriorative processes. The carotenoid protective mechanism could take the form of the direct transfer of energy from excited chlorophyll pigments to the carotenoids for dissipation, or that the carotenoids function as quenchers of singlet oxygen. Pallet and Dodge (1979) found that the absence of oxygen in experiments where detached flax cotyledons were treated with monuron led to a delay in the breakdown of chlorophyll and carotenoids. In the presence of oxygen however, excited triplet chlorophyll could react with triplet oxygen to produce singlet oxygen. It is now clear that singlet oxygen can react directly with unsaturated fatty acids to produce lipid hydroperoxides (Rawls and van Santen, 1970) which would lead to the breakdown of cell membranes by a deteriorative chain reaction (Pallet, 1978). This latter view is supported by the results of a study of the mechanism of linuron toxicity in maize (Muschinek, Garab, Mustardy and Faludi-Daniel, 1979). It thus appears that the photochemical events outlined above could well account for the mechanism of the toxicity of substituted phenylureas in plants.

Non-Photosystem II Effects of the Substituted Phenylureas

Jagendorf and Margulies (1960) and Asahi and Jagendorf (1963) have reported that at high concentrations (10^{-4}M) monuron can be shown to inhibit PS I mediated reactions such as PMS-catalysed cyclic photophosphorylation. However, as the substituted phenylureas are potent

inhibitors of PS II-linked reactions at concentrations lower than 10^{-4}M, it is highly unlikely that their herbicidal action results from inhibition of electron transport processes not associated with PS II.

Although it now seems beyond doubt that the substituted phenylureas exert their phytotoxic effect through an inhibition of PS II, certain other processes within the plant have been reported to be affected. However, it must be emphasised that these additional effects are either elicited by considerably higher concentrations of the herbicide than are necessary to block photosynthesis or represent secondary effects resulting from the disturbance of metabolism caused by inhibition of electron flow through PS II. There are a number of reports in the literature of studies on the effects of substituted phenylureas on various respiratory activities of mitochondria. Oxygen uptake by cabbage mitochondria was shown to be inhibited by 15% at a monuron concentration of 10^{-3}M whereas phosphate esterification was inhibited some 48% (Lotlikar, Remmert and Freed, 1968). Moreland and Boots (1971) using the R- and S-isomers of N-(α-methylbenzyl)-3-(3,4-dichlorophenyl)urea (MBPU) observed a stimulation of state four respiration and an inhibition of state three respiration with either malate or succinate as substrates, though these effects were less marked when NADPH was the substrate used. A similar result was obtained more recently by Tluczkiewicz (1974a,b) who found that chlortoluron inhibited succinate oxidation by barley embryo mitochondria, depressed the esterification of inorganic phosphate and uncoupled oxidative phosphorylation. These results support the earlier work of McDaniel and Frans (1969) who studied the effect of the related phenylurea fluometuron

(N'-(m-trifluoromethylphenyl)-N,N-dimethylurea) on mitochondria from soybean (Glycine max L.). These workers reported a 50% uncoupling of oxidative phosphorylation at concentrations of 5.6×10^{-5}M with malate as substrate and 4.6×10^{-5}M with succinate as substrate. Though the precise site of action of the substituted phenylureas studied in these investigations were not determined, Convent and Briquet (1978) recently localised the site of diuron inhibition in mitochondria of Saccharomyces cerevisiae to be between cytochromes b and c_1.

An inhibition of glutamate-pyruvate and glutamate-oxaloacetate transamination following treatment of wheat with metoxuron (N'-(3-chloro-4-methoxyphenyl)-N,N-dimethylurea) was noted by Decleire, Van Roey and Bastin (1974). As part of a study on the effect of phenylureas on non-photosynthetic processes, Sumida and Ueda (1973) investigated the effect of diuron on the biosynthesis of complex lipids in Chlorella ellipsoidea. Their results indicated that diuron substantially impaired the incorporation of acetate into fatty acid residues. It was argued, however, that since biosynthesis of fatty acids from acetate units requires the co-factors ATP, co-enzyme A and NADPH, it was reasonable to assume that shortages of ATP and NADPH resulting from inhibition of photosynthesis was probably responsible for the observed inhibition of acetate assimilation into lipids. Another explanation, however, is that in conditions where ATP supply was reduced lipids, particularly galactolipids, might be metabolised as an energy source. The final possibility of a direct effect of the herbicide on enzyme(s) involved in lipid biosynthesis still remains. Moreland, Mulhotra, Gruenhagen and Shokraii (1969) in a study of the effects of substituted phenylureas on RNA and

protein synthesis, found that diuron was moderately active as an inhibitor of these processes, as measured by the incorporation of radioactively labelled ATP or orotate and leucine respectively. The physiological and biochemical processes required for plant growth and development are ultimately driven by energy derived from ATP. The observed inhibition of RNA and protein synthesis could thus be explained as a result of inhibition of cellular energy production, though the possibility of a direct effect cannot be ruled out. Thus there is evidence to suggest that the substituted phenylureas disrupt several processes either directly or as a secondary effect due to inhibition of photosynthesis, though electron transfer associated with PS II remains the primary site of action of this group of herbicides.

Selectivity

When applied at the same dosage to different plant species, herbicides usually give rise to differing responses. A herbicide can be said to be selective when the weeds being treated are inhibited in growth or killed, while the crop plants remain relatively unharmed. Factors which contribute to the selectivity of the substituted phenylurea herbicides include differences in absorption and distribution and in the morphological, physiological and biochemical characteristics of crop and weed species.

The earliest substituted phenylurea herbicides to be used commercially for weed control were relatively non-selective and were usually applied to the soil as general soil sterilants (Minshall, 1954; Fang et al, 1955). Later reports, however, claimed that monuron, for example,

exhibited a degree of selectivity which was attributed to physical phenomena such as placement in the soil (Rogers and Funderburk, 1967). Such an explanation, however, does not adequately explain the differences in susceptibility of tolerant and susceptible plants to the more recently developed substituted phenylureas. In an investigation of the metabolism of chloroxuron (N'-(4-(p-chlorophenoxy)-phenyl)-N,N-dimethylurea) in plants showing different degrees of tolerance (Geissbuhler, Haselbach, Aebi and Ebner, 1963b) it was found that although all plants studied were capable of metabolising the herbicide, there were differences in the rate of metabolism between susceptible and tolerant species. These workers consequently concluded that differences in the rate of metabolism represented a possible basis for the selective action of chloroxuron. Kuratle (1968) and Kuratle, Rahn and Woodmansee (1968) reported a greater uptake of linuron by susceptible common ragweed (Ambrosia artemisiifolia L.) compared to tolerant carrot (Daucus carota L.). In addition, the metabolites of linuron detected in common ragweed retained considerable phytotoxicity, whereas those extracted from carrot were essentially non-phytotoxic. On the basis of these results it was suggested that differences in uptake and metabolism of the herbicide and in the phytotoxicity of its metabolites may all contribute to selectivity.

A similar conclusion was reached by Nashed, Katz and Ilnicki (1970) from a study of the selective action of chlorbromuron (N-(3-chloro-4-bromophenyl)-N-methoxy-N-methylurea) in tolerant maize (Zea mays L.) and susceptible cucumber (Cucumis sativa L.). A degree of binding of the herbicide away from the site of action was reported in both species,

but cucumber was found to absorb more of the herbicide than did maize. In addition, maize degraded the herbicide more efficiently than did cucumber. Butts and Foy (1973) found that the differential tolerance of prickly sida (Sida spinosa L.) and cotton to methazole (2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione) could be explained by differential uptake and differences in the ability of the respective plants to degrade the herbicide to non-toxic compounds.

In a study of the effect of fluometuron and its metabolites on the rate of photosynthesis in resistant cotton (Gossypium hirsutum L.) and susceptible redroot pigweed (Amaranthus retroflexus L.) and foxtail (Setaria spp.), Rubin and Eshel (1971) found that cotton was not affected by fluometuron concentrations which were toxic to foxtail and severely reduced the growth of redroot pigweed. The N-monodesmethylated derivative, though less active, elicited a similar response. These results indicated that selectivity may result from differential susceptibility to the herbicide metabolites, in addition to differences in the rate of degradation of the herbicide. Feeny, Parochotti and Colby (1974) studied the mode of selectivity of chloroxuron in resistant soybean and susceptible tall morning glory (Ipomea purpurea L.). They found that selectivity was due to a greater accumulation of chloroxuron in the foliage of tall morning glory than soybean, and suggested that the roots of the soybean in some way hindered acropetal transport of the herbicide. A study of the intracellular distribution of the absorbed herbicide demonstrated a 2-5 fold greater amount of chloroxuron associated with chloroplasts isolated from tall morning glory compared to soybean. However, these authors were unable to detect any differences in the metabolism of the herbicide which could be related

to the differential tolerance exhibited by those particular species. In an attmept to elucidate the mechanism of selectivity of linuron in resistant parsnip (Peucedonum sativum. Benth) and susceptible tomato (Lycopersicum esculentum. Mill) Hague and Warren (1968) found that whereas tomato absorbed the herbicide readily and subsequently translocated it to the leaves, parsnip retained large amounts in the roots. In addition, in parsnip the herbicide was metabolised to a greater extent than in tomato. Thus the selective action of linuron was attributed to differences with respect to accumulation of the herbicide in the leaves and in its subsequent metabolism.

From a study of the effects of diuron on cotton (resistant) and soybean, oat (Avena sativa L.) and corn (susceptible), Smith and Sheets (1967) suggested that the rate of herbicide metabolism was largely responsible for the tolerance of cotton to diuron, though the differences in degree of susceptibility amongst the other three species appeared to be related more to herbicide absorption. Eshel (1969) has pointed out, however, that although cotton is capable of rapidly degrading diuron, this need not necessarily be the sole factor involved in the tolerance of this species. Results obtained by Rogers and Funderburk (1968) suggested that the tolerance of cotton to fluometuron mainly results from a rapid degradation of the herbicide, whereas differences amongst various species with respect to susceptibility to diuron appeared to be related more to differential absorption. In particular these workers found that cotton retained the greatest percentage of the absorbed herbicide in the roots, though some differences in metabolism of the absorbed diuron were detected, cotton containing lower amounts of phytotoxic material than susceptible cucumber. Recently a preliminary study of isoproturon

selectivity has been undertaken by McIntosh, Robertson and Kirkwood (1981), in resistant wheat (Triticum aestivum L.) and wild oats (Avena spp.) and blackgrass (Alopecurus myosuroides Huds.) (susceptible). There appeared to be no obvious relationship between the efficiency of absorption and distribution and selectivity. However, it was apparent that wheat was capable of a more rapid degradation of isoproturon than wild oats or blackgrass and displayed a lower level of binding of ^{14}C-isoproturon in the chloroplast. It was suggested that these two factors contribute to the selectivity of isoproturon between wheat, and wildoats and blackgrass.

Osgood, Romanowski and Hilton (1972) were the first workers to report intra-varietal differences to the substituted phenylureas. These workers found that Hawaiian sugarcane varieties, which are interspecific hybrids within the genus Saccharum, differed substantially with respect to their tolerance to diuron. ^{14}C-diuron was shown to be degraded in both tolerant (H50-7209) and susceptible (H53-263) cultivars though metabolism of diuron was more extensive in the resistant variety. The distribution of radioactivity fourteen days after treatment was almost identical in both varieties when expressed as total activity, however, on the basis of activity per unit dry weight, there was a greater concentration of radioactivity in the younger leaves of H53-263 than H50-7209, this situation being reversed in the roots. Thus the varietal differences in response may be explained on the basis of a combination of differences in the distribution and metabolism of the absorbed herbicide. In a study some years later, Lii-Chyuan, Shimabukuro and Nalewaja (1978) concluded that the differential tolerance of two sugarcane cultivars, PR980

(resistant) and PR1048 (susceptible) to diuron was due to differences in the metabolism of the absorbed diuron. In that although both cultivars metabolised diuron by progressive N-demethylation, it was more rapid in the resistant variety.

An extensive study of the varietal susceptibility of wheat cultivars to metoxuron (N'-(3-chloro-4-methoxyphenyl)-N,N-dimethylurea) has been described by Emami-Saravi (1979). In this study it was found that a differential rate of herbicide uptake contributed to the varietal response. However, the resistant varieties demonstrated a greater rate of degradation of metoxuron than did the susceptible varieties, resulting in the production of appreciably larger amounts of non-phytotoxic material in the resistant rather than the susceptible varieties.

There is now considerable evidence for inter-varietal differences in the response to a particular herbicide (Griffiths and Ummel, 1970; Hubbard and Livingstone, 1974; Brkic and Zekovic, 1975). Of considerable importance have been the reports that varieties of cereals differ markedly with respect to their tolerance of metoxuron, and more importantly, chlortoluron, both in the field and in glasshouse trials (Holroyd and Bailey, 1970; Maas, 1971). These observations have given rise to some concern in recent years as several of the newer high-yielding varieties suffer an unacceptable degree of damage at the herbicide dosage required for adequate weed control. A major weed problem in cereals is caused by grass weeds, principally wild oat (Avena fatua L., Avena ludoviciana. Durieu, Avena sterilis L. and Avena barbata Pott.) and blackgrass (Alopecurus myosuroides Huds.).

There is now extensive information available on the degree of infestation of these weeds, provided largely by the surveys of Thurston (1954), Perring and Walters (1962), a collaborative study by Rothamsted Experimental Station and the Agricultural Department Advisory Service (1967; 1968), Pfeiffer (1968), Waterson and Davis (1973) and Phillipson (1973). Such studies suggest that some 405-607 thousand hectares in the United Kingdom are infested, and that many more may be threatened. A. myosuroides Huds. constitutes a particularly difficult control problem, in that germination can occur at any time between sowing and harvesting of the crop. Some 80% may remain to germinate the following season, with sporadic germination in the years following. Studies reported by Wormell (1972) have indicated that infestations of blackgrass for a period as short as one week can drastically reduce the ultimate yield. Wild oats poses a similar problem in that both winter and spring germinating types of wild oat may occur in the same field. An additional problem is that seed is shed before the cereal crop has ripened, thus a reservoir of infection can accumulate in the soil.

A. myosuroides Huds. and Avena spp. thus represent a serious weed problem, and because of the economic importance of cereals, a large number of chemicals have been tested for the ability to selectively control these weeds (Baldwin and Livingstone, 1972). Among the chemicals specifically recommended for the control of these two weeds are chlortoluron and isoproturon (Hewson, 1974). The efficacy of chlortoluron and isoproturon for weed control in cereals has been investigated in considerable detail (Van Hiele, Hommes and Vervelde,

1970; Skorda, 1974; Guillement, 1975; Scheller and Zemp, 1975). In view of the high level of control of Avena spp. and A. myosuroides, and the corresponding large increase in yield that could be achieved in cereals by the use of chlortoluron or isoproturon, the observation (Holroyd and Bailey, 1970; Maas, 1971) that a number of commercially important and high-yielding wheat varieties were damaged by chlortoluron stimulated a thorough investigation of the differences in varietal response of cereal cultivars to these herbicides. Van Hiele et al (1970) compared the response of selected cultivars of winter wheat to chlortoluron in glasshouse trials. Although several cultivars were extensively damaged by treatment with the herbicide, one cultivar showed considerable tolerance. Similar results were obtained by Hewson (1974) and Hubbard and Livingstone (1974) following treatment of wheat varieties with chlortoluron in field trials. Susceptible varieties were severely scorched at various stages of growth, but these effects were not apparent in the resistant varieties. Interestingly, there was no evidence of varietal susceptibility to the related isoproturon. This latter observation was confirmed by Tottman, Holroyd, Lupton,Oliver, Barnes and Tysoe (1975) in a study of the tolerance of chlortoluron and isoproturon by winter wheat varieties. Responses to isoproturon showed no clear-cut grouping of varieties, any differences being attributed to the expression of several genetic characters interacting with the environment. In the case of chlortoluron, however, the varieties segregated into two distinct groups, one tolerant and the other susceptible. Inheritance studies showed a strict segregation in the progeny of crosses between tolerant and susceptible varieties, implying that the tolerance was simply inherited. On the basis of these results, it was suggested that resistance to chlortoluron might be explained in terms of a simply

inherited detoxifying mechanism in addition to genetic and environmental factors similar to those affecting isoproturon tolerance. Evidence for a genetic basis for the differences in susceptibility of wheat cultivars to phenylurea herbicides has also been obtained by Strykers, Van Himm, Persijn and Bulck (1974). These workers concluded that the property of susceptibility to substituted phenylurea compounds was derived from a susceptible parent line, while tolerance was similarly dependent on the parent line. Where intermediate responses were observed, these were attributed to genetically determined differences in physiology and/or morphology rather than to an ability to detoxify the applied herbicide.

The Molecular Fate of the Substituted Phenylurea Herbicides

Many studies have been carried out on the degradation of substituted phenylurea herbicides, often because of a requirement to satisfy government regulations concerning the fate of the herbicide in the environment, but also to extend our knowledge of the degradation of xenobiotics so that it may be possible to design more effective herbicides for the future.

Results obtained by several investigators have indicated that soil micro-organisms play an important role in the degradation of herbicides. Early studies made by Muzik et al (1954) showed that conditions which increase microbial activity also enhance the rate of inactivation of applied phenylurea herbicides. Comparison of the degradation of phenylureas in sterilised and non-sterilised soils (Hill, McGahen, Baker, Finnerty and Bingeman, 1955; Sheets and Crafts, 1957;

Geissbuhler et al, 1963a; and Cserhati, Janos and Keckes, 1979) invariably showed that degradation occurs at a greater rate in the non-sterilised soils. The first systematic search for organisms capable of degrading phenylurea herbicides was carried out by Hill et al (1955) who succeeded in isolating a pseudomonad capable of utilising monuron as the sole carbon source. A subsequent study (Hill and McGahen, 1955) showed that other species of common soil bacteria, for example Xanthomonas spp, Sarcina spp and Bacillus spp were also able to degrade monuron. In a study of the decomposition of seven phenylurea herbicides by Bacillus sphaericus, Wallnofer (1969) found that the organism was specific for the methoxy substituted herbicides monolinuron, linuron and metobromuron, degrading them by removal of CO_2 from the ureido portion of the molecule thereby producing the corresponding chloroaniline moieties. Schroeder (1970) has examined the ability of some species of fungi and bacteria to degrade linuron, monolinuron, monuron and diuron. The results obtained demonstrated N-demethylation, N-demethoxylation, hydroxylation and decarboxylation reactions with the extensive occurrence of the chloroaniline moiety. Ross and Tweedy (1973) observed that microbial populations from two types of soils were capable of degrading both fluometuron and chloroxuron by successive N-demethylation reactions. However, N-demethylation represented only a minor pathway in the degradation of the methoxymethylphenylurea herbicides, metobromuron and chlorbromuron, for which direct hydrolysis to polar aromatic compounds predominated. The ability of various soil bacteria to further metabolise the aniline product of phenylurea degradation has been investigated by a number of workers. For example, Walker and Harris (1969) obtained evidence to suggest that the aniline derivatives may

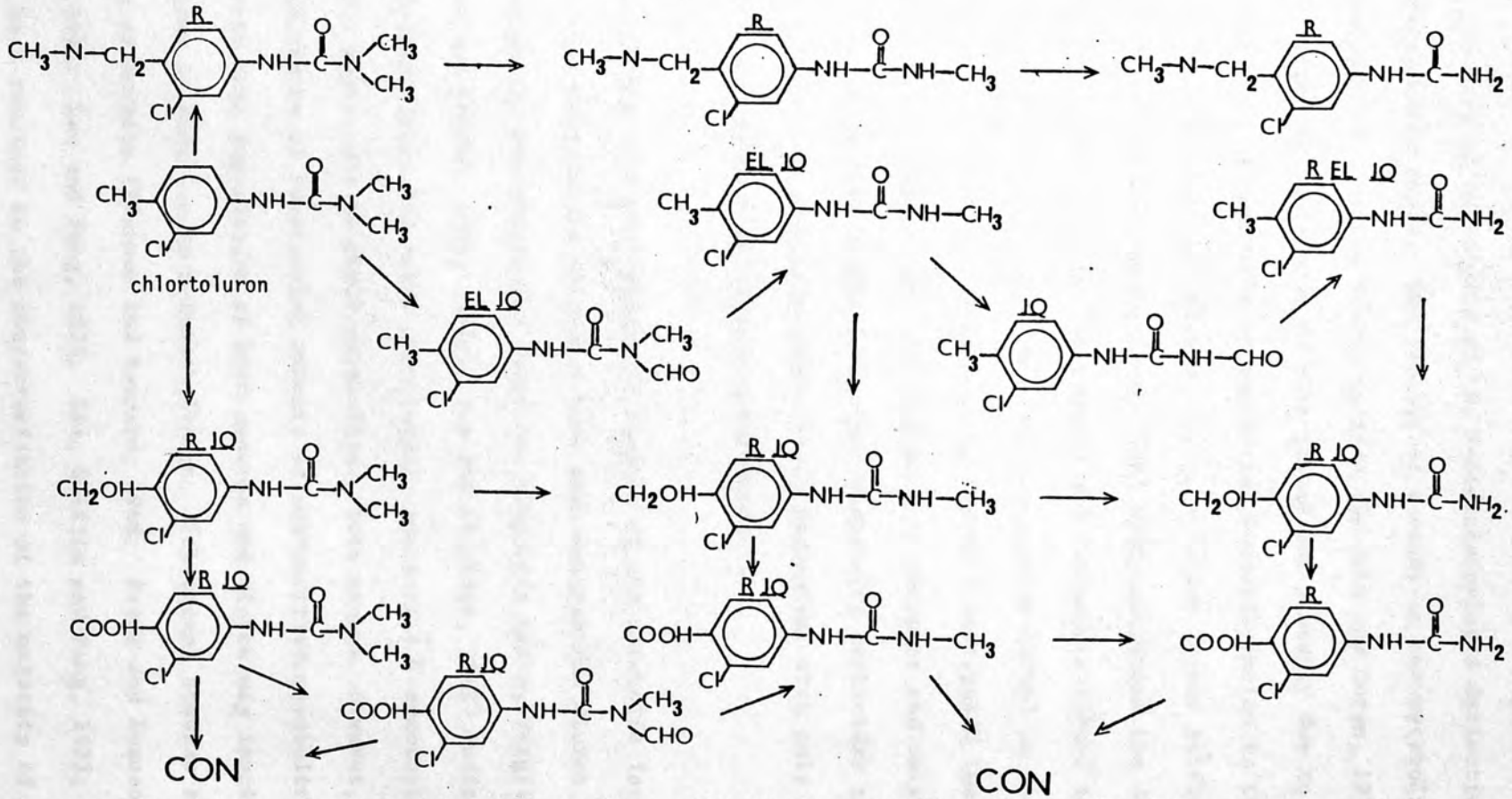
be further oxidised to catechol, whereas Briggs and Walker (1973) demonstrated that the growth of Alcaligenes faecalis with 4-chloroaniline as the sole carbon source was accompanied by the production of a chlorinated phenoxazinone. In addition, Briggs and Ogilvie (1971) have detected the production of an azobenzene derivative from the aniline moiety of metoxuron. In contrast in a study of the degradation of metobromuron (N'-(p-bromophenyl)-N-methoxy-N-methylurea) (Tweedy, Loeppky and Ross, 1970) no azobenzene products were identified, the major product being p-bromoacetanilide. However, it was noted that high concentrations of the urea or aniline derivatives could lead to azobenzene formation although this was thought unlikely to occur under normal soil conditions.

Of particular relevance to the present thesis are the results of studies (Smith and Briggs, 1978) on the metabolic fate of chlortoluron in soil. The only metabolite identified in this study was the N-desmethylated derivative, although it was suggested that the aniline derivative could be directly formed from this compound by cleavage of the amide bond. In contrast, results of an independent study (Gross, Lannio, Dupuis and Esser, 1979) on the metabolism of the same phenylurea in soil indicated degradation by ring-methyl oxidation in addition to a more extensive N-demethylation than reported by Smith and Briggs (1978) in that a variety of N-demethylated and 4-hydroxymethylphenyl and 4-carboxyphenyl derivatives were observed. An earlier study (Fournier, Reudet and Soulas, 1975) demonstrated that the related phenylurea herbicide isoproturon was subjected only to N-demethylation in soil, the major product being the N-monodesmethylated derivative. Information in the literature concerning the fate of substituted

phenylurea herbicides in animals is rather limited. However, the studies which have been reported indicate that the urea moiety is hydrolysed to only a small extent. The routes of metabolism reported in the past include demethylation, ring hydroxylation and conjugation. One of the first studies undertaken was that of Ernst and Böhme (1965) on the fate of monuron, monolinuron, diuron and linuron in the rat. The majority of the metabolites detected were products of sequential N-demethylation or ring hydroxylation. In addition, a study of the metabolic fate of fluometuron in the rat (Boyd and Fogleman, 1967) indicated that the major metabolites were conjugates of N-monodesmethyl and N-didesmethyl compounds. Of particular relevance to the present investigation are the results of a study (Muecke, Menzer, Alt, Richter and Esser, 1976) on the metabolism of chlortoluron in the rat. These authors found that N-demethylation and oxidation of the methylphenyl group to give hydroxymethylphenyl and carboxymethylphenyl derivatives represented the major degradation pathways (Fig. 3). Both mechanisms apparently proceeded simultaneously so that the metabolites produced represented all combinations of N-demethylation and ring-methyl oxidation. In addition, N-formyl derivatives were detected as minor metabolites, it being postulated that such compounds may be intermediates in N-demethylation reactions. When a higher dose of chlortoluron was used additional metabolites in which the methylphenyl group had been converted to a methylthio-methyl derivative were detected.

In addition to their studies on the metabolic fate of chlortoluron in soil, Geissbuhler, Hasselbach, Aebi and Ebner (1963a) also investigated the degradation of this herbicide by several plant species. As had been demonstrated with bacteria, N-demethylation appeared to be the

Fig. 3: Proposed pathway of the metabolism of chlortoluron in animal systems after Hinderer and Menzer (1976b), Lin et al (1976) and Muecke et al (1976)



R, rat. EL, human embryonic lung cell culture. JQ, japanese quail. CON, conjugate.

primary detoxification mechanism, a slow release of $^{14}CO_2$ suggested only a low rate of hydrolysis of the N-didesmethylated derivative to the corresponding amine. The results of a study on the metabolic fate of fluometuron in various Citrus species (Menashe and Goren, 1973) suggested that the observed resistance of such plants was probably due to a rapid degradation of the herbicide by sequential N-demethylation to the N-mono and N-didesmethylated derivatives, and to a lesser extent trifluoromethylaniline following hydrolysis. This study confirmed the degradation pathway previously described by Neptune and Funderburk (1969) in etiolated corn and wheat tissue, and by Voss and Geissbuhler (1966) in cotton and wheat. In a related study Nashed et al (1970) investigated the time-course of the degradation of chlorbromuron in cucumber and maize. These authors found that in common with other phenylurea herbicides sequential N-demethylation was the major degradation mechanism, with only a small amount of the aniline derivative being detected.

The herbicides used most widely in studies of the metabolic fate of the substituted phenylureas in plants have been monuron and diuron. Early studies on the degradation of these two compounds (Fang, Virgil, Freed, Johnson and Coffee, 1955; Onley, Yip and Aldridge, 1968) indicated that as described later for other phenylureas, sequential N-demethylation played a major role in their metabolism. Both groups, however, noted the occurrence of substantial amounts of unidentified metabolites which suggested that degradation of both monuron and diuron may involve other reactions in addition to N-demethylation. Subsequent studies by several groups of workers (Swanson and Swanson, 1968; Frear and Swanson, 1971, 1972, 1974; Lee and Fang, 1973; Lee, Griffin and Fang, 1973; Dawson, 1975) have resulted in the characterisation of the majority of the

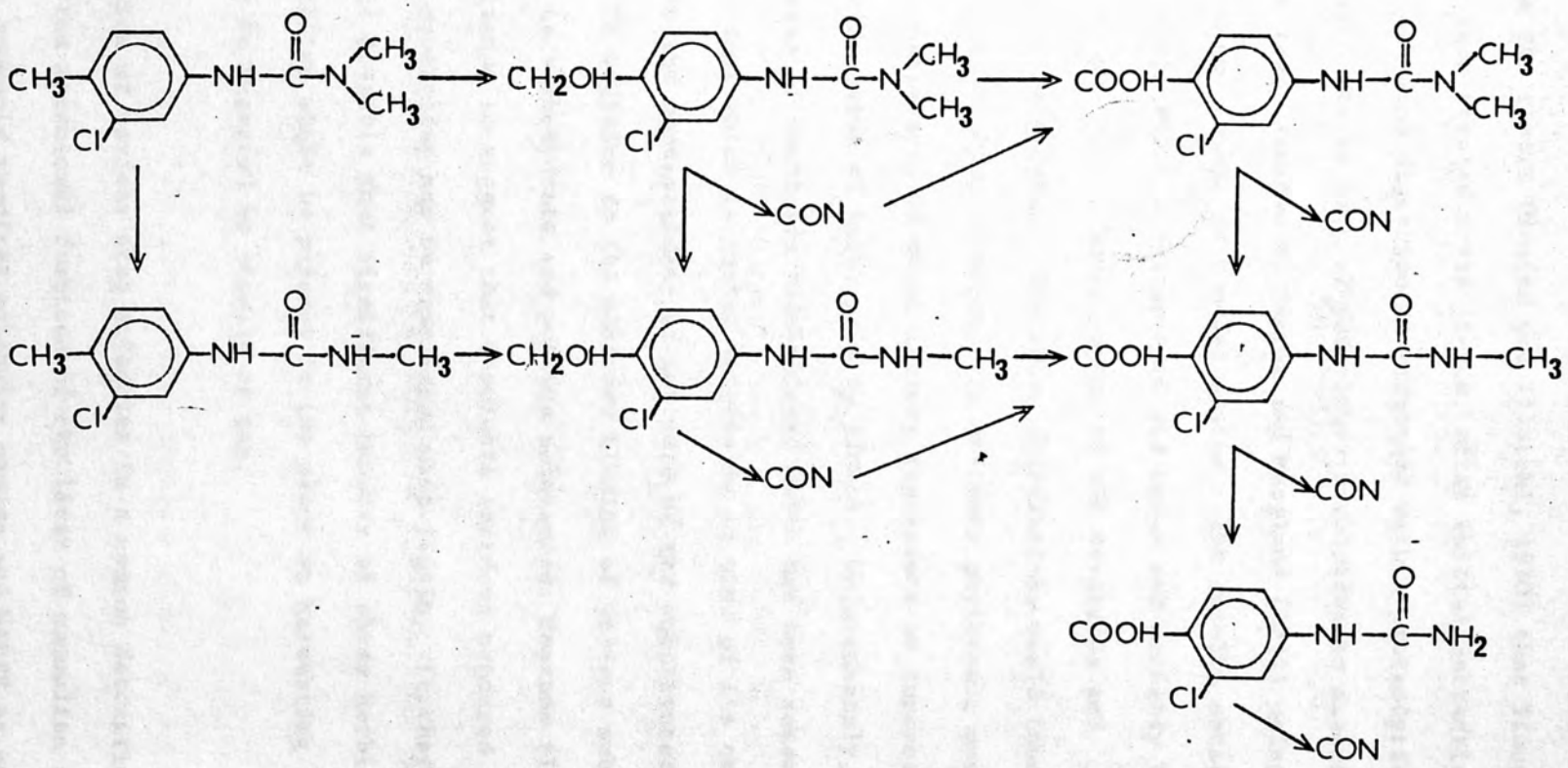
metabolites of monuron and diuron. The initial pathway of degradation was determined to be sequential N-demethylation. In the degradation of monuron, N'-(4-chlorophenyl)-N-hydroxymethyl-N-methylurea and the corresponding didesmethylated derivative were found to be intermediates of N-demethylation. These compounds invariably occurred as β-D-glucosides, and provided evidence for the formation of N-hydroxymethyl intermediates in the N-demethylation of the substituted phenylureas. Frear and Swanson (1972) proposed that the N-hydroxymethyl compounds might represent important intermediates in the degradation of monuron as their removal by conjugation would prevent the accumulation of appreciable amounts of free p-chlorophenylurea which has been shown to inhibit monuron degradation. In addition to degradation by N-demethylation, Lee and Fang (1973) demonstrated parallel ring hydroxylation by the introduction of a hydroxyl group at the ortho position. Various water soluble metabolites were also detected and shown to consist of polypeptide complexes of monuron and monodesmethylated monuron, and β-D-glucosides of the hydroxylated metabolites. In a more recent study on the metabolism of monuron by Citrus sinensis L.Dawson (1975) demonstrated the occurrence of a number of water soluble metabolites, which unlike those described by previous authors, were dehalogenated in addition to being ring hydroxylated and conjugated. In contrast to previous studies in which the glucoside conjugates involved β-D-glucose, those latter metabolites were apparently conjugated to either fructose or O-methylglucuronic acid. However, these compounds were not identified unambiguously and thus their true nature remains somewhat uncertain.

A study of considerable relevance to the present thesis is that of Gross, Lannio, Dupuis and Esser (1979) on the metabolic fate of chlor-

toluron in wheat. Unlike the substituted phenylureas investigated previously, this latter herbicide was shown to be degraded by two independent mechanisms involving, (i) oxidation of the 4-methylphenyl group to give 4-hydroxymethylphenyl and 4-carboxyphenyl derivatives, and (ii) sequential N-demethylation (Fig. 4). Although the stepwise N-demethylation pathway established for a number of substituted phenylureas was employed to some extent, oxidation of the methylphenyl group yielding benzylalcohol derivatives represented the major route for chlortoluron degradation. All the metabolites produced were also identified as polar conjugates with, it was suggested, β-D-glucose.

As has been indicated previously, a key role in the detoxification of the substituted phenylureas is that of conjugation as this invariably leads to a complete loss of toxicity. A large number of naturally occurring compounds are available for conjugation with phenylurea herbicides and their metabolites. However, a survey of the literature rapidly reveals that for plants the only conjugates fully characterised have been shown to be glycosides of the pesticide or its metabolites. Although the studies described by Frear and Swanson (1972) and Lee, Griffin and Fang (1973) demonstrated the presence of β-D-glucose as the glycone moiety, Frear (1979) has suggested that further investigations may reveal the presence of O-glucosides, S-glucosides, glucose esters and gentiobioside metabolites. It has been suggested (Climie and Hutson, 1979; Mumma and Hamilton, 1979) that conjugation with amino acids and proteins could occur. Fang et al (1955) observed the formation of a monuron complex after treatment of french bean (Phaseolus vulgaris L.) which on hydrolysis with acid yielded the unchanged herbicide. The

Fig. 4: Proposed pathway of the metabolism of chlortoluron in wheat after Gross et al (1974)



component complexing with the herbicide was later considered to be of a protein or peptide nature (Freed et al, 1961). This suggestion gains support from the report (Nashed and Illnicki, 1970) that linuron could be released from treated maize tissue, after initial extraction with acetone, by alkaline digestion or treatment with a proteolytic enzyme such as ficin. The ability of phenylurea herbicides to associate with proteins has been examined by Camper and Moreland (1971) using diuron and bovine serum albumin as a model system. The results obtained indicated that binding to the protein did occur and probably involved the amide nitrogen and carbonyl oxygen of the herbicide and free amino groups of the protein. The above observations would thus seem to indicate that binding of herbicides or their phytotoxic metabolites to various constituents of plant tissues represents an important mechanism in the detoxification of herbicides by plants. Unfortunately, however, this is an area of herbicide biochemistry which has been somewhat neglected, a fact which is rather surprising in view of its obvious importance in the understanding of the fate of the substituted phenylureas in plants. In addition to the observed binding of various substituted phenylureas to carbohydrate and peptide substances, Neptune (1970) has provided evidence to suggest that insoluble residues produced during fluometuron metabolism may be associated with lignin. It, therefore, appears quite possible that significant amounts of these herbicides or their metabolites might be present in the plant at harvesting and may consequently be ingested by animals or man.

The degradation of various drug molecules is a common detoxification reaction of the microsomal fraction of the liver of mammalian species. This process commonly requires molecular oxygen and NADPH as co-factors

(Brodie, Gellete and La Du, 1958; Shuster, 1964) as in the demethylation of N-methyl barbiturates (McMahon, 1963), oxidation of cisanilide (cis-2,5-dimethyl-1-pyrrolidine carboxanilide) (Frear and Swanson, 1976), O-demethylation of p-nitrophenetole (Shigematsu, Yamano and Yoshimura, 1976), and the hydroxylation and N-dealkylation of certain dinitroaniline herbicides (Nelson, Kearney, Plimmer and Menzer, 1977). In many cases treatment of the animal with the pesticide will induce the microsomal electron transport system. Indeed, Bankowska and Bojanowska (1972, 1973, 1974) and Carthay, Medilanski and Benakis (1977) have demonstrated induction of demethylase activity of mammalian liver by the phenylurea herbicides linuron, diuron and chlortoluron. In a study of the metabolism of chlortoluron, fluometuron and metobromuron in human embryonic lung cell cultures, in addition to metabolites resulting from N-demethylation, Lin, Menzer and North (1976) detected the presence of N-formyl-N-methylurea and N-formylurea derivatives which they considered to be intermediates of the N-demethylation pathway. More recently Hinderer and Menzer (1976a, b) made an in vitro study of chlortoluron metabolism using postmitochondrial, microsomal and supernatant fractions prepared from liver, testis, lung and kidney tissue of the rat and from the liver of japanese quail. The postmitochondrial fraction from quail liver homogenates was the only preparation found to degrade chlortoluron, the metabolites detected being those previously characterised in cultures of human embryonic lung tissue (Lin, Menzer and North, 1976) with the additional presence of metabolites resulting from ring-methyl oxidation (Fig. 3). Degradation of pesticides has been much less extensively studied in in vitro preparations from plant tissue though a number of reports have appeared in the literature. A number of investigations on the degradation

of aldrin (1,2,3,4,10,10-hexachloro-1α,4α,4aβ,5a,8a,8aβ-hexahydro-1,4:5,8-dimethanonaphthalene) and dieldrin (3,4,5,6,9,9-hexachloro-1aα,2β,2aα,3β,6β,6aα,7β,7aα-octahydro-2,7:3,6-dimethanonapht h 2,3-b -oxirene) by in vitro preparations from Pea (Pisum sativum L.) and French bean (Lichtenstein and Corbett, 1969; Orloffs, 197C; Yu, Kiigemagi and Terriere, 1971; Mehendale, 1973; McKinney and Mehendale, 1973) have indicated that such studies could represent a very useful avenue of research, complementing experiments in vivo. The earliest study to supply direct evidence for the degradation of substituted phenylureas in vitro was supplied by Frear (1968) in a study of monuron metabolism in cotton. This author was able to isolate an NADPH and molecular oxygen dependent microsomal enzyme system capable of N-demethylating monuron. An extension of the study to include plantain (Plantago L.), buckwheat (Fagopyrum esculentum. Moench) and broadbean (Vicia faba L.) revealed that the leaves of these species also contained an active microsomal monuron N-demethylase system, the enzyme being specific for substituted N'-phenyl-N-methylurea compounds (Frear, Swanson and Tanaka, 1969). The observed inhibition of the enzyme by carbon monoxide, ionic detergents, sulphydryl reagents, chelating agents and electron acceptors, and the presence of cytochrome b_5 and NADPH-cytochrome c reductase have been taken as confirmatory evidence for the involvement of a microsomal electron transport system. Results of preliminary experiments on the metabolism of fluometuron by a microsomal preparation from citrus rootlets led Menashe and Goren (1973) to suggest that this latter tissue contains a similar N-demethylase system to that reported by Frear (1968) in cotton. In a related study, Young and Beevers (1976) examined the mixed function oxidase activity of microsomal preparations from germinating castorbean (Ricinus communis L.) endosperm and found that an active N-demethylase

specific for N-methylarylamines was also dependent on NADPH and molecular oxygen for activity. A mixed function oxidase from avocado pear (Persea gratissima L.) that catalysed the N-demethylation of p-chloro-N-methyl-aniline has been reported (McPherson, Markham, Bridges, Hartman and Parke; 1975), in this study the O-demethylation of p-nitroanisole was also reported. A recent study has also shown that 2,4-D is hydroxylated by an in vitro mixed function oxidase system from cucumber (Makeev, Makoveichuk and Chkanikov, 1977).

Efforts to isolate enzyme systems capable of degrading the substituted phenylureas have met with limited success. In a survey of some nineteen genera of weeds for enzymes capable of metabolising amide, urea and carbamate herbicides, Hoagland and Graf (1972a) found that although fourteen yielded preparations capable of degrading propanil(3,4-dichloro-propionanilide) only one (Echinocystis lobata L.) demonstrated an ability to metabolise urea or carbamate herbicides. It has however proved possible to isolate enzyme systems capable of degrading pesticides, for example, an aryl acylamidase from Bacillus sphaericus which was shown to hydrolyse various phenylamide herbicides including linuron (Englehardt, Wallnoeffer and Plapp, 1973; Englehardt and Wallnoefer, 1976), a glutathione-s-transferase from pea involved in the degradation of fluorodifen (2,4-dinitro-4-trifluoromethyldiphenylether) (Frear and Swanson, 1973) and an aryl acylamidase from rice (Oryza sativa L.) and tulip (Tulipa L.) (Frear and Still, 1968; Hoagland and Graf, 1972b). The enzyme systems capable of catalysing individual reactions of the major pathways of substituted phenylurea herbicide metabolism, discussed above, have yet to be isolated and characterised.

MATERIALS AND METHODS

Growth of Plants

Seeds of all the plant species used were supplied by Ciba-Geigy, Agrochemicals Division, Cambridge, U.K. or by Ciba-Geigy A.G, Basle, Switzerland. The varieties and their response to chlortoluron, CGA43057 (N'-(3-methyl-4-chlorophenyl)-N,N-dimethylurea) and diuron are listed below.

	VARIETY	RESPONSE TO CHLORTOLURON	CGA43057	DIURON
Triticum aestivum L.	Atou	R	S	S
	Bouquet	R	S	S
	Probus	R	S	S
	Maris Kinsman	S	S	S
	Maris Huntsman	S	S	S
Hordeum vulgare L.	Astrix	R	S	S
	Sonja	R	S	S
	Maris Otter	MR	S	S
	Katy	S	S	S
Avena fatua L.	(type U.S.A.)	S	S	S
Avena sativa L.	Flemish Crown	S	S	S
Lolium perenne L.		S	S	S
Alopecurus myosuroides Huds.		S	S	S
Bromus tectorum L.		S	S	S
Gossypium hirsutum L.	Delta Pine	MR	MR	MR

R: resistant S: susceptible MR: moderately resistant

For experiments requiring untreated plants, seeds of the different varieties were separately sown in 10 cm diameter pots containing John Innes No. 2 compost. For plants treated with herbicide via the soil, the pots contained soil type 'stein' with the following composition: pH 7.65; organic content, 5.3%; water holding capacity, 36.8%; clay, 18.6%; loam, 25%; sand, 56.4%. In certain experiments plants were grown in nutrient solution, in this case seeds were soaked in deionised water for

three hours prior to germination in moist autoclaved vermiculite. Three days after germination, the plants were transferred to a nutrient culture (Table 2) solution of composition similar to that described by Hewitt (1965). The pH of the solution was adjusted to 5.7 with 0.1M-H_2SO_4 when required.

TABLE 2: Nutrient Solution Composition

SALT	CONCENTRATION (mg/L)
KNO_3	202.0
$Ca(NO_3)_2$	656.0
$NaH_2PO_4.2H_2O$	208.0
$MgSO_4.7H_2O$	441.6
$C_6H_5O_7Fe.5H_2O$	38.4
$CH_2.N(CH_2.COONa)_2 .2H_2O$	44.0
$MnSO_4.4H_2O$	2.22
$CuSO_4.5H_2O$	0.24
$ZnSO_4.7H_2O$	0.28
H_3BO_3	1.98
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.035
$(Al)_2SO_3.12H_2O$	0.18
$Ga(NO_2)_3$	0.005
$CoSO_4.7H_2O$	0.028;
$NiSO_4.7H_2O$	0.028

All plants except cotton were grown in either a Conviron (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) or Brown-Boveri (BBC Brown-Boveri A.G, Baden, Switzerland) controlled environment cabinet maintaining day and night temperatures of 20°C and 14°C respectively and a 16 hour day regime with a light intensity of 10,000 lux. In addition, the Brown-Boveri cabinet maintained the relative humidity at 60%. Cotton was grown in a high temperature controlled environment room,

maintaining day and night temperatures of 28°C and 22°C, and relative humidity of 80% and 60% respectively with a 16 hour day regime of light intensity 10,000 lux. Plants were used for experimental studies at various times after germination, the growth stage identified according to the scale of Zadoks, Chang and Konzak (1974).

Application of herbicides to intact plants

For studies of the absorption of chlortoluron, or the timecourse of chlortoluron degradation, nutrient solution grown plants at growth stage 11 were treated with 3.1 ppm carbonyl ^{14}C-chlortoluron (sp. act. 0.21 or 6.502 μCi/μmole) respectively. Nutrient solution grown plants from which chloroplasts were subsequently extracted to study the Hill activity of chloroplasts isolated from pretreated plants, were treated at growth stage 12 with 3.1 ppm chlortoluron or isoproturon for four days prior to harvest.

Certain experiments were undertaken to quantitate the metabolism of chlortoluron, diuron and CGA43057 in soil grown cereals, cereal weeds and cotton. The cereals and cotton were treated with 1.2 mg/pot and the cereal weeds with 0.6 mg/pot carbonyl ^{14}C-chlortoluron (sp. act. 6.502 μCi/μmole) corresponding to field rates of 1.5 and 0.75 kg/ha respectively. Carbonyl ^{14}C-CGA43057 (sp. act. 7.416 μCi/μmole) and carbonyl ^{14}C-diuron (sp. act. 8.018 μCi/μmole) were applied at 0.6 mg/pot to all plants. To facilitate the treatment, seed of the varieties used were placed on soil in 10 cm diameter pots and carefully covered to a depth of 2 cm with 60 g of soil containing the radio-labelled herbicide. Where the intracellular distribution of absorbed chlortoluron or the identity of conjugated metabolites of chlortoluron was examined, soil grown plants were treated as described

above with carbonyl ^{14}C-chlortoluron (sp. act. 6.502 or 2.379 μCi/μmole) respectively. In studies where the oxygen evolution of plants treated with chlortoluron was monitored, nutrient solution grown plants were treated for 24 hours with 3.1 or 8.0 ppm chlortoluron.

Isolation of Chloroplasts

During chloroplast isolation all operations were carried out at 2-4°C, the method used being essentially that described by Owen (1971). Small fragments of leaves were homogenised in a MSE Atomix Blender (MSE Ltd., England) using 40 ml of ice cold homogenising medium per 6-10 g of material. The blender was operated at maximum speed for three 10 second intervals, allowing a 10 second interval between each homogenisation to prevent a rise in temperature. The leaf homogenate was strained through two layers of washed muslin, the leaf debris being discarded. The resulting filtrate was centrifuged at 200 x g for 90 second in a MSE bench centrifuge, the pellet of cell debris was discarded and the supernatant centrifuged at 800 x g for 10 minutes. The chloroplast pellet obtained was washed in 35 ml of the homogenising medium and resuspended in 1 ml of the same medium after sedimentation at 800 x g for 8 minutes. After chlorophyll estimation the chloroplast preparation was diluted so that the suspension contained 600-800 μg chlorophyll per ml. The medium used throughout the extraction procedure consisted of 0.5M sucrose, 0.05M $KHCO_3$ adjusted to pH 7.5. The chloroplast suspensions were maintained in an ice bath and used as quickly as possible.

Though chloroplast preparations of the type obtained by this extraction procedure are sometimes designated as "whole chloroplasts", microscopic

examination has revealed the presence of variable quantities of broken chloroplasts, as well as some contamination by other cell organelles (Owen, 1971). In the present study the chloroplast preparations were not subjected to microscopic examination, but it is likely that they contained similar proportions of whole chloroplasts as those described by Appelqvist, Stumpf and von Wettstein (1968) for sucrose media. For experiments involving the incubation of chloroplast preparations with herbicides, the chloroplast preparation was subjected to osmotic shock by washing once with a small volume of deionised water. By causing complete disruption of the chloroplasts, a uniformly broken chloroplast preparation was obtained in order to facilitate even access of the herbicides to the thylakoid membranes.

Application of substituted phenylurea herbicides and their metabolites to isolated chloroplasts

To determine the inherent sensitivity of chloroplasts to isoproturon, chlortoluron and possible metabolites of chlortoluron, chloroplasts were isolated from cereals and cereal weeds and treated in vitro with these compounds. The solutions of substituted phenylureas referred to in studies where these herbicides were added to chloroplast preparations, were derived from stock solutions in aqueous 5% methanol. The control assays incorporated an equal volume of 5% methanol not containing herbicide. The herbicides in the incubation medium were in a range of concentrations from 2.82×10^{-3}M to 2.82×10^{-10}M. Where metabolites of chlortoluron were added to the incubation medium, the concentrations employed were equal to the I_{50} values for chlortoluron inhibition of Hill reaction activity in the particular cereal variety or cereal weed. In all assays the substituted phenylurea concentration employed was related to the chlorophyll content of the chloroplast preparations.

Determination of chlorophyll

The chlorophyll content of tissue or chloroplast preparations was determined spectrophotometrically by the Arnon (1949) modification of the method of Mackinney (1941). This method is based on the absorption of light by aqueous 80% acetone extracts of chlorophyll using the specific extinction coefficients for chlorophyll a and b given by Mackinney (1941) and subsequently checked by Arnon (1949) and Bruinsma (1961).

For chloroplast preparations, 0.1 ml of the chloroplast suspension was added to 0.9 ml of deionised water and 4 ml of acetone. After vigorous shaking the chloroplast debris and precipitated protein were removed by centrifugation at maximum speed (2000 x g) in a MSE bench centrifuge. The extinction at 645 nm and 663 nm was determined using a Pye-Unicam SP500 spectrophotometer (Pye Unicam, Cambridge, U.K.), in a 1 cm pathlength cuvette using 80% acetone as blank. The chlorophyll content of the chloroplast suspension was calculated by substitution of the extinction values in the following equation.

$$C = \frac{(20.2 \times E645 + 8.02 \times E663)}{20} =$$ mg chlorophyll/ml of chloroplast preparation

where C = concentration
E645 = optical density at 645 nm
E663 = optical density at 663 nm

For the estimation of chlorophyll in plant tissue, the leaf material was homogenised in 80% acetone using an ultra-turrax homogeniser (Janke Kunkel A.G, West Germany) operated at maximum speed for 30 seconds. The extract was adjusted to a volume of 25 ml and stored in the dark at -5°C for 1 hour to facilitate extraction of the chlorophyll. The suspension was clarified and assayed as previously described for the chloroplast

preparations, the chlorophyll content being calculated by substitution of the extinction values in the equation

$$C = \frac{(20.2 \times E645 + 8.02 \times E663)}{40} = \text{mg chlorophyll/25 ml}$$

Protein determination

Protein was determined in suspensions of organelle fractions by the method of Lowry, Rosebrough, Farr and Randall (1951). The reagents used were :

A. 2% Na_2CO_3 in 0.1M NaOH

B. 0.5% $CuSO_4.5H_2O$, 1% sodium or potassium tartrate

C. 50 ml A, 1 ml B

D. Folin and Ciocalteau reagent diluted 1:1 with deionised water

Protein was precipitated from a known volume of the organelle preparation by the addition of trichloroacetic acid (TCA) to a final concentration of 10%. The sample was centrifuged at maximum speed in a MSE bench centrifuge for 1 hour and the supernatant discarded. The precipitated protein was dissolved in 0.5 ml of 0.5M NaOH and the solution neutralised with 0.5 ml of 0.5M HCl. To this sample was added 5 ml of reagent C and the mixture allowed to stand for 10 minutes at room temperature. 0.5 ml of reagent D was then added with rapid mixing and after 30 minutes the blue colour measured at 750 nm using a Pye Unicam SP500 spectrophotometer. A control not containing protein was prepared on each occasion, this solution being used as the blank. Protein content was quantified by reference to a standard curve prepared using crystalline bovine serum albumin.

Spectrophotometric assay of electron transport in chloroplasts

In 1937 it was discovered that a leaf homogenate could evolve oxygen when illuminated in the presence of ferric oxalate (Hill, 1937). Later, Warburg

and Luttgens (1944) demonstrated that p-benzoquinone could be reduced, with concommitant oxygen evolution upon illumination of a crude chloroplast preparation. Some years later Vishniac and Ochoa (1951), Tolmach (1951) and Arnon (1951) independently demonstrated photosynthetic NADP reduction by chloroplasts.

The Hill reaction can be considered as the coupling of electron transport from H_2O to the photoreduction of an acceptor. The final Hill acceptor in photosynthesis *in vivo* is CO_2, whilst in whole chloroplasts *in vitro* the physiological Hill reaction is the photoreduction of NADP. It has been found, however, that numerous artificial compounds such as the dye 2,6-dichlorophenol indophenol (DCPIP) can act as electron acceptors. It should be noted, however, that the complete electron transfer system may not necessarily be operating when a non-physiological acceptor is reduced. In theory an electron transfer chain could reduce an electron acceptor at any of several points in the system, the site of reduction being determined by the redox potential and the accessibility of the acceptor. The photoreduction of indophenol dyes such as DCPIP may be followed by the decline in extinction at 620 nm, an assay which represents one of the more sensitive and convenient methods for measuring Hill reaction activity.

Assay of DCPIP reduction

All assays of DCPIP photoreduction reported in the present thesis were carried out in 1 cm light path glass cuvettes illuminated by white light of an approximate intensity of 15,000 lux. Illumination was provided by a 375 W photographic lamp (Atlas PL/6, E27/27) placed 15 cm from the cuvettes in line with their light paths. A heat filter comprised of a microscope slide staining trough filled with water was incorporated

between the cuvettes and the light source to ensure that the temperature of the assay medium was maintained at 17°C. The assay medium was made up directly in 1 cm light path cuvettes from stock solutions of the following :

A. 0.05M KH_2PO_4, 0.01M KCl, adjusted with KOH to pH 6.5

B. 3.2×10^{-4}M DCPIP

To each cuvette was added 2.5 ml solution A, 0.5 ml solution B and 0.05 ml of the chloroplast suspension. Each reaction cuvette thus contained KH_2PO_4, 125 μmoles; KCl, 25 μmoles; DCPIP, 0.1 μmoles and chloroplasts equivalent to 30-40 μg chlorophyll in a total volume of 3.05 ml. Each experiment incorporated the appropriate control containing 0.5 ml of H_2O in place of the DCPIP. The reaction cuvettes were kept covered with aluminium foil prior to illumination. Following inversion of the cuvettes to obtain thorough mixing of the contents, the initial extinction at 620 nm was measured with a Pye Unicam SP500 spectrophotometer using the minus DCPIP as reference. The cuvettes were illuminated for a series of 30 second or 60 second intervals, the reduction in extinction at 620 nm being determined at the end of each illumination period. The rate of reduction of DCPIP by the chloroplast preparation, expressed as μmoles dye reduced per mg chlorophyll per hour, was determined from the reduction after the first 30 or 60 second period. Reduction proceeded linearly during this period.

The rate of reduction of DCPIP was calculated using the equation :

$$\frac{E \times 1.23}{\varepsilon} \times \frac{3.05}{1000} \times \frac{1000}{\mu g \text{ chlorophyll}} \times 10^6 \times 60 = \mu \text{moles DCPIP reduced per hour per mg chlorophyll}$$

where, ΔE = change in absorbance per minute

ε = molar extinction of DCPIP (22,400), $(l.moles^{-1}\ cm^{-1})$

3.05 = assay medium volume (ml)

1.23 = correction factor, obtained from data given by Punnet (1959) on the effect of pH on the absorbance of solutions of DCPIP (pK = 5.7)

The theoretical extinction at zero time can be calculated from :-

$$E = \varepsilon c\, l$$

where, E = extinction

ε = extinction coefficient, $(l.moles^{-1}\ cm^{-1})$

c = concentration, (moles/l)

l = path length, (cm)

The theoretical extinction is therefore 1.17. In practice the initial optical densities of the reaction cuvettes fell within the range 0.95 to 0.98, so that each cuvette actually contained between 0.129 to 0.133 μmoles DCPIP. The discrepancy from the theoretical value of 0.16 moles can be attributed to the purity of the dye used, which was supplied by BDH Ltd. (Poole, U.K.).

Manometric methods used in studies of gas exchange

In addition to data obtained with isolated chloroplasts, studies on the effects of herbicides on photosynthetic activity in intact plants or sections of plant tissue can provide much relevant information. In contrast to other types of herbicidal action, inhibitory effects on photosynthesis can be easily assessed by measuring gas exchange (Van Oorschot, 1970). When a phenylurea herbicide is absorbed by plant tissue there is a progressive decline in photosynthetic activity. If subsequent uptake of the herbicide is prevented then any recovery from inhibition

of photosynthesis observed may indicate a capacity of the tissue to inactivate the herbicide. It was of considerable interest to determine whether the degree of recovery from inhibition of photosynthesis could be correlated with resistance or sensitivity to chlortoluron and/or isoproturon. The response of net gaseous exchange has in the past been used as a measure of relative tolerance to photosynthetic inhibitors (Van Oorschot, 1965, 1968, 1970; Shimabukuro and Swanson, 1969; Jensen, Stephenson and Hunt, 1977). Rates of recovery of photosynthesis in plants treated with phenylurea herbicides, related to those used in the present study, have been correlated with rates of N-demethylation to less toxic compounds (Swanson and Swanson, 1968a, 1968b; Neptune and Funderburk, 1969).

The basis for gas exchange measurements using Warburg manometry is the determination of pressure changes which occur at constant volume as a result of gas uptake or evolution in a sealed reaction flask connected to a manometer. In manometric studies of photosynthetic oxygen evolution at high levels of illumination a relatively high partial pressure of carbon dioxide is necessary if photosynthesis is not to be carbon dioxide limited, thus the rate of carbon dioxide utilisation must not exceed that of carbon dioxide availability.

In the present study 5 mm sections were cut with a sharp razor blade from the mid-area of the second fully expanded leaf of plants at growth stage 12. Experiments were conducted in a Warburg apparatus (B. Braun, Melsungen, W. Germany) fitted with a bank of incandescent lamps providing illumination of 10,000 lux. Five leaf segments were arranged in a single layer in the main compartment of the Warburg flasks with their adaxial

surfaces in contact with 2.5 ml of 0.05M phosphate buffer pH 7.0. Preliminary experiments had shown that this was the most appropriate number of leaf segments to use routinely, to obtain an easily measurable rate of oxygen evolution whilst ensuring that the carbon dioxide uptake did not exceed the capacity of the carbon dioxide buffer. In the centre well of each flask was placed a piece of convoluted, Whatman No. 42 filter paper and 0.6 ml of a CO_2 buffer, consisting of a diethanolamine/ potassium bicarbonate mixture (Pardee, 1949, modified by Umbreit, Stauffer and Harris, 1972) to maintain a CO_2 atmosphere of 1% at 25°C (the experimental temperature). Oxygen evolution was determined at intervals over a five hour period.

In some experiments leaf segments were incubated on 2 ml of 0.05M phosphate buffer pH 7.0 containing 5 ppm chlortoluron or isoproturon, or no herbicide, at 25°C for 45 minutes prior to determination of oxygen evolution in the Warburg apparatus. Preliminary experiments had indicated that this concentration of chlortoluron or isoproturon would produce a measurable inhibition of oxygen evolution in resistant varieties but not cause a complete cessation of oxygen evolution in the sensitive varieties.

Sample preparation for liquid scintillation counting

Liquid scintillation counting is the most widely used procedure for the measurement of weak β-emitters such as ^{14}C and ^{3}H. Consequently, there has been a rapid development of this technique and the literature on the subject is wide-spread. Reviews on the general principles of liquid scintillation counting, as well as practical aspects, are abundant and comprehensive (Birks, 1964; Rapkin, 1964; Horrocks and Peng, 1971; Peng, 1977).

For liquid scintillation spectrometry, toluene or xylene based solvents are the most efficient light transducers. However, the non-polar characteristics of these scintillators limit their use. Aqueous and polar samples are incompatible unless a non-ionic surfactant such as Triton X-100 is added, in which case an emulsion is formed. p-Dioxane based scintillators were introduced to meet the need for measuring aqueous samples. However, the best p-dioxane based scintillators only attain an efficiency of about 70% of the toluene based systems.

Insoluble materials present special problems, but can be counted with high efficiencies as fine powders suspended in thixotropic gels. Many insoluble labelled materials cannot, however, be counted as suspensions. In these cases the usual procedure is solubilisation or combustion of the sample. Sample solubilisation is most usually achieved with the aid of commercially available solubilisers. Although the composition of these solubilisers has not been revealed, they are normally quaternary ammonium bases incorporating a compatible primary scintillator. However, the most efficient method for counting insoluble samples containing ^{3}H, ^{14}C or ^{35}S is combustion of the sample. The liberated tritrated water, $^{14}CO_2$ or $^{35}SO_2$ can be collected directly and incorporated into a scintillation solution for counting. In this manner impurity quenching and chemiluminescence are significantly reduced or avoided completely.

A major practical problem encountered with liquid scintillation counting is that of quenching. This can take the form of chemical quenching, where there is interference of energy excitation of the scintillator, or colour quenching which involves interference of energy transfer from the excited

scintillator to the photocathode. The second major problem is that of chemiluminescence, which results from chemical reactions between additives or sample and the components of the scintillator. However, standard sample preparation techniques reduce these problems to an acceptable minimum.

Measurement of radioactivity

All measurements of radioactivity were performed with a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3390 or 3375 (Packard Inc., New Jersey, USA).

The following scintillation systems were used :

A. For organic plant extracts or NCS (Radiochemicals Centre Ltd., Amersham, U.K.) digests, 0.1 to 1.0 ml of sample in 10 ml of the cocktail composed of :-

Toluene	1L
PPO (2,5-diphenyloxazole)	5 g

B. For organic and aqueous plant extracts, 0.1 to 1.0 ml of sample in 10 ml of the cocktail composed of :-

Dioxane	1L
Ethyleneglycol monoethylether	200 ml
Naphthalene	60 g
PPO	12 g
POPOP (1,4-bis-2(5-phenyloxazolyl)benzene)	0.6 g

C. For aqueous extracts, 0.85 to 1.0 ml of sample in 10 ml of the cocktail composed of :-

Toluene	1L
Triton-X100 (Hopkin and Williams, Chadwell, U.K.)	500 ml
PPO	5 g

When using the above systems, quench correction was achieved by using the automatic external standard (AES) method. Quench correction curves were prepared by adding increasing amounts of chloroform to the above scintillation cocktails which contained a known amount of ^{14}C-hexadecane. Thus the percentage efficiency of the system could be related to the AES.

D. For suspensions of silica gel removed from thin layer chromatography plates and large aqueous samples the scintillation cocktail consisted of :-

Instagel (Packard Inc., New Jersey, USA)	10 ml	or
PCS (Radiochemicals Centre Ltd., Amersham, U.K.)	10 ml	
H_2O	5 ml	

E. For $^{14}CO_2$, 10 ml of the CO_2 absorption solution plus 10 ml of a scintillation cocktail consisting of :-

Toluene	1L
Ethyleneglycol monoethylether	500 ml
PPO	8.25 g

When using systems D or E, quench correction was made by the internal standard method using ^{14}C-hexadecane.

For sample combustion, up to 100 mg of the material was combusted in a Packard Tri-Carb Sample Oxidiser. The $^{14}CO_2$ was collected in a carbo-sorb/permafluor monophase mixture (Packard Inc.) and automatically injected into a scintillation vial containing the scintillation cocktail. Sample solubilisation was achieved by using NCS, which was added to samples in a ratio of 1 ml/10 mg of tissue. Samples were subsequently maintained at 50°C for two hours after which time the mixtures were neutralised with 0.03 ml of glacial acetic acid and stored at 4°C for 24 hours to reduce chemiluminescence prior to scintillation counting.

Absorption and Distribution of Chlortoluron in Resistant and Sensitive Plants

There is now general agreement that all substituted phenylureas so far examined are primarily absorbed from nutrient solution and soil via the root system (Bucha and Todd, 1951; Donaldson, 1967; Nishimoto, Appleby and Furtick, 1967; Prenderville, Eschel, Schreiber and Warren, 1967). Substantial indirect evidence has been presented in the literature to indicate that translocation of phenylureas is restricted to the apoplast (Minshall, 1956; Crafts and Yamaguchi, 1958; Crafts, 1959; Geissbuhler, Haselbach, Aebi and Ebner, 1963; Strang and Rogers, 1971). In the past, movement of phenylureas within plants has largely been investigated with the aid of biological and gross autoradiographic techniques (Minshall, 1956; Bayer and Yamaguchi, 1965). This latter technique has been refined by Strang and Rogers (1971) who used microautoradiography to study the movement of diuron in cotton at the subcellular level.

Uptake and distribution of chlortoluron in the present study was investigated following exposure of plants at growth stage 11 to 2.57 μCi of (carbonyl-^{14}C)

chlortoluron (sp. act. 0.21 μCi/μmole) in 1L of nutrient solution (see p.49). Plants were harvested at 0, 0.5, 1, 1.5, 2, 3, 3.5 and 6 days after exposure to the herbicide. At each harvest the roots were carefully washed and the rinsings returned to the culture containers. Plant fresh weights were determined and the volume of the culture solutions carefully measured. Ten 5 ml aliquots were subsequently taken for determination of radioactivity (p.61). Nutrient solutions in culture vessels not containing plants were also sampled so that any losses of radioactivity due to volatilisation or decomposition could be determined.

Intracellular distribution of absorbed chlortoluron

Since it is now well documented that the mode of action of the phenylurea herbicides can be explained in terms of their potency as inhibitors of photosynthetic electron transport, significant concentrations of herbicide would be expected to accumulate in the chloroplast fraction of the cell following uptake.

There have been relatively few studies on the intracellular distribution of herbicides. However, one such study by Boulware and Camper (1973) using isolated cells and protoplasts of tomato fruit, indicated that fluorodifen (p-nitrophenyl-α, α, α -trifluoro-2-nitro-p-tolyl ether) an inhibitor of photosynthetic electron transport was concentrated in the chloroplast/nucleus fraction. In contrast, the same authors found that trifluralin (α, α, α -trifluro-2,6-dinitro-N,N-dipropyl-p-toluidine) which uncouples oxidative phosphorylation, was evenly distributed between the mitochondrial and chloroplast/nucleus fractions. Since various herbicides may thus show different patterns of distribution between

subcellular organelles, it was of interest in the present study to determine whether the same herbicide, in this case chlortoluron, might behave differently in this respect in resistant and sensitive varieties.

Plants were grown in soil treated with 36.72 μCi (wheat, variety atou) or 18.36 μCi (A. fatua, USA) of (carbonyl-^{14}C)chlortoluron (sp. act. 6.50 μCi/μmole). The plants were harvested at growth stage 12 and the root and shoot tissue separately homogenised in an atomix (MSE, U.K.) at maximum speed for 10 seconds with 5 ml per gram fresh weight of a buffer containing sorbitol, 0.3M; KCl, 0.01M; tricine, 0.05M; adjusted to pH 7.3. The resulting homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at 200 x g for 5 minutes. The resulting supernatant fraction was subjected to differential centrifugation for 10 minutes at 1500 x g (shoots only), 20 minutes at 9000 x g and finally 80 minutes at 100,000 x g. Each fraction was washed in 10 ml of the homogenising buffer and resedimented. All fractions were freeze-dried and subsequently exhaustively extracted with 80% aqueous methanol prior to separation and quantification of the radioactivity by TLC and liquid scintillation counting (p. 68 and p.60 respectively). The non-extractable radioactivity was quantitated by liquid scintillation counting after NCS digestion of the extracted tissue (p.60).

Organelle characterisation

The distribution of chloroplasts amongst fractions obtained by differential centrifugation of the plant homogenates was ascertained on the basis of their chlorophyll content. The distribution of mitochondria was determined by assay of succinate-cytochrome c oxidoreductase and succinate-DCPIP oxidoreductase, using the spectrophotometric methods of Martin and Morten

(1956) and Brooker and Russel (1975) respectively. Microsomal containing fractions were characterised by the assay of NADPH-DCPIP oxidoreductase, NADPH-cytochrome c oxidoreductase and NADH-cytochrome c oxidoreductase using the spectrophotometric procedures of Dignam and Strobel (1975), Brooker and Russel (1975) and Donaldson, Tolbert and Scharrenberger (1972) respectively. Rates of marker enzyme activity were expressed as μmoles/mg protein/hour. The data obtained enabled the distribution and percentage contamination of each fraction to be determined.

Metabolism of Chlortoluron, Diuron and CGA43057

Although herbicides have been in use for many years, the attention paid to their degradation by plants has not been great until comparatively recently (Naylor, 1977). The few excellent reviews which have appeared (Ashton and Crafts, 1973; Frear, Swanson and Tanaka, 1974; Kearney and Kaufman, 1975) have served to demonstrate that such degradation is of great significance to the mode of action, the resistance of the plants and the residues remaining in the plants at harvest.

It has been demonstrated for a number of herbicides that differences in their rates of degradation by plant species may offer at least a partial explanation for their differential susceptibility (Geissbuhler et al 1963a; Swanson and Swanson, 1968; Frear, Swanson and Tanaka, 1969; Shimabukuro, Lamoureux, Frear and Bakke, 1971; Osgood, 1972; Muller, Frahm and Sanad, 1977). The major pathway for the degradation of substituted phenylureas has in the past been found to consist of a stepwise N-demethylation and/or demethoxylation, often followed by conjugation of

intermediates or reaction products (Naylor, 1977). More directly relevant to the present investigation, however, are the results of a study on the metabolism of chlortoluron in plant (wheat) and soil systems (Gross et al, 1979), which clearly demonstrated that in addition to N-demethylation, oxidation of the 4-methylphenyl group was a major degradative route for the herbicide.

In the present study, experiments were conducted to compare the nature and extent of the metabolism of chlortoluron and the related phenylureas diuron and CGA43057 in chlortoluron resistant and sensitive plants. For most experiments seed of the varieties under study was placed on soil (type stein) contained in 10 cm diameter pots and carefully covered with soil containing the radio-labelled herbicide. The cereal varieties and cotton were treated with 1.2 mg and the cereal weeds with 0.6 mg/pot ^{14}C-chlortoluron (sp. act. 6.502 μCi/μmole). ^{14}C-Diuron (sp. act. 8.018 μCi/μmole) and ^{14}C-CGA43057 (sp. act. 7.416 μCi/μmole) were applied to all plants at the rate of 0.6 mg/pot. Soil containing no plants was also treated with chlortoluron to determine if any breakdown of chlortoluron in the soil occurred during the course of the experiment. Thus the possibility of the entry of herbicide metabolites into plants under study could be assessed.

In experiments designed to study the time course of the degradation of chlortoluron in resistant and sensitive plants, nutrient solution grown plants were exposed to 3.1 ppm ^{14}C-chlortoluron (sp. act. 6.502 μCi/μmole) for two days after which they were transferred to nutrient solution not containing herbicide. This study was subsequently extended to determine whether the roots or the shoots were the primary site of chlortoluron

degradation. For this purpose root and shoot segments of nutrient solution grown plants were exposed to 3 μCi of ^{14}C-chlortoluron (sp. act. 6.502 μCi/μmole) in 5 ml of 0.05M phosphate buffer pH 7 containing 1% sucrose, for two hours. At the end of the incubation period the root and shoot segments were transferred to 5 ml of 0.05M phosphate buffer pH 7 containing 1% sucrose and 250 μg of streptomycin.

Routinely, soil grown plants were harvested ten days after sowing, nutrient solution grown plants zero, one, two, three and seven days, and root and shoot segments 0, 4, 8, 12 and 24 hours, after cessation of exposure to ^{14}C-chlortoluron. All plants, with the exception of those selected for autoradiography were washed and separated into roots and shoots prior to determination of fresh weight. The parent herbicide and metabolites were normally extracted from root and shoot tissue by homogenising in an Omni-Mix with 80% aqueous methanol (50 ml/gm tissue) at maximum speed for one minute. After filtration under vacuum through a sintered glass funnel (pore size G.3), the volume of the methanolic extract was reduced by vacuum distillation at 35^{o}C in a rotary evaporator (Buchi Glassaparatefabrik, Switzerland). In the case of root and shoot segments the tissue was lyophilised over night and subsequently ground to a powder prior to extraction with 80% aqueous methanol (50 ml/gm tissue). The methanolic extract was centrifuged at 30,000 x g for 20 minutes and the plant residue washed by shaking with a further volume (20 ml/gm tissue) of 80% aqueous methanol followed by centrifugation at 30,000 x g for 20 minutes. In all cases the aqueous and methanolic extracts were adjusted to a known volume and total extractable radioactivity determined by liquid scintillation counting of aliquots of the extracts. The extracts were then

reduced to dryness and the residues dissolved in a small volume of 80% aqueous methanol. A known amount of each sample was subsequently subjected to thin layer chromatography on pre-coated plates (silica gel F_{254} with a layer thickness of 0.25 mm) irrigated with one of the following solvent systems :-

for chlortoluron and metabolites

1. first dimension: chloroform, ethanol 9:2, v/v
   second dimension: acetonitrile, water, ammonia 40:9:1, v/v/v

2. first dimension: chloroform, ethanol, acetic acid 9:1:1, v/v/v
   second dimension: chloroform, ethanol, acetone 9:2:1, v/v/v

3. first dimension: ether, ethanol, acetone, acetic acid 9:2:1:0.1%, v/v/v + v
   second dimension: chloroform, ethanol, acetic acid 9:2:1, v/v/v

4. chloroform, ethanol 1:1, v/v

5. chloroform, ethanol 9:2, v/v

6. chloroform, ethanol, acetic acid 9:1:1, v/v/v

for CGA43057 and metabolites

1. first dimension: chloroform, ethanol, acetic acid 9:1:1, v/v/v
   second dimension: chloroform, ethanol, acetone 9:2:1, v/v/v

2. first dimension: ether, ethanol, acetone, acetic acid 9:2:1:0.1%, v/v/v + v
   second dimension: chloroform, ethanol, acetic acid 9:2:1, v/v/v

for diuron and metabolites

1. benzene, acetone 2:1, v/v
2. chloroform, ethyl acetate 1:1, v/v
3. chloroform, ethanol 1:1, v/v

Herbicides and their metabolites were identified by comparison to R_f values of authentic reference compounds applied to adjacent zones on the TLC plates and by co-chromatography in several solvent systems. After development of the chromatograms the radioactive zones were located using an LKB-2105 radiochromatogram camera and the areas to be eluted defined with the aid of an Optiskop-20 image projector (LKB, Broma, Sweden). Alternatively radioactive zones were both located and defined with a Birchover sparkchamber (Birchover Instruments Ltd., Letchworth, U.K.). Radioactivity was quantitated by liquid scintillation counting as described on page 61. In addition to non-polar metabolites, a number of water soluble conjugates were found to be present in the plant extracts. The non-polar metabolites were separated from these latter compounds by preparative thin layer chromatography on pre-coated plates (silica gel F_{254} with a layer thickness of 0.5 mm) irrigated with ether, ethanol, acetone, acetic acid 9:2:1:0.1% (v/v/v + v). The conjugated radioactive material was then concentrated as a narrow band slightly removed from the origin by development with methanol for 2 cm. The zone containing the conjugated radioactive material was eluted from the TLC plates with 80% aqueous methanol, reduced to dryness and redissolved in 5 ml of 1M phosphate-citrate buffer pH 4.5, prepared immediately prior to use. The conjugated material was hydrolysed by incubation with 3-5 mg of almond β-glucosidase (Fluka, A.G., Switzerland) at 37°C in a shaking water bath

for four hours. The incubation solution was repeatedly partitioned with ether in a Kutscher-Steudel apparatus (Buchi, Glassaparatefabrik, Switzerland) to extract the non-polar metabolites produced. The degree of cleavage of the conjugated radioactivity was assessed by determining the amount of radioactivity still remaining in the aqueous phase. The ether phase was subsequently reduced to dryness and the residue dissolved in a small volume of 80% aqueous methanol for characterisation and quantification as described above.

In the case of data obtained for plants treated with radioactive material via the soil, any involvement of soil degradation in the production of the observed metabolites was assessed by characterisation and quantification of the radioactivity extracted from soil of control pots not containing plants. Such soil samples were extracted with ten volumes of 80% aqueous methanol for four hours. Non-extractable radioactivity associated with both soil and plant material was determined by combustion and liquid scintillation counting (p. 62).

In the study of the quantitative degradation of chlortoluron, diuron and CGA43057 in chlortoluron resistant and sensitive plants, a number of plants of each variety were selected for autoradiography. These plants were placed in contact with Structurix D7 Röntgenfilm (Agfa-Gaevert, Antwerp, Belgium) for a period of two weeks.

Characterisation of the glycone moiety of the polar metabolites of chlortoluron

Until recently most pesticide metabolism studies emphasised the isolation, identification and toxicity of primary metabolism products. Recently,

however, increased interest has been focused on the nature and significance of a variety of conjugated pesticide metabolites (Menzer, 1963; Frear and Swanson, 1973, 1975; Lamoureux, Stafford and Tanaka, 1971; Lee, Griffin and Fang, 1973) including a number of glycosides. Increasing evidence suggests that the rate and extent of glycoside formation is a significant factor in regulating the biological activity and selectivity of pesticides and their metabolites. However, relatively few studies have reported the isolation and identification of pesticide metabolites as glycosides. The continuing search for more selective and less persistent pesticides, together with concern over pesticide residues, suggest that basic studies on glycoside metabolism should provide needed insights into the behaviour and fate of pesticides. Thus it was of interest to characterise the cellular component(s) conjugated to metabolites of chlortoluron in the present study.

For this purpose soil grown plants of one wheat variety (atou) were treated with carbonyl ^{14}C-chlortoluron (sp. act. 2.379 μCi/μmole) as described on p. 49; untreated (control) plants were also included in the study. Plants were harvested and extracted as described above. Following removal of the methanol from the 80% aqueous methanol extracts, the resulting aqueous extract was partitioned for eight hours in a liquid/liquid partition unit (Corning Laboratory Division, Staff., U.K.) to remove non-conjugated metabolites. The aqueous phase was subsequently subjected to ultra-filtration using an Amicon model 202 fitted with a type UM-10 membrane (declared cut off - 10,000) operating at a pressure of 3.103 megapascals. The membrane was washed after use and the washings were combined with the filtrate. The extract was then desalted using the mixed ion exchange resin Zerolite DM-F (BDH, Poole, U.K.) previously activated with 10%

acetic acid. The extracts were then subjected to gel filtration using Sephadex G-15 (Pharmacia, Uppsala, Sweden) in a column of dimensions 80 cm x 1.5 cm. Deionised water at a flow rate of 25 ml/hour was used as eluant and 5 ml fractions were collected. Samples were taken from each fraction and subjected to liquid scintillation counting to determine the location of the conjugated chlortoluron metabolites. Protein was removed from the extracts by the addition of ethanol to a final concentration of 70%, the ethanolic solutions were subsequently centrifuged at 2000 x g in a MSE bench centrifuge to remove the precipitates.

The conjugates were separated from contaminating plant sugars by preparative chromatography on Whatman No. 3 paper, with a mobile phase of ethyl acetate/acetic acid/water (9:2:2, v/v/v). The conjugates were located with a Birchover Spark Chamber, whereas the plant sugars and co-chromatographed sugar standards were visualised with the p-aminobenzoic acid reagent of Menzies, Mount and Wheeler (1978). The zones corresponding to the conjugated metabolites were exhaustively eluted with water, the eluant being filtered through a Whatman glass microfiber filter (GF/F). At each stage of the above clean-up procedure samples of the extracts were subjected to thin layer chromatography and liquid scintillation counting, thus the recovery of the conjugated metabolites could be determined.

Aliquots of the aqueous extracts were hydrolysed with either 0.1M trifluoroacetic acid for one hour at 100°C in a sealed reaction flask, or, β-glucosidase at 37°C for five hours in McIlvaine buffer pH 4.4. The reaction solutions were subsequently reduced to dryness under vacuum and

redissolved in 100 µl of deionised water. Initial identification and quantification of the released glycone moieties was achieved using the thin layer chromatography procedure of Menzies et al (1978). Suitable sugar standards, unhydrolysed extract and 5-10 µl of the hydrolysed samples containing lactose as the internal marker, were applied as 1.5 cm bands to silica gel F1800 plastic backed thin layer chromatography plates with a layer thickness of 0.25 mm. The plates were subsequently developed three times using ethyl acetate/pyridine/acetic acid/water (6:3:1:1 v/v/v/v) as the mobile phase. Sugars were located with the p-aminobenzoic acid reagent and quantified using a double-beam recording, integrating densitometer (Joyce-Loeble, West Germany) fitted with a quartz-iodine light source and blue filter operating with a slit width of 5 x 0.55 mm. Peak heights were measured from the scan profiles and corrected by applying the internal marker factor. Confirmatory quantification and identification was obtained by the glucose oxidase assay (Fleming and Pegler, 1963) and further thin layer chromatography with mobile phases of ethyl acetate/ethanol/pyridine/acetic acid/water (6:2:1:1:1 v/v/v/v/v) or ethyl acetate/pyridine/acetic acid/water (7:1.5:1:1 v/v/v/v). The aglycone moieties were identified and quantified by two dimensional thin layer chromatography and liquid scintillation counting as described previously.

Studies on the sub-cellular localisation of the chlortoluron metabolising enzyme system(s)

In vivo studies of the metabolism of phenylurea herbicides by higher plants have shown that the herbicides can be subjected to oxidation, reduction, hydrolysis and conjugation reactions (Ashton and Crafts, 1973). Furthermore,

a number of studies have revealed that the selective action of many phenylureas is related to differential metabolism by plants of resistant and sensitive species (Geissbuhler et al, 1963; Smith and Sheets, 1967; Swanson and Swanson, 1968). However, the use of in vitro systems has received little attention even though there could be many advantages to be gained from such an approach (Frear, Swanson and Tanaka, 1972). Indeed, as it is now well known that the microsomal fraction of animal systems is capable of metabolising xenobiotics (Williams, 1959; Conney, 1967; Sum and Cho, 1976; Nelson, Kearney, Plimmer and Menzer, 1977; Powis, Lyon and McKillop, 1977) and that the microsomal fractions of both animal and plants share several biochemical similarities. It is possible that many of the enzyme systems responsible for pesticide metabolism in plants may reside in the microsomal fraction.

The only previous reports of the metabolism of substituted phenylurea herbicides by a plant microsomal system are those of Frear and co-workers (Frear, 1968; Tanaka, Swanson and Frear, 1972a,b). These authors succeeded in isolating from cotton and certain other species a microsomal mixed function oxidase system capable of N-demethylating monomethyl- and dimethyl-substituted phenylureas. The N-demethylation reaction was dependent on the presence of NADPH and molecular oxygen, and was most active in a smooth endoplasmic reticulum preparation.

The method employed for the isolation of subcellular organelle fractions from plants used in the present study was an adaption of the procedure of Frear (1968). Plants at several growth stages were employed, most usually at growth stage 11 to 12. Routinely, 10 g of excised leaf or

root tissue was washed with deionised water and then ground to a fine powder in a pestle and mortar with liquid nitrogen and 2 g of acid washed sand. The frozen leaf powder was then slurried with 50 ml of 0.3M potassium phosphate buffer, pH 7.5 containing 0.3M mannitol, 0.1M sodium isoascorbate, 1 mM sodium cyanide and 2 g Polyclar AT (insoluble polyvinyl polypyrrolidone). After standing for 15 minutes with occasional stirring, the slurry was squeezed through cheesecloth and subjected to differential centrifugation. The cell free extract (supernatant after centrifugation at 300 x g for 5 minutes) was centrifuged at 1500 x g for 10 minutes, 9000 x g for 20 minutes and 100,000 x g for 80 minutes. The pellets were washed by resuspension in 10 ml of the homogenising medium minus Polyclar AT followed by resedimentation. The pellets were finally resuspended in a small volume of 0.01M potassium phosphate buffer, pH 7.5 containing 1 mM NaCN and were used for assays as soon as possible. All enzyme extraction and centrifugation procedures were carried out at $4^{o}C$.

Enzyme activity was assayed by determining the amount of substrate remaining at the end of the incubation period. The standard reaction mixture, adjusted to pH 7.5, contained 6.99 nm (carbonyl ^{14}C)chlortoluron (sp. act. 6.502 μCi/μmole), 0.05μmole NaCN, 50μmole K_2HPO_4 and an NADPH generating system consisting of 2.0μmole NADP, 2.0μmole glucose-6-phosphate and 0.2 units glucose-6-phosphate dehydrogenase. The NADPH generating system was incubated separately for 5 minutes at $25^{o}C$. Reactions were initiated by the addition of the NADPH generating system, the reaction vessels being maintained in a shaking water bath for a period of time between 30 minutes and 2 hours. Each experiment incorporated control assays from which the NADPH generating system was omitted or the

enzyme preparation boiled. The reactions were terminated by rapid freezing in a dry ice-acetone bath followed by lyophilisation. The lyophilised reaction mixtures were subsequently extracted with a small volume of 80% aqueous methanol and the reaction products characterised and quantitated by thin layer chromatography and liquid scintillation counting as described previously (p. 68 and p. 60 respectively). Insoluble radioactivity was quantitated by liquid scintillation counting after NCS digestion (p. 60).

RESULTS AND DISCUSSION

Effect of Chlortoluron and Isoproturon on Electron Transport in Photosynthesis

The Hill activity of chloroplasts isolated from intact plants and treated with herbicide in vitro

The Hill activity of isolated chloroplasts as measured by the photoreduction of dichlorophenol indophenol (DCPIP) was assessed by the procedure described in the appropriate sections of the preceeding chapter. Chloroplasts were isolated from resistant and susceptible varieties of wheat and barley, and a susceptible wild oat. Their Hill activity was subsequently assayed in the presence of various concentrations of chlortoluron and isoproturon.

It is well documented that phenylureas interfere with the light dependent phase of photosynthesis (Wessels and Van der Veen, 1956; Gingras and Lemasson, 1965) being potent inhibitors of electron transport (Dicks, 1978; Ducruet and Lavorel, 1974; Lee and Fang, 1972). The present investigation was initiated to ascertain whether the resistance or susceptibility of varieties could be correlated with inherent differences in the sensitivity of the chloroplast to chlortoluron. Isoproturon is structurally related to chlortoluron and has a qualitatively similar herbicidal spectrum, it was therefore included for comparative purposes.

The pI50 values for individual varieties calculated by probit analysis of the depression of DCPIP photoreduction induced by various concentrations of the herbicides (Fig. 5a 5b, 6a 6b) are presented in Table 3. Although there are differences in the dose response curves of the varieties, and differences in the log concentration of the herbicide which causes a 50% inhibition of DCPIP photoreduction (pI50), these do not correlate with the known resistance or susceptibility of the varieties. This indicates that resistant and susceptible varieties are not distinguishable on the

basis of any differences in the inherent sensitivity of isolated chloroplasts to chlortoluron. The similarity of the response to isoproturon, which generally does not elicit a differential response from cereals supports this view.

Table 3: Inhibition of photoreduction of DCPIP by chloroplasts treated in vitro with chlortoluron or isoproturon

	Variety		Chlortoluron pI50	Isoproturon
Triticum aestivum L.	Atou	R	$\overline{6}.238$	$\overline{6}.697$
	Bouquet	R	$\overline{7}.130$	$\overline{6}.987$
	M. Huntsman	S	$\overline{6}.277$	$\overline{6}.121$
	M. Kinsman	S	$\overline{6}.959$	$\overline{6}.909$
Hordeum vulgare L.	Astrix	R	$\overline{6}.509$	$\overline{6}.213$
	Sonja	R	$\overline{6}.920$	$\overline{6}.653$
	M. Otter	MR	$\overline{6}.281$	$\overline{6}.152$
	Katy	S	$\overline{6}.933$	$\overline{5}.406$
Avena fatua L.	USA	Si	$\overline{7}.012$	$\overline{6}.458$

Reactions were carried out in spectrophotometric cuvettes. Each reaction cuvette contained (μmoles): KH_2PO_4, 125; KCl, 25; DCPIP, 0.1; chloroplasts equivalent to 30-40 μg chlorophyll; and various concentrations of chlortoluron, isoproturon or aqueous methanol where required.

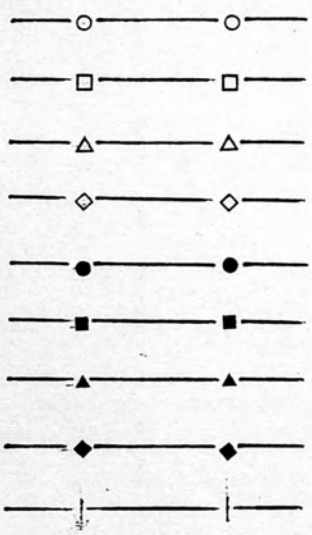
R = Displays resistance to chlortoluron and isoproturon

MR = Displays medium resistance to chlortoluron and resistance to isoproturon

S = Displays susceptibility to chlortoluron and resistance to isoproturon

Si = Displays susceptibility to both chlortoluron and isoproturon.

The reaction conditions were as shown for Table 3.



Atou

Bouquet

Kinsman

M. Huntsman

Astrix

Sonja

M. Otter-

Katy

Wild Oat

Inhibition of DCPIP photoreduction of chloroplasts by chlortoluron

Fig. 5a: Wheat

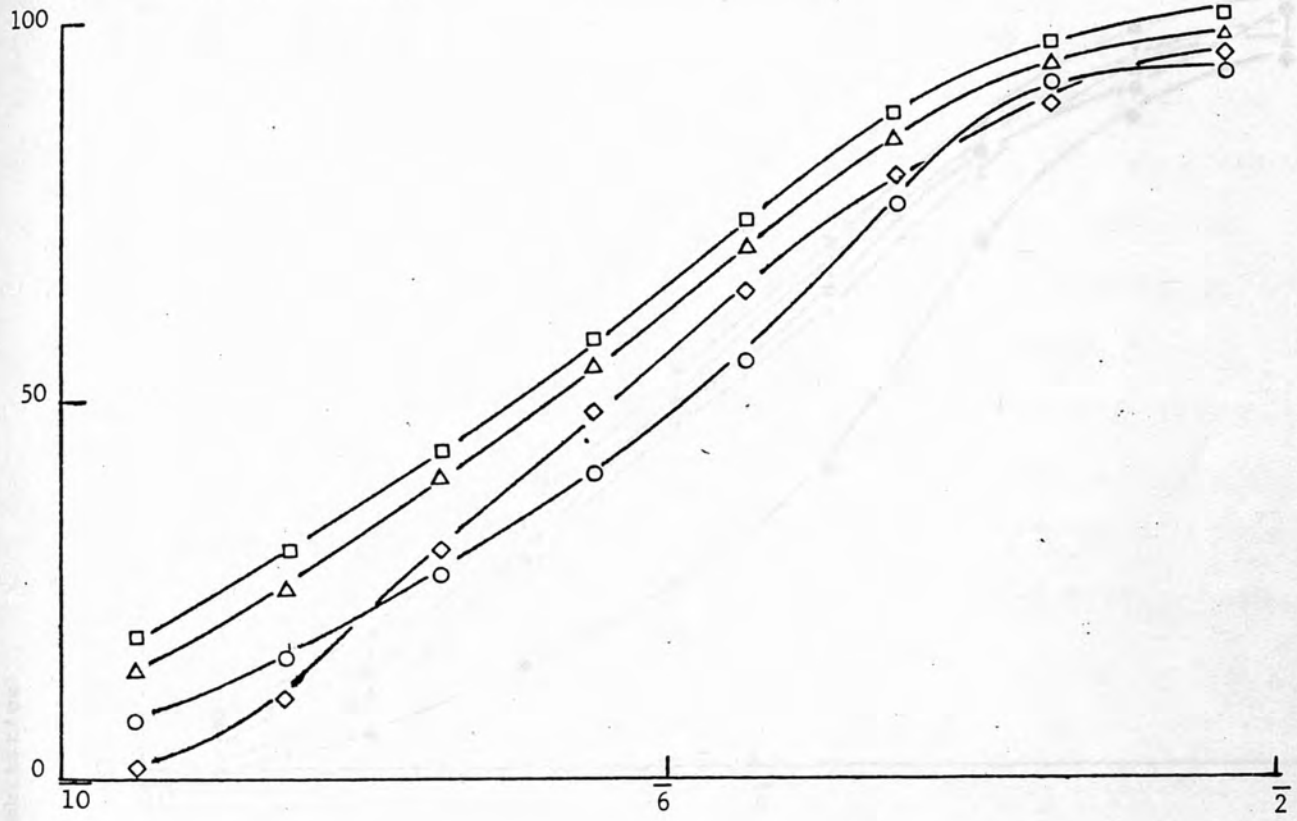
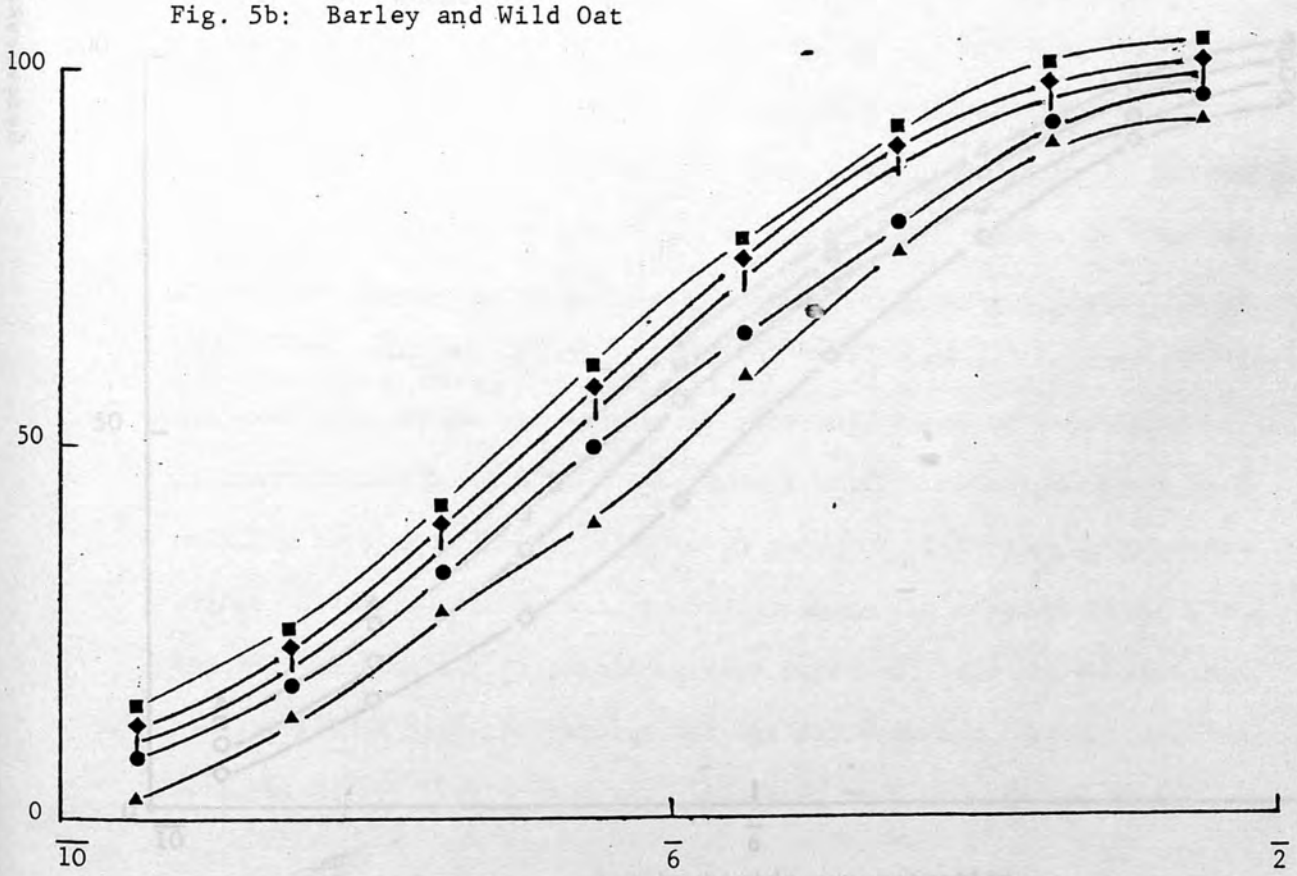


Fig. 5b: Barley and Wild Oat



percentage inhibition

Inhibition of DCPIP photoreduction of chloroplasts by isoproturon

Fig. 6a: Barley and Wild Oat

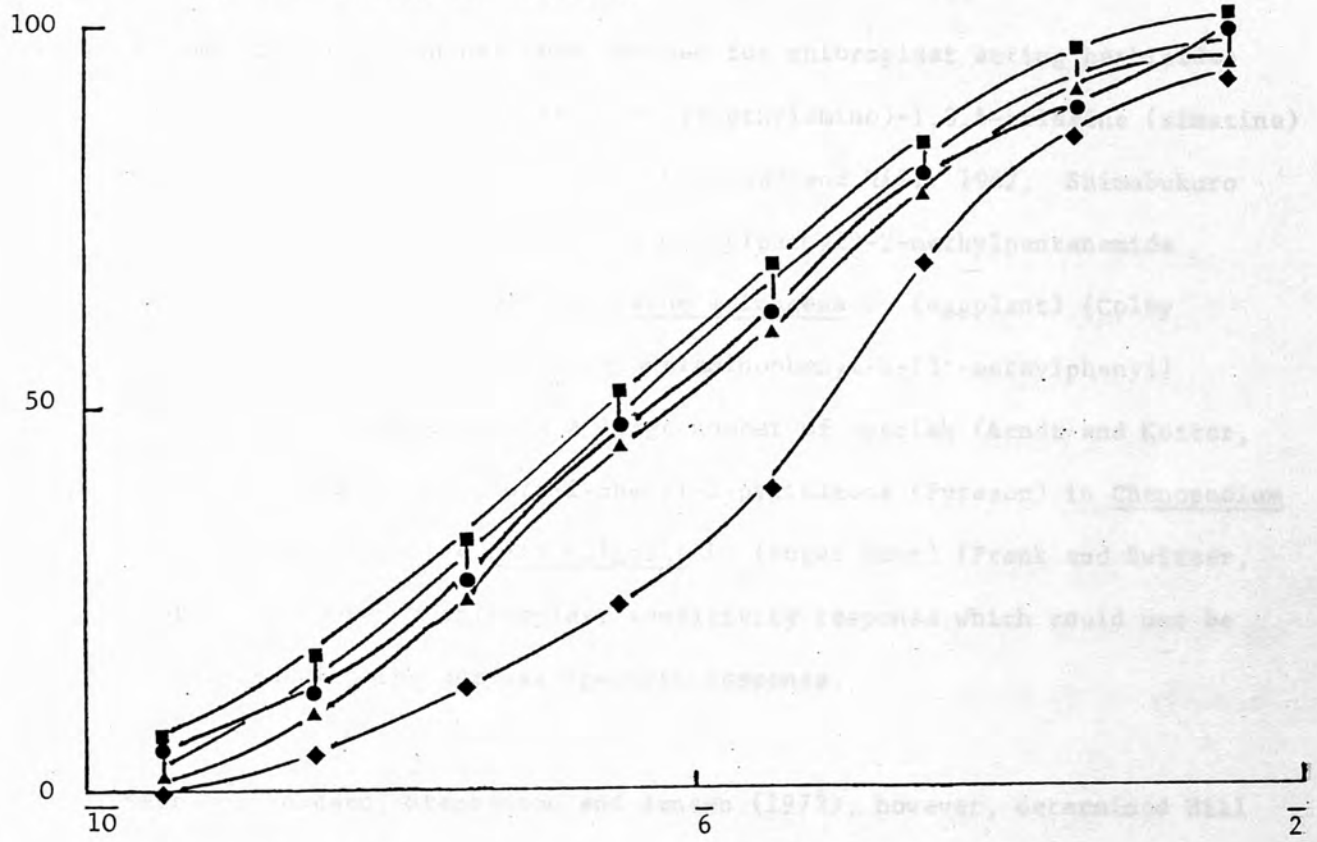
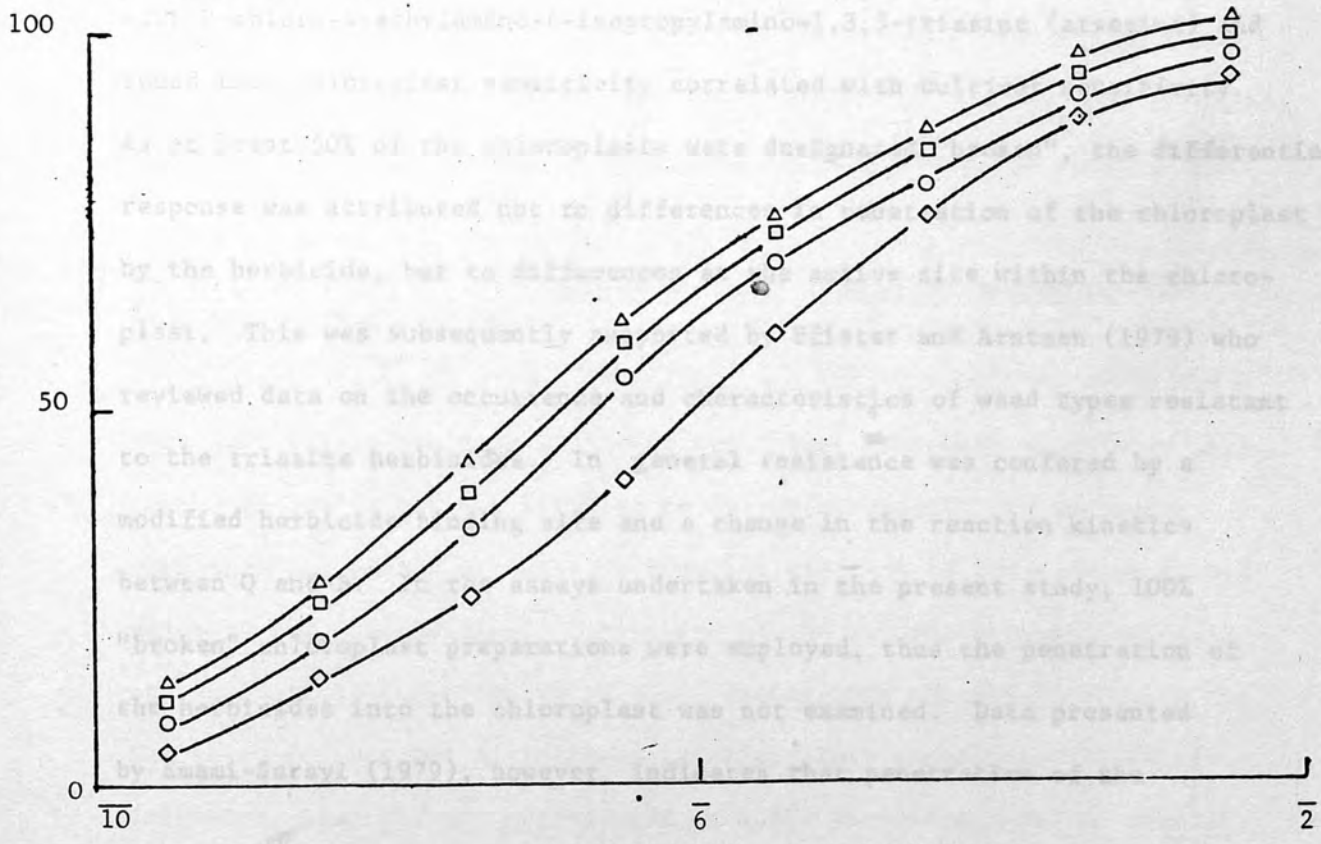


Fig. 6b: Wheat



percentage inhibition

log herbicide concentration

A similar conclusion has been reached for chloroplast acting herbicides in various species; 2-chloro-4,6-bis(ethylamino)-1,3,5-triazine (simazine) in Brassica spp, pea and sorghum (Moreland and Hill, 1962; Shimabukuro and Swanson, 1969), N-(3-chloro-4-methylphenyl)-2-methylpentanamide (Pentanochlor) in tomato and Solanum melongena L. (eggplant) (Colby and Warren, 1965), 3-methoxycarbonylaminophenyl-N-(3'-methylphenyl) carbamate (Phenmedipham) in a large number of species (Arndt and Kotter, 1968), and 5-amino-4-chloro-2-phenyl-3-pyridazone (Pyrazon) in Chenopodium album L. (fat hen) and Beta vulgaris L. (sugar beet) (Frank and Switzer, 1969), all showed a chloroplast sensitivity response which could not be correlated with the species specific response.

Machado, Bandeen, Stephenson and Jensen (1977), however, determined Hill reaction inhibition in isolated chloroplasts of fat hen biotypes, treated with 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (atrazine) and found that chloroplast sensitivity correlated with cultivar sensitivity. As at least 50% of the chloroplasts were designated "broken", the differential response was attributed not to differences in penetration of the chloroplast by the herbicide, but to differences at the active site within the chloroplast. This was subsequently supported by Pfister and Arntzen (1979) who reviewed data on the occurrence and characteristics of weed types resistant to the triazine herbicides. In general resistance was confered by a modified herbicide binding site and a change in the reaction kinetics between Q and B. In the assays undertaken in the present study, 100% "broken" chloroplast preparations were employed, thus the penetration of the herbicides into the chloroplast was not examined. Data presented by Emami-Saravi (1979), however, indicates that penetration of the

chloroplast by the closely related herbicide metoxuron, is similar in resistant and susceptible cultivars.

There are limitations which must be recognised when investigating the action of herbicides via the use of isolated chloroplasts. Compared to the relatively simple situation in isolated chloroplasts, that in intact plants is many fold more complicated. The accumulation of a herbicide in the chloroplast is determined by the physical and chemical properties of the herbicide and by the plants physiological and biochemical characteristics. Thus, in the present in vitro studies in which chlortoluron and isoproturon have arrived at the sites of action within the chloroplast by mainly physical processes, no distinction exists between resistant and susceptible varieties. This result does not, however, discount the possibility that chloroplasts isolated from chlortoluron or isoproturon treated plants, in which physiological phenomena are also involved in the subsequent distribution of the absorbed herbicide, may show different degrees of inhibition of photosynthetic activities.

The Hill activity of chloroplasts isolated from chlortoluron or isoproturon treated plants

Chloroplasts isolated from chlortoluron and isoproturon treated plants some days after treatment will have herbicidal compounds associated with them. It was of obvious interest to determine whether the herbicidal compounds associated with the chloroplast fraction of the treated plants affected the photosynthetic activities of the chloroplasts of resistant and susceptible varieties to an equal extent. As the previous study has indicated no inherent differences in chloroplast sensitivity to chlortoluron or isoproturon, differences in DCPIP photoreduction in the present study would indicate variations in the total amount of active

herbicidal material present in chloroplasts from resistant and susceptible varieties.

In the present investigations, chloroplasts were isolated from varieties of wheat, barley and a wild oat cultivar. The percentage inhibition of DCPIP photoreduction by chloroplasts isolated from resistant and susceptible plants is presented in Table 4. These results indicate that the photochemical activity of chloroplasts isolated from susceptible cereal varieties treated with chlortoluron is depressed to a substantially greater extent than that of chloroplasts isolated from resistant cereal varieties. The largest percentage inhibition of DCPIP photoreduction occurs in chloroplasts

Table 4: Percentage inhibition of DCPIP photoreduction of chloroplasts isolated from chlortoluron and isoproturon treated plants

	Variety		Chlortoluron treated	Isoproturon treated
			Percentage inhibition of DCPIP photoreduction	
Triticum aestivum L.	Atou	R	13.8	7.1
	Bouquet	R	7.5	8.7
	M. Huntsman	S	40.6	3.2
	M. Kinsman	S	44.3	7.3
Hordeum vulgare L.	Astrix	R	0	0
	Sonja	R	5.3	1.2
	M. Otter	MR	22.3	20.4
	Katy	S	38.3	27.7
Avena fatua L.	USA	Si	60.4	70.1

The reaction conditions were as given previously in Table 3 with the exception that no herbicide was added to the reaction vessels. The photoreduction of DCPIP by chloroplasts isolated from untreated plants were in the range 55 - 71.9 μmole DCPIP reduced/mg chlorophyll/hour. R, MR, S and Si are as presented for Table 3.

isolated from the wild oat cultivar. As regards chloroplasts isolated from isoproturon treated plants, there is less inhibition of DCPIP photoreduction in all the cereal varieties. There remains, however, a considerable inhibition in chloroplasts isolated from the wild oat cultivar. M. Otter appears to be intermediate in sensitivity towards chlortoluron. In general the susceptible barley varieties are less sensitive to chlortoluron than the susceptible wheat varieties. This situation is reversed when the response to isoproturon is examined. The above results thus demonstrate a clear distinction between resistant and susceptible varieties in response to treatment with chlortoluron. The cereal varieties were relatively resistant to isoproturon, whereas the wild oat cultivar displayed extreme sensitivity. This reflects the varietal differences noted in field and glasshouse trials (Hewson, 1974; Van Hiele, Hommes and Vervelde, 1970; Lelley, 1972; Langeluddeke, 1976) where the application rate of chlortoluron recommended for the control of wild oats could prove toxic to certain cereal varieties. The control of wild oats by isoproturon, however, was more satisfactory seldom eliciting a toxic response from any of the cereal varieties. The results of the present study also support field observations that the response of wheat varieties is more extreme than that of barley varieties (Hewson, 1974). It is of interest that the difference in percentage inhibition of DCPIP photoreduction between the wild oat cultivar and the cereal varieties after isoproturon treatment is larger than the concomitant difference in chloroplasts isolated from chlortoluron treated plants. This may well explain the greater selectivity noted in the field for isoproturon.

The results from the present study, when taken in conjunction with the data from the previous study, indicate that there is less herbicidal material present in the chloroplasts of chlortoluron treated resistant plants four days after treatment, than in chlortoluron treated sensitive plants. In vitro experimentation, however, provides no information on the method by which a lower amount of herbicidal material is accumulated in the chloroplasts of resistant plants than in those of sensitive plants. Such information could only be gained from a study of intact plants, or to a lesser extent plant tissue.

Effect of Chlortoluron and Isoproturon on Oxygen Evolution

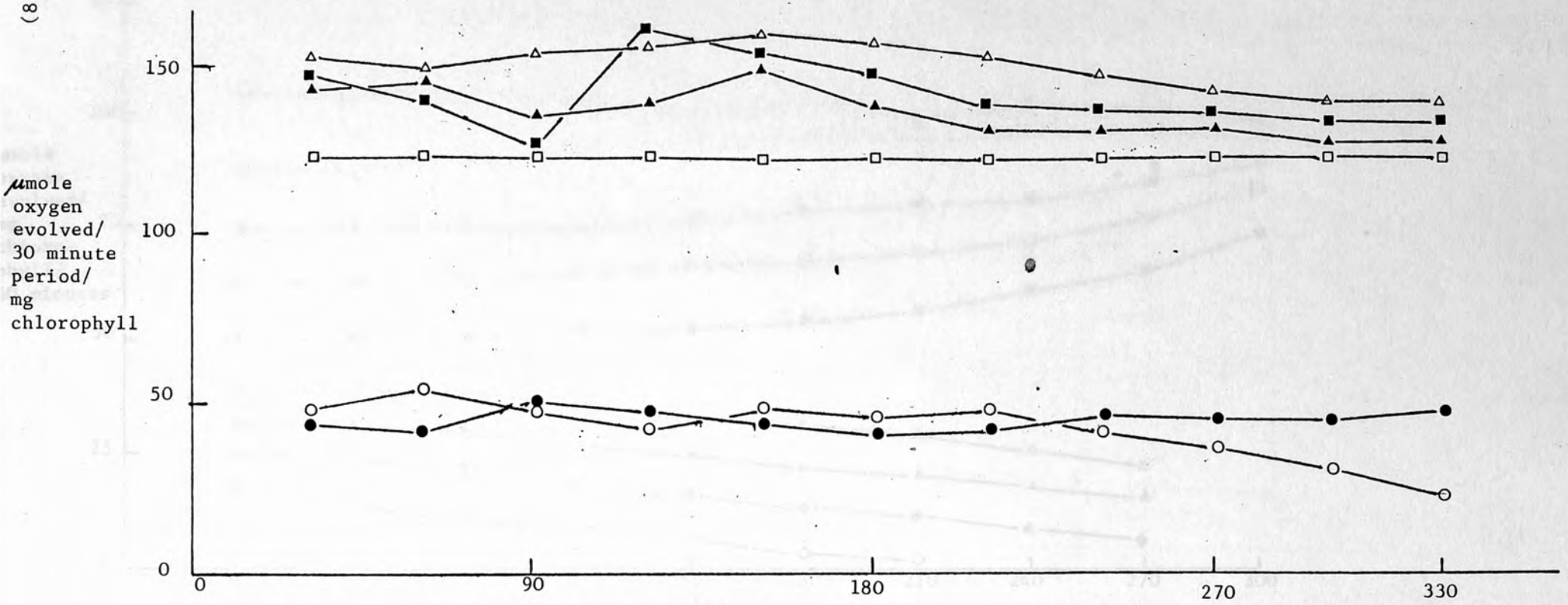
Oxygen evolution of chlortoluron and isoproturon treated leaf segments

In addition to information obtained from studies of the affects of photosynthesis inhibiting herbicides on isolated chloroplasts, useful information can also be gained from in vivo studies. In contrast to other types of herbicide action, inhibitory effects on photosynthesis can easily be assessed by measuring gas exchange. Indeed, as the principal mode of action of the substituted phenylurea herbicides is an interference with chloroplast electron transport, a positive correlation between the rate of recovery of oxygen evolution after herbicide treatment, and the conversion of the herbicide to less toxic or non-toxic compounds should exist. In the present study leaf segments of chlortoluron resistant and sensitive varieties were incubated on a phosphate buffer containing chlortoluron or isoproturon. After 45 minutes all the segments were rinsed and transferred to fresh buffer not containing herbicide, and oxygen evolution determined over a five hour period using a Warburg apparatus.

Preliminary experiments to determine the optimum amount of leaf tissue and concentration of herbicide to use in subsequent studies were conducted. The data for these experiments are presented in Fig. 7 and Figs. 8 and 9 respectively. As has been demonstrated by Sestak, Catsky and Jarvis (1972), large amounts of tissue may cause variations in the CO_2 content of the reaction flask as the CO_2 absorbed during photosynthesis exceeds the rate of CO_2 evolution from the CO_2 buffer. This is quite possibly the explanation for the variability in oxygen evolution measurements obtained for reaction flasks containing large amounts of tissue (Fig. 7). The variation observed where small amounts of tissue are employed could be due to a high error of determination, which is inversely related to the size of the sample as it is largely dependent on the reading error. Thus, the optimum amount of leaf tissue would appear to be in the region of 4-6 leaf segments. An optimum concentration of herbicide is one that would produce a significant reduction in oxygen evolution by tissue of resistant varieties while not totally inhibiting oxygen evolution by sensitive varieties. It is apparent that the optimum concentration is 5 ppm chlortoluron or isoproturon, other concentrations proving to be either too toxic or relatively non-toxic (Figs. 8 and 9).

Four cereal varieties and one wild oat cultivar were employed for the determination of oxygen evolution by leaf segments after treatment with chlortoluron or isoproturon. The results are presented in Figs. 10 and 11. It is apparent from the data that the recovery of oxygen evolution over the period of the experiment occurs to a greater extent in the resistant cereal varieties than in the sensitive cereal varieties.

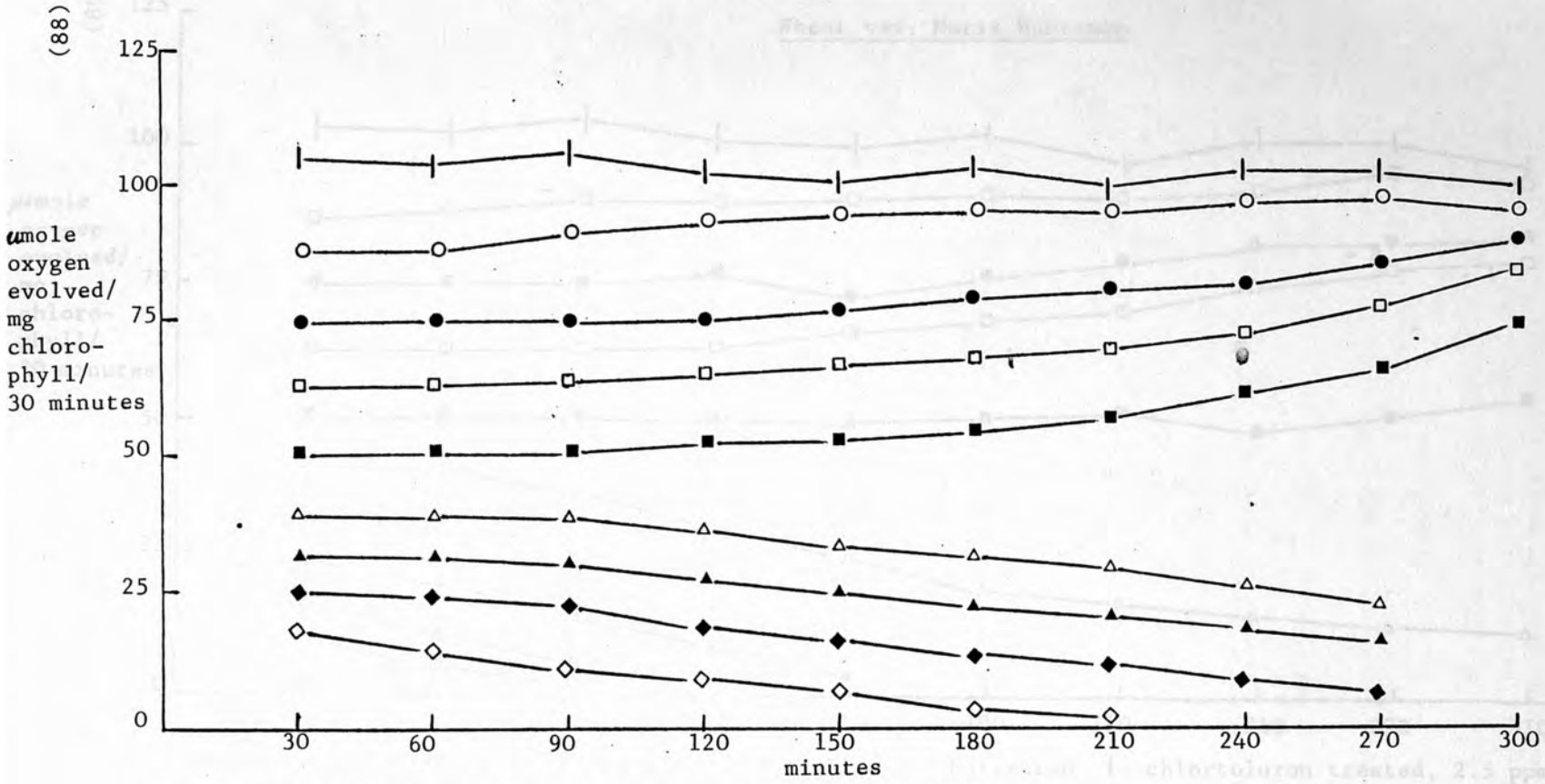
Fig. 7: Oxygen evolution by leaf segments



Leaf segments were incubated on 0.05M phosphate buffer pH 7.5 in a Warburg apparatus. The centre wells of the flasks contained a CO_2 buffer maintaining a CO_2 atmosphere of 1% at 25°C.

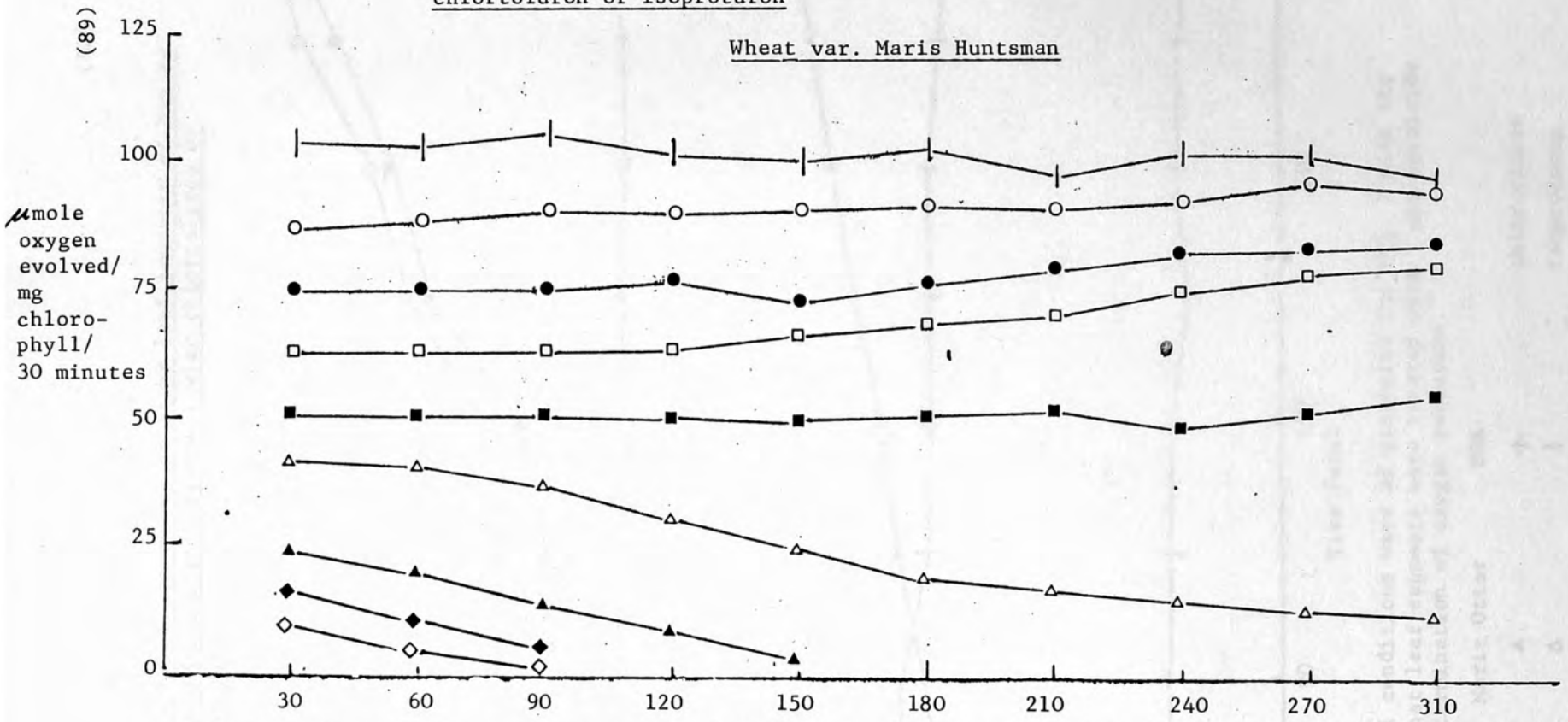
Number of leaf segments:	1	2	4	8	10	13
	○	●	□	△	▲	■

Fig. 8: Oxygen evolution by leaf segments after treatment with various concentrations of chlortoluron or isoproturon Wheat var. Atou



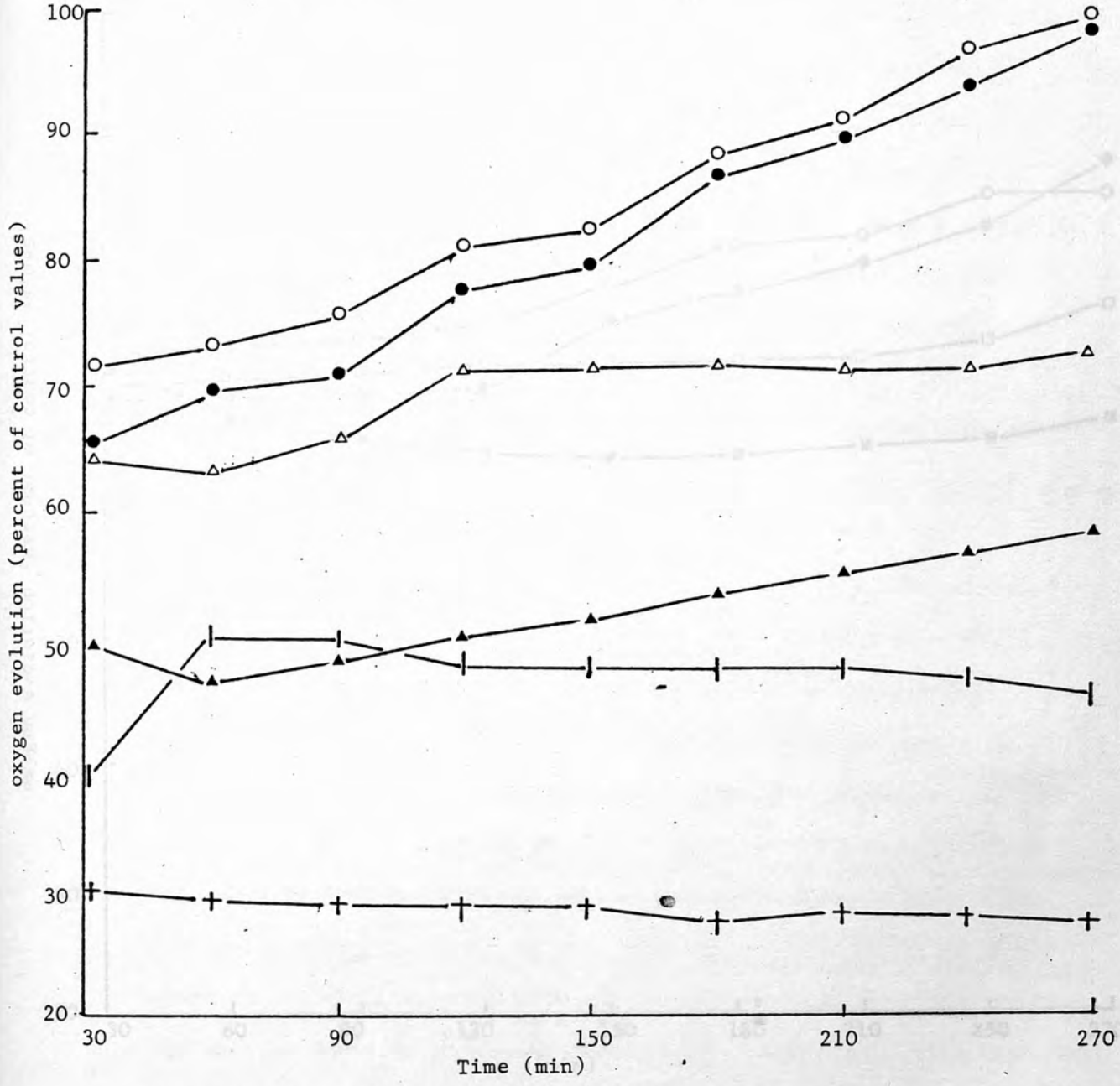
Experimental conditions were as presented in Fig. 7. Additionally leaf segments were incubated for 45 minutes on 0.05M phosphate buffer pH 7.0 containing various concentrations of isoproturon or chlortoluron prior to determination of oxygen evolution in the Warburg apparatus. Untreated, ↕ . chlortoluron treated, 2.5 ppm ● . 5 ppm ■ . 7.5 ppm ▲ . 10 ppm ◆ . Isoproturon treated, 2.5 ppm ○ . 5 ppm □ . 7.5 ppm △ 10 ppm ◇ .

Fig. 9: Oxygen evolution by leaf segments after treatment with various concentrations of chlortoluron or isoproturon



Experimental conditions were as presented in Fig. 8. Untreated, 1. chlortoluron treated, 2.5 ppm ● . 5 ppm ■ . 7.5 ppm ▲ . 10 ppm ◆ . Isoproturon treated, 2.5 ppm ○ . 5 ppm □ . 7.5 ppm △ . 10 ppm ◇ .

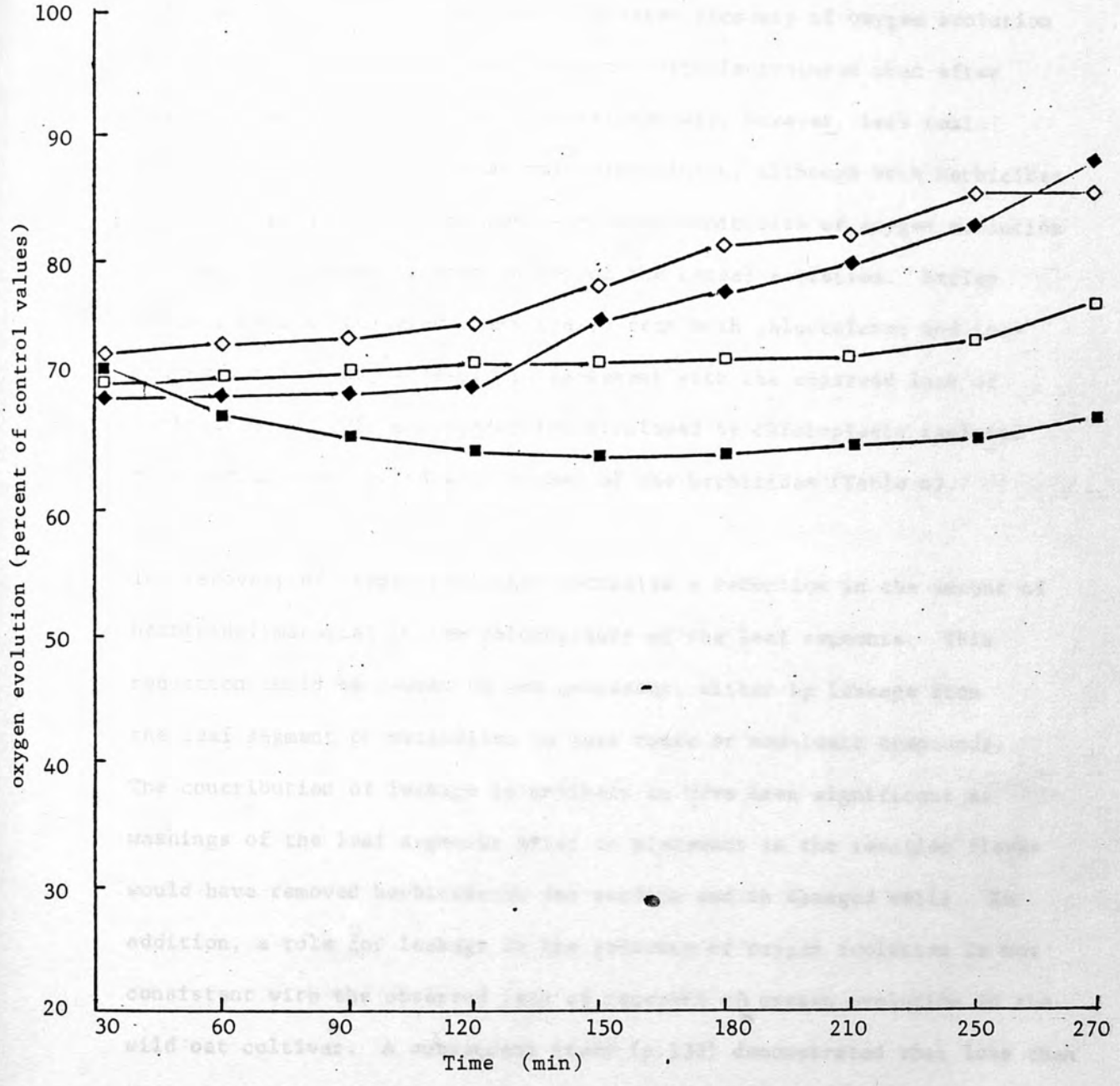
Fig. 10: Recovery of oxygen evolution by leaf segments of barley and wild oat after treatment with chlortoluron or isoproturon



Experimental conditions were as presented for Fig. 7 with the exception that leaf segments were treated with 5 ppm herbicide prior to determination of oxygen evolution.

Astrix	Maris Otter	USA	
●	▲	+	chlortoluron
○	△	┤	isoproturon

Fig. 11: Recovery of oxygen evolution by leaf segments of wheat after treatment with chlortoluron or isoproturon



Experimental conditions were as presented for Fig. 10.

Maris Huntsman	Atou	
■	◆	chlortoluron
□	◇	isoproturon

There is no evidence for a recovery of oxygen evolution in the wild oat cultivar. Overall there was a greater recovery of oxygen evolution after treatment of cereal leaf segments with isoproturon than after treatment with chlortoluron. Isoproturon was, however, less toxic to the wild oat cultivar than was chlortoluron, although both herbicides demonstrated a greater and more sustained inhibition of oxygen evolution in these leaf segments than in any of the cereal varieties. Barley variety Astrix recovered dramatically from both chlortoluron and isoproturon treatment, a result in agreement with the observed lack of inhibition of DCPIP photoreduction displayed by chloroplasts isolated from Astrix pre-treated with either of the herbicides (Table 4).

The recovery of oxygen evolution indicates a reduction in the amount of herbicidal material in the chloroplasts of the leaf segments. This reduction could be caused by two processes, either by leakage from the leaf segment or metabolism to less toxic or non-toxic compounds. The contribution of leakage is unlikely to have been significant as washings of the leaf segments prior to placement in the reaction flasks would have removed herbicide on the surface and in damaged cells. In addition, a role for leakage in the recovery of oxygen evolution is not consistent with the observed lack of recovery of oxygen evolution in the wild oat cultivar. A subsequent study (p.132) demonstrated that less than 5% of the absorbed herbicide was released after transfer of leaf segments to an incubation medium not containing herbicide.

The recovery of oxygen evolution would appear to be due to detoxification of the herbicides. As the component of the herbicide which causes inhibition of the Hill reaction is reversibly bound within the chloroplast (Izawa and Good, 1965; Lee and Fang, 1972), a reduction in the extra-chloroplast herbicide concentration would affect the chloroplast herbicide:cell herbicide equilibrium, resulting in the establishment of a new equilibrium with a reduced amount of herbicide within the chloroplast. Van Oorschot (1965) demonstrated the recovery of CO_2 uptake after an initial period of depression in resistant plants treated with such photosynthesis inhibiting herbicides as simazine and monuron. The recovery of photosynthesis in leaf discs, with the concomitant metabolism of monuron and diuron was demonstrated by Swanson and Swanson (1968a, b). A similar relationship was noted in sorghum (Shimabukuro and Swanson, 1969) clearly implicating metabolism of the herbicides in the recovery of photosynthesis. Thus from the present study it is not unreasonable to suggest that the recovery of oxygen evolution is due to detoxification of the herbicide, and that differences in the abilities of the varieties and cultivar to detoxify chlortoluron or isoproturon are reflected in the rates of recovery of oxygen evolution. However, the present study does not provide information on the contribution of differences in uptake of the herbicides to selectivity, and is of necessity of a short term nature.

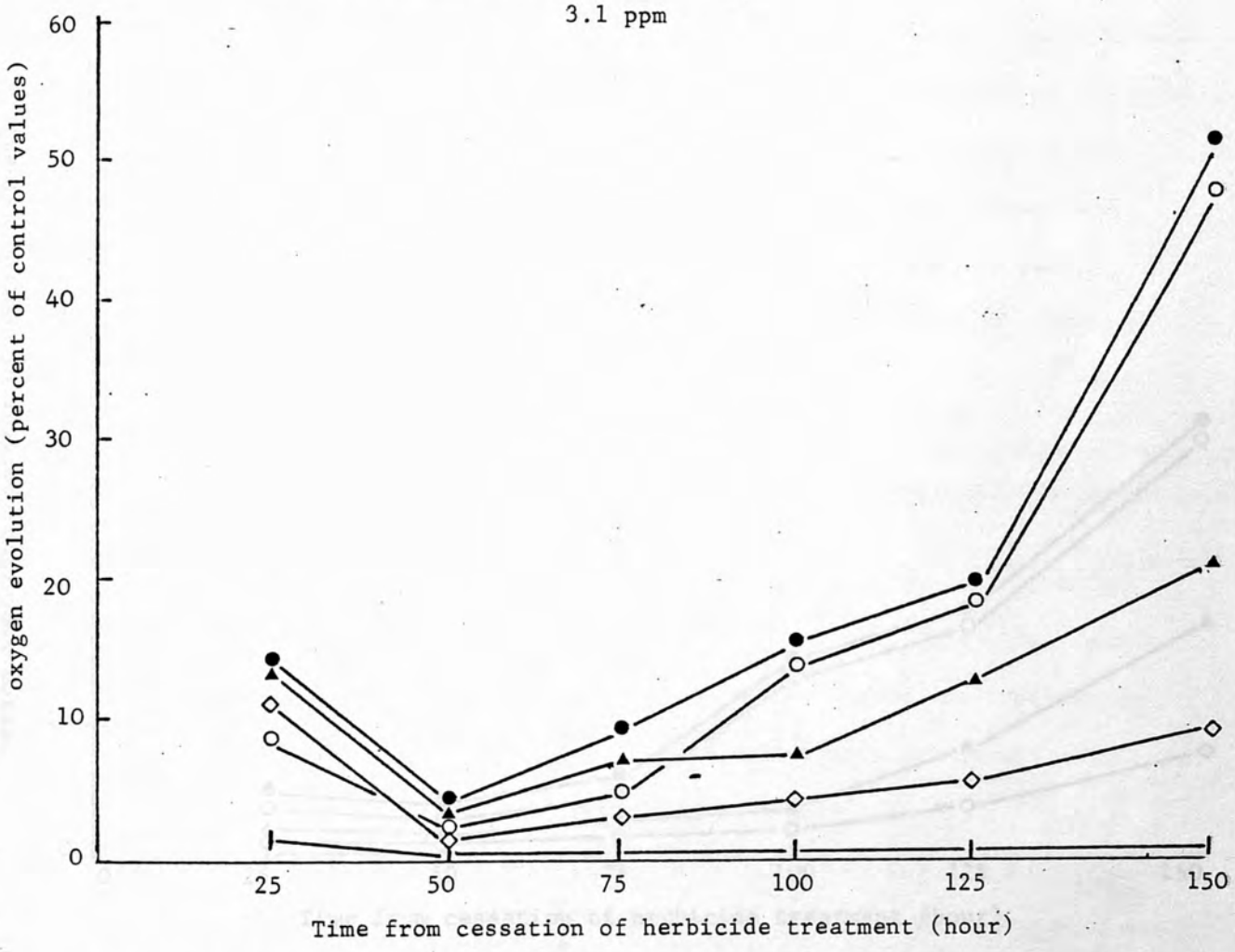
Whole-plant oxygen evolution after treatment with chlortoluron

Where the recovery of whole-plant oxygen evolution after a period of treatment with chlortoluron is studied, the effects of differences in absorption and distribution in addition to degradation of the herbicide,

on selectivity can be examined. In the present study resistant and sensitive plants were treated in vivo with chlortoluron for 24 hours and the oxygen evolution of leaf segments subsequently measured. The results presented in Figs. 12 and 13 demonstrate a rapid onset of inhibition of oxygen evolution in all varieties and the cultivar, indicating absorption and translocation of the herbicide. After the removal of the herbicide from the treatment solution, the recovery of oxygen evolution did not commence immediately. This was probably due to herbicide within the plant, but not at the chloroplast, being transported to the chloroplast and reinforcing inhibition.

The recovery of oxygen evolution was related to the reactions of cultivars in field and glasshouse trials. There was a more rapid recovery of oxygen evolution in the resistant varieties (Astrix and Atou) than in the sensitive or moderately resistant varieties (M. Huntsman and M. Otter). Recovery did not occur at either treatment level in the wild oat cultivar. As would be expected, recovery of oxygen evolution by the cereals was more rapid after treatment with 3.1 ppm than 8.1 ppm. The greater initial depression and subsequent lower oxygen evolution in the wild oat cultivar can be explained by the presence of a larger amount of toxic material in the chloroplasts. This could be due either to a greater rate of absorption and translocation of the herbicide or a greater ease of transport through the symplast to the active site, coupled to a low rate of detoxification. The results indicate that a more rapid detoxification of the herbicide occurs in resistant varieties, as measured by the rate of recovery of oxygen evolution, than in the sensitive varieties or cultivar.

Fig. 12: Recovery of oxygen evolution by leaf segments from plants treated with chlortoluron



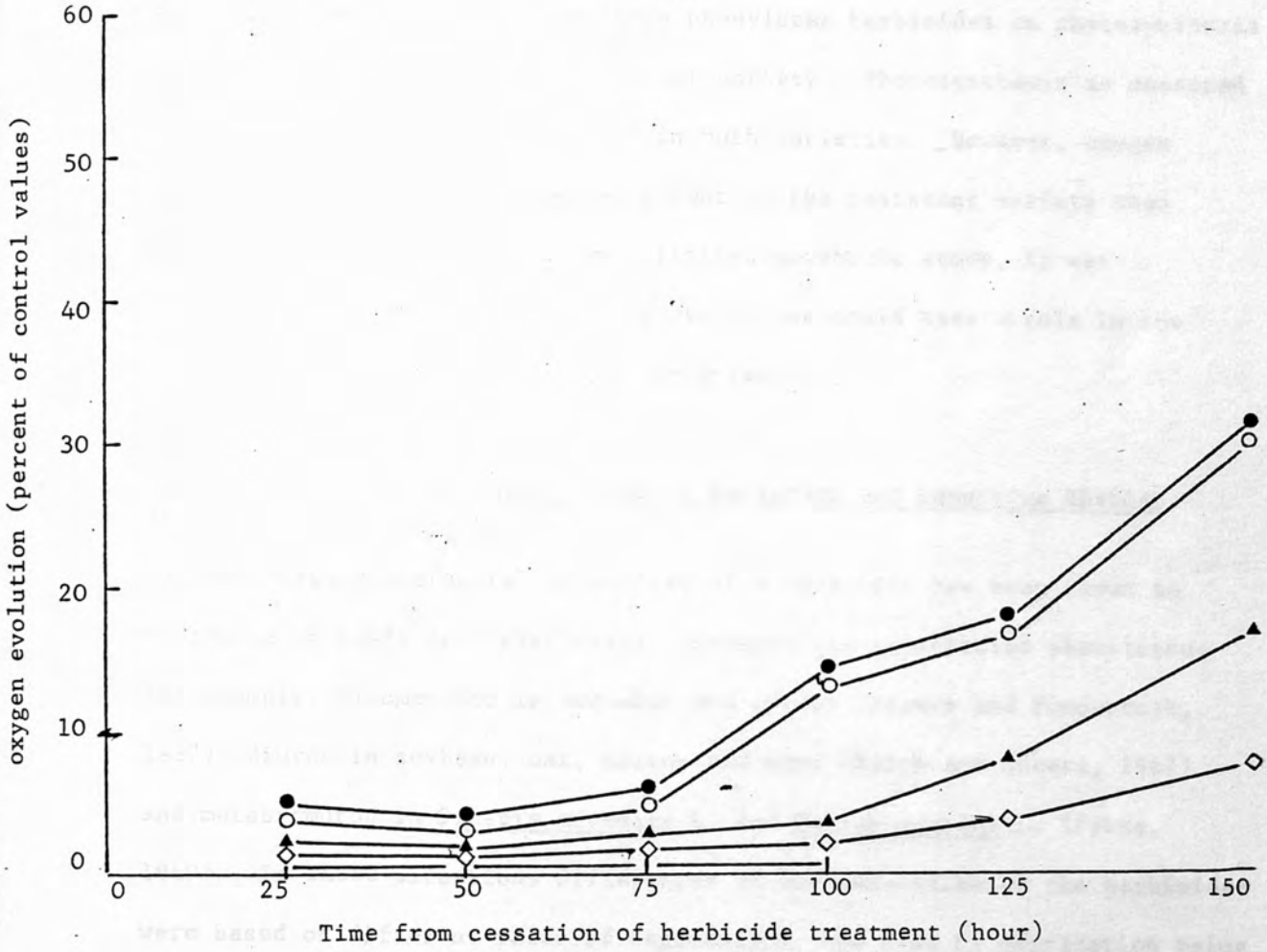
Plants were grown in nutrient solution and at growth stage 12 treated with chlortoluron for 24 hours. After this time the nutrient solution was replaced and leaf tissue harvested at intervals. Oxygen evolution was determined via Warburg manometry.

Atou ○ Astrix ● USA I

Maris Huntsman ◇ Maris Otter ▲

Fig. 13: Recovery of oxygen evolution by leaf segments from plants treated with chlortoluron

8.1 ppm



Experimental conditions were as presented in Fig. 12.

Atou ○ Astrix ● USA I

Maris Huntsman ◇ Maris Otter ▲

This conclusion is encouraged by the results of Muller and Sanad (1975) who determined the effects of three phenylurea herbicides on photosynthesis in a resistant, and a sensitive wheat variety. Photosynthesis as measured by oxygen evolution was depressed in both varieties. However, oxygen evolution recovered to a greater extent in the resistant variety than in the sensitive variety. From a limited metabolic study, it was postulated that degradation of the herbicides could have a role in the differential response of the two varieties.

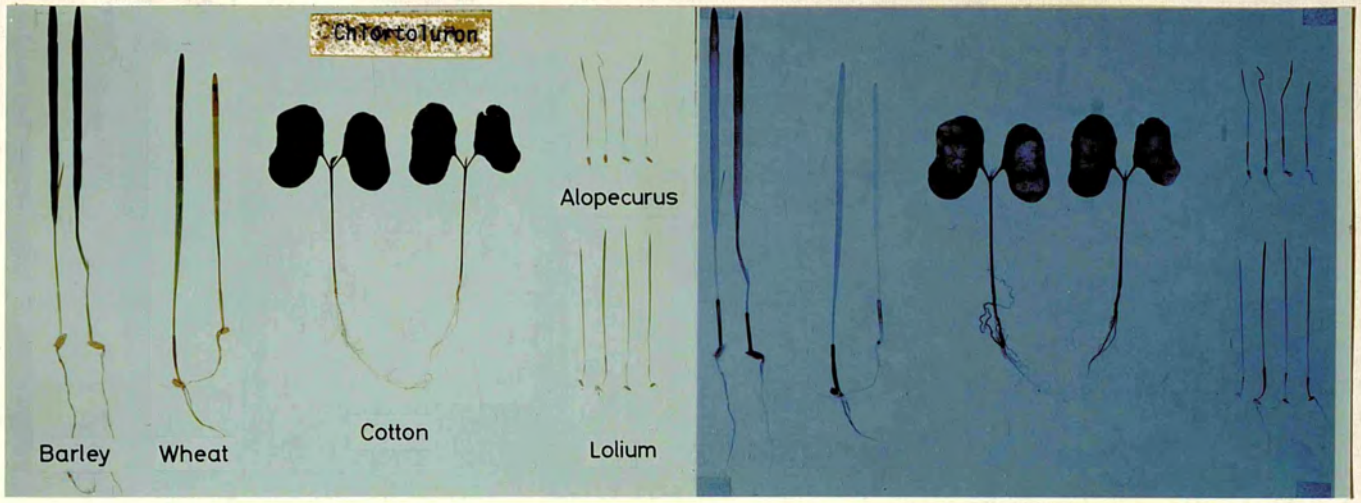
The Metabolic Fate of Chlortoluron in Resistant and Sensitive Species, Varieties and Cultivars

In many cases differential metabolism of a herbicide has been shown to be the major basis for selectivity. Amongst the substituted phenylureas for example, fluometuron in cucumber and cotton (Rogers and Funderburk, 1967), diuron in soybean, oat, cotton and corn (Smith and Sheets, 1967) and metabromuron in Sinapis arvensis L. and Galium aparine L. (Fykse, 1969). In these situations differences in the metabolism of the herbicides were based on different rates of degradation, the type of degradation being identical. Stepwise N-demethylation, often followed by conjugation of the intermediates has generally been considered the major degradation pathway for dimethylphenylureas in plants. However, a recent study (Gross et al, 1979) of the metabolic fate of chlortoluron in wheat clearly demonstrated that N-demethylation did not represent the major pathway of degradation and that oxidation of the 4-methylphenyl group to give benzyl alcohol and benzoic acid derivatives a proportion of which were conjugated, was quantitatively more important.

In the present investigation, a detailed study of the quantitative metabolism of chlortoluron was carried out in both resistant and sensitive species, varieties and cultivars. Plants were grown in soil supplemented with either 0.6 mg (oat and cereal weeds) or 1.2 mg (cereal varieties and cotton) of (carbonyl ^{14}C) chlortoluron (sp. act. 6.502 μCi/μmole), all plants being harvested ten days after planting. These treatments corresponded to field applications of 0.75 and 1.5 kg a.i/ha respectively. Preliminary control experiments indicated that at these rates chlortoluron was not phytotoxic to the varieties and cultivars under study, thus the metabolic status of treated plants was expected to be normal. The soil used was not sterilised as experience indicated no significant breakdown of chlortoluron would occur over the time of the experiment, a conclusion supported by Smith and Briggs (1978). Guth (personal communication) has shown that chlortoluron is leached slowly from soil under field conditions, thus it is unlikely that leaching would result in any appreciable removal of chlortoluron in the present investigation. Absorbed radioactivity was extracted, identified and quantitated as described in the relevant section of the preceeding chapter.

The total radioactivity detected in the plants after application of ^{14}C-chlortoluron, and the partitioning of the radioactivity between roots and shoots is presented in Table 5 and Fig. 14. The results indicate that any differences in absorption of the herbicide by the cereal varieties does not correlate with the cultivar specific response. There are however indications that the cereal weeds absorbed proportionally more chlortoluron than the cereals, in as much as the oat and cereal weeds which were treated with 0.6 mg of ^{14}C-chlortoluron and would thus be expected to absorb approximately half that absorbed by the wheat and barley varieties

Fig. 14: Distribution of radioactivity in ^{14}C-chlortoluron treated plants



Soil grown plants were harvested ten days after application of ^{14}C-chlortoluron.

Autoradiograms were prepared with Structurix D7 Röntgenfilm using an exposure period of two weeks

Table 5: Absorption and distribution of ^{14}C-chlortoluron in soil grown plants

Species	Variety/Cultivar		dpm x 10^{-5}/ g fresh weight plant	Distribution (%)	
				Root	Shoot
T. aestivum L.	Atou	R	3.7	17.8	82.2
	Bouquet	R	2.76	5.5	94.5
	Probus	R	2.83	7.5	92.5
	M. Huntsman	S	2.76	6.9	93.1
	M. Kinsman	S	1.95	11.5	88.5
H. vulgare L.	Astrix	R	2.09	6.2	93.8
	Sonja	R	2.02	5.8	94.2
	M. Otter	MR	2.09	6.9	93.1
	Katy	S	2.09	5.5	94.5
A. sativa L.	Flemish Crown	S	1.68	6.3	93.7
A. fatua L.	USA	Si	1.68	6.1	93.9
B. tectorum L.		Si	2.29	14.9	85.1
L. perenne L.		Si	2.02	7.3	92.7
A. myosuroides Huds.		Si	2.56	7.9	92.1
G. hirsutum L.	Delta Pine	MR	3.23	15.1	84.9

R, MR, S, Si are as described for Table 3.

Plants were grown in soil containing 36.72 µCi or 18.36 µCi ^{14}C-chlortoluron. After harvesting roots and shoots were separately extracted prior to quantification and identification of radioactivity by thin layer chromatography and liquid scintillation counting.

Table 6: The quantitative metabolism of chlortoluron in wheat

Variety	Part	1	Metabolites					Conjugates of					Un-known	Non-extr-actable
			11	1V	V	VI	VII	11	1V	V	VI	VII		
Atou (R)	Shoot	24.4	1.1	2.7	0.4	1.6	2.6	nd	36.9	8.6	nd	nd		
	Root	6.5	0.2	0.6	nd	0.1	nd	nd	9.2	nd	nd	nd		
	Total	30.9	1.3	3.3	0.4	1.7	2.6	nd	46.1	8.6	nd	nd	0.5	4.3
Bouquet (R)	Shoot	24.2	2.4	3.4	0.9	1.0	1.6	2.7	32.6	9.1	nd	nd		
	Root	3.7	0.2	0.2	nd	0.1	nd	0.7	7.8	1.2	nd	nd		
	Total	27.9	2.6	3.6	0.9	1.1	1.6	3.4	40.4	10.3	nd	nd	0.6	7.7
Probus (R)	Shoot	24.1	2.5	3.8	0.7	1.4	2.2	1.9	35.9	6.3	0.6	0.2		
	Root	1.8	0.2	0.3	0.05	0.1	0.2	0.4	8.5	nd	nd	nd		
	Total	25.9	2.7	4.1	0.75	1.5	2.4	2.3	44.4	6.3	0.6	0.2	0.2	8.8
M. Huntsman (S)	Shoot	37.0	6.4	1.6	1.0	nd	nd	2.5	24.7	7.2	nd	nd		
	Root	4.9	0.3	nd	nd	nd	nd	0.7	6.3	1.1	nd	nd		
	Total	41.9	6.7	1.6	1.0	nd	nd	3.2	31.0	8.3	nd	nd	0.4	5.8
M. Kinsman (S)	Shoot	30.3	4.8	1.4	0.9	nd	nd	2.8	28.3	7.8	nd	nd		
	Root	6.0	0.6	nd	nd	nd	nd	0.5	6.8	1.6	nd	nd		
	Total	36.3	5.4	1.4	0.9	nd	nd	3.3	35.1	9.4	nd	nd	0.6	7.5

Results expressed as the percentage of radioactivity/gram fresh weight plant. nd = non detectable.

Experimental conditions were as described in Table 5.

The structures of 1 - V1 are presented in attachment 1.

Table 7: The quantitative metabolism of chlortoluron in barley and cotton

Variety	Part	1	Metabolites						Conjugates of						Un-known	Non-extr-actable
			11	111	1V	V	V1	V11	11	111	1V	V	V1	V11		
Astrix (R)	Shoot	26.6	4.2	nd	4.7	1.5	1.2	1.5	1.6	nd	31.4	10.9	5.4	nd		
	Root	1.6	0.2	nd	0.3	nd	nd	nd	0.1	nd	1.9	0.6	0.3	nd		
	Total	28.2	4.4	nd	5.0	1.5	1.2	1.5	1.7	nd	33.3	11.5	5.7	nd	1.2	3.7
Sonja (R)	Shoot	23.9	5.2	nd	4.1	0.9	0.7	0.9	3.5	nd	28.9	10.4	2.8	1.9		
	Root	1.4	0.3	nd	0.2	nd	nd	nd	0.7	nd	6.3	2.2	0.8	0.4		
	Total	25.3	5.5	nd	4.3	0.9	0.7	0.9	4.2	nd	35.2	12.6	3.6	2.3	0.4	3.6
M. Otter (MR)	Shoot	25.4	4.0	nd	8.5	2.2	2.0	1.9	3.4	nd	24.9	8.5	3.6	0.9		
	Root	1.5	0.2	nd	0.5	nd	nd	nd	0.6	nd	5.8	2.1	0.6	0.1		
	Total	26.9	4.2	nd	9.0	2.2	2.0	1.9	4.0	nd	30.7	10.6	4.2	1.0	0.2	4.2
Katy (S)	Shoot	37.1	5.0	nd	7.8	2.1	1.3	1.1	3.1	nd	18.9	7.1	2.3	1.2		
	Root	2.1	0.3	nd	0.4	nd	nd	nd	0.3	nd	4.9	1.2	0.3	0.2		
	Total	39.2	5.3	nd	8.2	2.1	1.3	1.1	3.4	nd	23.8	8.3	2.6	1.4	0.2	3.7
Delta Pine (MR)	Shoot	21.6	6.4	6.4	0.4	0.5	0.5	0.4	3.6	3.5	8.1	5.1	6.2	2.1		
	Root	4.8	1.2	1.2	0.06	0.1	nd	0.1	1.9	2.0	3.2	1.4	2.3	1.1		
	Total	26.4	7.6	7.6	0.46	0.6	0.5	0.5	5.5	5.5	11.3	6.5	8.5	3.2	12.5	2.7

Results expressed as the percentage of radioactivity/gram fresh weight plant. nd = non-detectable

Experimental conditions were as described in Table 5.

The structures of 1 - V11 are presented in attachment 1.

treated with 1.2 mg of ^{14}C-chlortoluron did in fact contain rather more than this amount of radioactivity.

The amounts of unchanged chlortoluron and its metabolites expressed as a percentage of total radioactivity recovered per gram plant fresh weight from plants of the various species are presented in Tables 6, 7 and 8. The Rf values of chlortoluron and its metabolites in the various solvent systems employed for characterisation are recorded in Table 9. These data show that in general the overall degradation of chlortoluron correlated well with the sensitivity of the plant, the herbicide being more efficiently degraded in the tolerant wheat and barley varieties and cotton than in sensitive cereal varieties and cereal weeds. Chlortoluron was shown to be degraded by both N-demethylation and ring-methyl oxidation pathways, reactions which took place independently from each other with the result that a variety of ring-methyl oxidation derivatives, N-demethylation derivatives and derivatives displaying characteristics of both pathways could be detected (metabolites II-VII). Ring-methyl oxidation derivatives predominated in the non-polar metabolite fraction of resistant varieties of wheat and of barley, 1-(3-chloro-4-hydroxymethylphenyl)-3,3-dimethyl-urea (IV) being the major metabolite. In contrast to barley and wheat, however, in the other plants N-demethylation activity predominated over ring-methyl oxidation. In addition, varying proportions of most of the metabolites were also extracted as polar conjugates. With the exception of cotton, the amount of uncharacterised radioactive material extracted from treated plants was generally small.

Table 8: The quantitative metabolism of chlortoluron in oat and cereal weeds

	Part	1	Metabolites					Conjugates of					Unknown	Non-extr-actable
			11	1V	V	V1	V11	11	1V	V	V1	V11		
Avena fatua L. (USA) (Si)	Shoot	36.5	18.7	1.1	nd	nd	nd	6.1	8.2	8.1	4.9	nd		
	Root	2.9	3.7	nd	nd	nd	nd	1.1	2.1	2.4	nd	nd		
	Total	39.4	22.4	1.1	nd	nd	nd	7.2	10.3	10.5	4.9	nd	0.5	3.2
Avena sativa L. (Flemish Crown) (Si)	Shoot	38.4	19.6	0.9	nd	nd	nd	3.5	10.5	7.1	4.3	nd		
	Root	2.5	2.7	nd	nd	nd	nd	0.9	2.1	1.5	nd	nd		
	Total	40.9	22.3	0.9	nd	nd	nd	4.4	12.6	8.6	4.3	nd	1.7	3.8
Lolium perenne L. (Si)	Shoot	44.1	5.5	1.7	0.6	0.3	0.1	9.5	14.6	4.4	0.07	0.3		
	Root	3.4	0.4	0.1	0.05	nd	nd	2.3	3.2	1.0	nd	nd		
	Total	47.5	5.9	1.8	0.65	0.3	0.1	11.8	17.8	5.4	0.07	0.3	0.2	7.8
Alopecurus myosuroides Huds. (Si)	Shoot	36.7	5.4	1.9	1.3	0.4	0.7	10.5	16.3	4.5	0.4	nd		
	Root	3.3	0.5	0.1	0.1	nd	nd	2.7	4.1	0.9	nd	nd		
	Total	40.0	5.9	2.0	1.4	0.4	0.7	13.2	20.4	5.4	0.4	nd	0.1	9.8
Bromus tectorum L. (Si)	Shoot	40.8	4.8	1.4	0.7	1.4	1.0	1.0	26.1	5.4	nd	nd		
	Root	7.3	0.9	0.3	nd	0.2	nd	0.2	3.3	0.5	nd	nd		
	Total	48.1	5.7	1.7	0.7	1.6	1.0	1.2	29.4	4.6	nd	nd	1.5	2.4

Results expressed as the percentage of radioactivity/gram fresh weight plant. nd = non-detectable

Experimental conditions were as described in Table 5. The structures of 1 to V11 are presented in attachment 1.

The major metabolites produced by the resistant plants (with the exception of cotton) were products of ring methyl oxidation. Atou, Bouquet, Probus (Table 6) and Astrix, Sonja and to a less extent Maris Otter (Table 7) produced greater amounts of metabolite 1V and overall ring-methyl oxidation derivatives than did Maris Huntsman and Kinsman (Table 6) and Katy (Table 7). As the sensitivity of the plants increased there was a parallel decrease in the total quantity of degradation products of chlortoluron. Of the derivatives that were present, products of N-demethylation assumed an increasingly important proportion. Where the very sensitive plants (Table 8) are examined the major degradation products are no longer due to ring-methyl oxidation, but have in some cases (for example Avena spp) been exceeded by products of N-demethylation. Secondary ring-methyl oxidation leading to the production of the benzoic acid (metabolites V1 and V11) occurred only to a small extent, most notably in the resistant plants.

Polar metabolites were detected in extracts from all plants and appeared to consist solely of conjugates, all of which contained a pesticide moiety subjected to ring-methyl oxidation as a major component. There are indications from the tables that the ability to conjugate the metabolites of chlortoluron contributes to the field-observed resistance. The total amount of material conjugated declined as the sensitivity of the plants increased, additionally metabolite 1V would appear to be the metabolite most susceptible to conjugation. In extracts where the major free metabolite was not 1V, for example in extracts from cotton (Table 7), 1V appeared as the major conjugated metabolite.

Table 9: rf values for chlortoluron and metabolites

Solvent System	1		2		3	
Compound	First dimension	Second dimension	First dimension	Second dimension	First dimension	Second dimension
1	0.65	0.91	0.87	0.86	0.64	0.84
11	0.47	0.89	0.79	0.79	0.66	0.74
111	0.42	0.75	0.70	0.63	0.57	0.63
1V	0.30	0.85	0.55	0.52	0.59	0.53
V	0.18	0.82	0.38	0.31	0.60	0.43
V1	0.05	0.45	0.64	0.65	0.38	0.57
V11	0.05	0.42	0.40	0.43	0.40	0.45
conjugates	0	0-0.15	0-0.20	0-0.15	0-0.15	0-0.20

Solvent system	Dimension	
1	1	chloroform, ethanol 9:2
	2	acetonitrile, water, ammonia 40:9:1
2	1	chloroform, ethanol, acetic acid 9:1:1
	2	chloroform, ethanol, acetone 9:2:1
3	1	ether, ethanol, acetone, acetic acid 9:2:1 + 0.1%
	2	chloroform, ethanol, acetic acid 9:2:1

all ratios as v:v

In cotton the major degradative route was via N-demethylation, which proceeded to a greater extent than in any of the other plants examined, leading to the production of substantial quantities of the di-demethylated metabolite 111.

The amount of non-identified radioactivity was similar in all the plants examined with the exception of cotton. This unidentified radioactivity occurred as one compound with an R_f value greater than that of chlortoluron in all the solvent systems utilised. This compound did not co-chromatograph with any of the authentic standards available, and identification was not attempted. No degradation of chlortoluron occurred in the soil during the course of the study as defined by thin layer chromatography of soil extracts (Table 10). This finding eliminated the possibility of the plants absorbing chlortoluron metabolites from the soil and of the exudation of chlortoluron metabolites via the roots.

Table 10: Degradation of chlortoluron in the soil

	Extractable radioactivity	Non-extractable radioactivity	Chlortoluron
dpm x 10^5/ 50 g soil	145.7	3.4	145.6
percentage	97.7	2.3	97.6

The results presented above demonstrate a clear correlation between ring-methyl oxidation and tolerance to chlortoluron, indicating a mechanism of selectivity which depends on both the rate and type of degradation of the herbicide.

In vitro determination of the toxicity of metabolites of chlortoluron

The results presented in the preceeding section show a clear distinction between resistant and sensitive cereals and cereal weeds, with respect to both rate and type of metabolism of ^{14}C-labelled chlortoluron. However, because of the importance of the relationship between reactions at the ring-methyl group and resistance, it was deemed necessary to determine the relative toxicities of the metabolites produced by ring-methyl oxidation and N-demethylation of chlortoluron. In order to compare differences in phytotoxicity between chlortoluron and its metabolites, the effects of these compounds on the Hill activity of chloroplasts isolated from resistant and sensitive plants were investigated. The rates of DCPIP photoreduction were determined for chloroplasts treated with each metabolite at a concentration equivalent to the chlortoluron I_{50} value.

The results obtained (Table 11) show that a considerable ability to inhibit photosynthetic electron transport is retained in the N-mono-desmethylated metabolite (11) which was only some 10%-18% less inhibitory than chlortoluron (1), in the range of species studied. In contrast, products of N-didesmethylation (111) and ring-methyloxidation (1V, V1) were essentially non-inhibitory, as were metabolites resulting from both N-demethylation and ring-methyloxidation (V, V11). These findings have an important bearing on the selective response of plants to chlortoluron. As demonstrated in the preceeding study in addition to displaying a greater overall rate of degradation of chlortoluron, the resistant plants utilised the ring-methyloxidation pathway to a more

Table 11: Inhibition of DCPIP photoreduction by chlortoluron and its metabolites

	Plant	Chlortoluron and metabolites						
		1	11	111	1V	V	V1	V11
T. aestivum L.	Atou	52.7	66.9	93.3	92.3	101.2	96.9	95.9
	Bouquet	51.6	69.3	97.4	95.1	99.2	95.3	94.8
	M. Huntsman	49.3	67.8	94.2	93.4	98.9	96.6	96.2
	M. Kinsman	49.6	64.2	94.2	91.9	97.3	95.7	95.4
H. vulgare L.	Astrix	52.6	62.3	94.3	91.9	102.1	97.1	97.9
	Sonja	50.7	65.1	97.1	91.8	97.4	94.3	96.3
	M. Otter	52.5	64.2	92.9	94.6	97.4	96.3	98.2
	Katy	48.9	63.2	93.4	94.7	98.5	97.6	95.2
A. fatua L.	USA	50.3	65.8	96.3	93.2	97.9	97.6	94.1

Results are expressed as a percentage of control values at the concentration of chlortoluron calculated to give 50% inhibition of DCPIP photoreduction. The structures of compounds 1 to V11 are given on attachment 1. The experimental conditions were as described in Table 3.

pronounced extent than did the sensitive plants, thus it is now apparent producing immediately non toxic metabolites. As cotton produced large amounts of the didesmethylated metabolite (111) an explanation for its resistance is also apparent. These data on the phytotoxicity of products of N-demethylation are supported by Good (1961) who concluded that certain of the N-demethylated derivatives of diuron were likely to contribute to the phytotoxic action of the herbicide. Hansch and Deutsch (1966) suggested that phenyl substituents affect inhibition via the lipophilic properties of the molecule. Hansch (1969) suggested that to inhibit the Hill reaction a molecule requires a large lipophilic moiety and a polar function. Thus it can be suggested that ring-methyloxidation affects the lipophilic nature of the phenyl ring while N-didesmethylation affects the polar function provided by the urea group, the effect of N-monodemethylation not being substantial.

This study does not demonstrate the degree to which compounds 11 to V11 are less inhibitory than chlortoluron, as the maximum solubility of each compound in the assay media precluded the calculation of I_{50} values.

The comparative metabolism of chlortoluron, diuron and N-(3-methyl-4-chlorophenyl-N,N-dimethylurea (CGA 43057)

Stepwise N-demethylation, often followed by conjugation of the intermediates has generally been considered as the major degradation pathway for dimethylphenylureas in plants. However, the previous study has demonstrated that chlortoluron is differentially degraded by ring-methyloxidation in addition to N-demethylation which at least partially explains the selective action of chlortoluron in cereals. CGA 43057

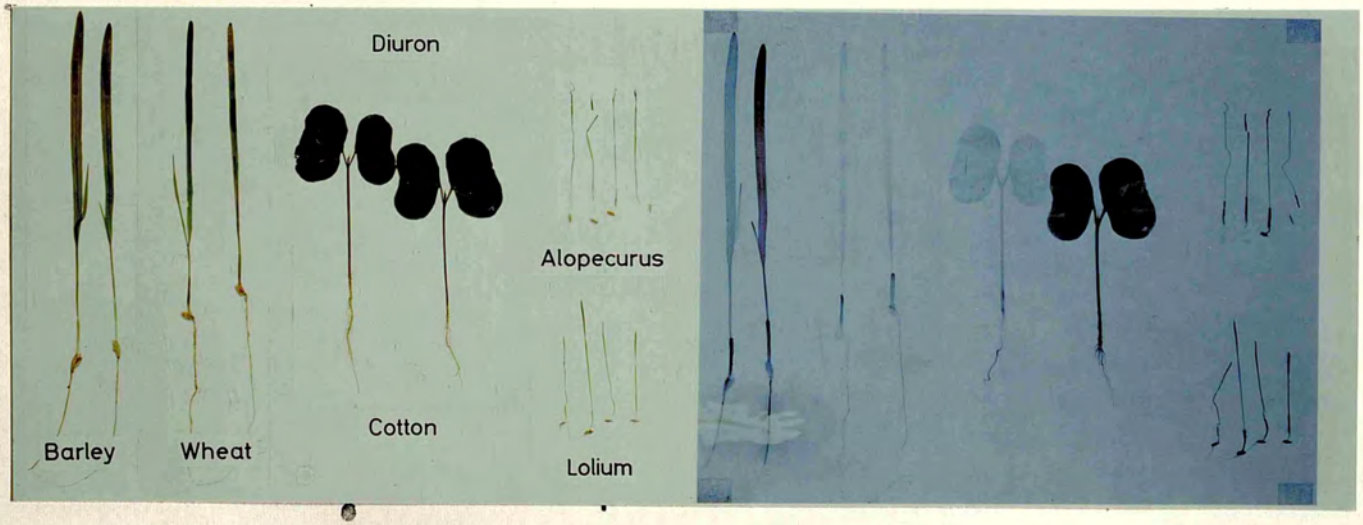
which differs from chlortoluron only in the positioning of the phenyl ring substituents, however, is neither as toxic nor as selective as chlortoluron. Diuron in which both phenyl ring substituents are chlorine atoms displays a high order of phytotoxicity combined with little selectivity. Although it has been utilised for weed control in certain crops such as cotton, selectivity depends on placement of the herbicide in addition to the physiological resistance of the crop.

In the present study the possibility that differential degradation is the basis for the observed differences in selectivity observed between chlortoluron, diuron and CGA 43057 was investigated.

As in the previous study plants were grown in soil supplemented with the radiolabelled compounds, in this case 0.6 mg of diuron or CGA 43057 per pot. Preliminary control experiments indicated that at this application rate there was no toxicity to any of the plants under study. The total radioactivity detected in the plants after application of ^{14}C-diuron and ^{14}C-CGA 43057, and the partitioning of the radioactivity between roots and shoots are presented in Tables 12 and 13, and Figs. 15 and 16. The results indicate that differences in the absorption and distribution of CGA 43057 and diuron in the test species do not consistently correlate with the observed response.

The amounts of unchanged diuron and CGA 43057 and their metabolites expressed as a percentage of total radioactivity recovered per gram plant fresh weight from plants of the various species are presented in Table 14, and Tables 15 and 16 respectively. The rf values obtained

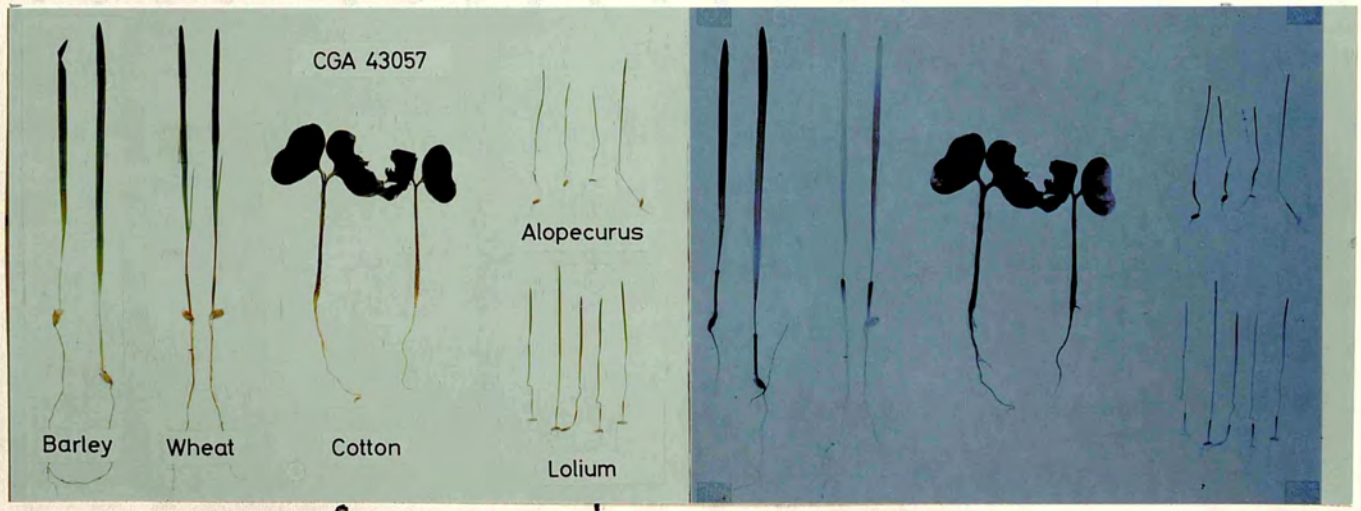
Fig. 15: Distribution of radioactivity in ^{14}C-diuron treated plants



Soil grown plants were harvested ten days after application of ^{14}C-diuron.

Autoradiograms were prepared with Structurix D7 Röntgenfilm using an exposure period of two weeks.

Fig. 16: Distribution of radioactivity in ^{14}C-N-(3-methyl-4-chlorophenyl)-N,N-dimethylurea (CGA 43057) treated plants



Soil grown plants were harvested ten days after application of ^{14}C-CGA 43057.

Autoradiograms were prepared with Structurix D7 Röntgenfilm using an exposure period of two weeks.

in various solvent systems are presented in Tables 17 and 18. These data indicate that the degradation of CGA 43057 proceeded more slowly than that of chlortoluron in all grass species studied. In such plants, CGA 43057 degradation was characterised by the presence of similar or slightly higher amounts of non-polar metabolites but much lower amounts of polar degradation products than with chlortoluron. The non-polar metabolite fraction consisted primarily of N-demethylated substances, metabolites X11 and X111 of which N-(3-methyl-4-chlorophenyl)methylurea (X11) predominated. It is also apparent from the data that the ring-methyl group of CGA 43057 is considerably more resistant to oxidation than that of chlortoluron in all species studied. These data are in agreement with previous observations that CGA 43057 was of little value as a selective herbicide in cereal crops (Ciba-Geigy, unpublished results). In contrast, however, degradation of CGA 43057 was much more extensive in cotton which possessed a more effective mechanism for N-demethylation than the grasses, as indicated by the presence in the extracts of a considerable amount of the N-didemethylated metabolite (X111), most of which was associated with the non-polar fraction (Table 16).

The results obtained for diuron (Table 14) indicate that with the exception of cotton, which is resistant to diuron, all species degraded this herbicide more slowly than chlortoluron, this being particularly evident in the case of Lolium perenne L., which retained some 78% of unchanged herbicide. The only metabolites characterised were products of N-demethylation (XX11 and XX111). In cotton however, not only was diuron more extensively degraded than in the grasses, as was the case for CGA 43057, but also N-demethylation appeared considerably more effective

Table 12: Absorption and distribution of ^{14}C-diuron in soil grown plants

Species	Variety/Cultivar		dpm x 10^5/ g fresh weight plant	Distribution (%) root	shoot
T. aestivum L.	Atou	S	3.03	11.3	88.7
H. vulgare L.	Astrix	S	2.15	16.6	83.4
B. tectorum L.		S	3.39	11.4	88.6
L. perenne L.		S	1.54	11.4	88.6
A. myosuroides Huds.		S	2.18	11.5	88.5
A. fatua L.	USA	S	2.39	9.9	90.1
G. hirsutum L.	Delta pine	MR	1.61	3.8	96.2

S, R are as described on Page 47.

Plants were grown in soil containing 20.64 μCi ^{14}C-diuron.

Experimental conditions were as described for Table 5.

Table 13: Absorption and distribution of ^{14}C-N-(3-methyl-4-chlorophenyl)-N,N-dimethylurea (CGA 43057) in soil grown plants

Species	Variety/cultivar		dpm x 10^5/ g fresh weight plant	Distribution (%) root	shoot
T. aestivum L.	Atou	S	2.66	8.4	91.6
H. vulgare L.	Astrix	S	2.34	13.9	86.1
B. tectorum L.		S	1.63	9.0	91.0
L. perenne L.		S	1.92	8.2	91.8
A. myosuroides Huds.		S	1.04	8.7	91.3
A. fatua L.	USA	S	2.61	13.3	86.7
G. hirsutum L.	Delta pine	MR	2.89	5.9	94.1

S, R are as described on Page 47.

Plants were grown in soil containing 20.94 µCi ^{14}C-CGA 43057.

Experimental conditions were as described for Table 5.

Table 14: The quantitative metabolism of diuron

Species	Variety/ Cultivar	Part	XX1	Metabolites XX11	Metabolites XX111	Conjugates of XX11	Conjugates of XX111	Un-known	Non-extr-actable
T. aestivum L.	Atou	Shoot	47.3	16.1	3.4	4.3	6.8		
		Root	8.1	0.7	0.1	0.1	0.1		
		Total	55.4	16.8	3.5	4.4	6.9	8.9	4.1
H. vulgare L. (S)	Astrix	Shoot	35.4	19.9	8.0	4.8	8.2		
		Root	7.1	4.1	1.6	0.9	1.8		
		Total	42.5	24.0	9.6	5.7	10.0	5.1	3.1
B. tectorum L. (S)		Shoot	54.6	10.8	1.6	2.9	0.7		
		Root	7.6	0.7	nd	0.4	0.1		
		Total	62.2	11.5	1.6	3.3	0.8	14.8	5.4
L. perenne L. (S)		Shoot	70.0	8.7	0.6	1.8	0.2		
		Root	7.5	0.6	nd	0.1	0.1		
		Total	77.5	9.3	0.6	1.9	0.3	6.9	3.5
A. myosuroides Huds. (S)		Shoot	37.4	12.9	13.4	6.8	5.9		
		Root	7.4	0.9	0.2	0.5	0.4		
		Total	44.8	13.8	13.6	7.3	6.3	7.7	6.4
A. fatua L. (S)	USA	Shoot	40.0	15.9	1.6	1.7	2.3		
		Root	3.2	4.2	1.6	1.3	1.7		
		Total	43.2	20.1	3.2	3.0	4.0	10.5	16.0
Cotton (MR)	Delta pine	Shoot	20.9	11.1	33.1	0.9	7.7		
		Root	2.4	0.3	0.1	0.1	0.1		
		Total	23.3	11.4	33.2	1.0	7.8	16.8	6.5

Results expressed as the percentage of radioactivity/gram fresh weight plant.
Experimental conditions were as described for Table 5. nd = non-detectable.
S = sensitive MR = moderately resistant.
Structures of XX1 - XX111 are given in attachment 3.

Table 15: The quantitative metabolism of N-(3-methyl-4-chlorophenyl)-N,N-dimethylurea in cereal weeds

Species	Variety/ Cultivar	Part	X1	Metabolites X11	X111	X1V	XV	XV1	XV11	Conjugates of X11	X111	X1V	XV	XV1	XV11	Un-known	Non-extr-actable
A. fatua L.	USA	Shoot	34.2	14.8	0.7	nd	0.3	nd	nd	1.1	1.1	0.3	1.8	0.2	nd		
(S)		Root	10.9	10.5	1.1	1.1	0.5	nd	nd	0.8	0.7	0.8	0.9	0.3	nd		
		Total	45.1	25.3	1.8	1.1	0.8	nd	nd	1.9	1.8	1.1	2.7	0.5	nd	3.2	14.7
A. myosuroides		Shoot	62.0	11.5	4.7	0.5	nd	0.5	nd	1.1	2.1	0.5	0.4	0.5	nd		
Huds.		Root	7.0	0.4	0.1	nd	nd	nd	nd	0.1	0.1	nd	nd	nd	nd		
(S)		Total	69.0	11.9	4.8	0.5	nd	0.5	nd	1.2	2.2	0.5	0.4	0.5	nd	2.4	6.6
L. perenne L.		Shoot	75.5	8.2	0.6	0.7	nd	nd	nd	0.6	0.4	0.1	0.1	0.1	0.05		
(S)		Root	7.1	0.3	nd	nd	nd	nd	nd	0.1	0.05	nd	nd	nd	nd		
		Total	82.6	8.5	0.6	0.7	nd	nd	nd	0.7	0.45	0.1	0.1	0.1	0.05	1.2	4.9
B. tectorum L.		Shoot	69.5	6.5	0.9	1.3	nd	0.6	nd	2.0	1.6	2.0	2.6	nd	nd		
(S)		Root	8.1	0.3	0.1	nd	nd	nd	nd	0.3	0.1	nd	nd	nd	nd		
		Total	77.6	6.8	1.0	1.3	nd	0.6	nd	2.3	1.7	2.0	2.6	nd	nd	1.1	3.0

Results expressed as the percentage of radioactivity/gram fresh weight plant.

Experimental conditions were as described for Table 5. nd = non-detectable

S = sensitive

Structures of X1 - XV11 are given in attachment 2.

Table 16: The quantitative metabolism of N-(3-methyl-4-chlorophenyl)-N,N-dimethylurea in cereals and cotton

Species	Variety/ Cultivar	Part	X1	Metabolites X11	X111	X1V	XV	XV1	XV11	Conjugates of X11	X111	X1V	XV	XV1	XV11	Un-Known	Non-extr-actable
T. aestivum L. (S)	Atou	Shoot	65.1	10.3	1.6	0.9	nd	nd	nd	2.2	1.6	2.4	3.8	nd	nd		
		Root	4.7	0.4	0.1	nd	nd	nd	nd	0.1	1.1	nd	nd	nd	nd		
		Total	69.8	10.7	1.7	0.9	nd	nd	nd	2.3	1.7	2.4	3.8	nd	nd	3.4	3.3
H. vulgare L. (S)	Astrix	Shoot	48.7	7.7	0.6	2.1	0.3	0.7	nd	4.4	1.7	3.1	13.4	1.3	nd		
		Root	8.3	1.2	0.1	0.2	nd	nd	nd	1.2	1.1	nd	0.2	nd	nd		
		Total	57.0	8.9	0.7	2.3	0.3	0.7	nd	5.6	2.8	3.1	13.6	1.3	nd	1.2	3.5
G. hirsutum L. (MR)	Delta pine	Shoot	29.7	9.2	24.9	0.2	0.6	0.7	nd	1.4	7.7	3.2	3.6	2.8	1.7		
		Root	4.2	0.3	0.1	nd	nd	0.2	nd	nd	0.2	nd	nd	nd	nd		
		Total	33.9	9.5	25.0	0.2	0.6	0.9	nd	1.4	7.9	3.2	3.6	2.8	1.7	5.6	3.7

Results expressed as the percentage of radioactivity/gram fresh weight plant.

Experimental conditions were as described for Table 5. nd = non-detectable

S = sensitive MR = moderately resistant

Structures of X1 - XV11 are given in attachment 2.

Table 17: R_f values for diuron and metabolites

Solvent System	1	2	3
XX1	0.56	0.50	0.69
XX11	0.43	0.33	0.47
XX111	0.25	0.15	0.24
Conjugates	0 - 0.1	0 - 0.1	0 - 0.1
Unknown a	0.60	0.55	0.76
Unknown b	0.31	0.22	0.34

Solvent system 1. benzene, acetone 2:1

2. chloroform, ehtylacetate 1:1

3. chloroform, ethanol 1:1

All ratios as v:v

Table 18: R_f values for N-(3-methyl-4-chlorophenyl)-N,N-dimethylurea (CGA 43057) and metabolites

Solvent System	1		2	
Compound	First dimension	Second dimension	First dimension	Second dimension
X1	0.85	0.79	0.89	0.89
X11	0.70	0.69	0.90	0.85
X111	0.65	0.67	0.82	0.75
X1V	0.47	0.46	0.76	0.64
XV	0.32	0.38	0.82	0.54
XV1	0.35	0.31	0.50	0.55
XV11	0.25	0.19	0.54	0.44
Conjugates	0 - 0.1	0 - 0.1	0 - 0.05	0 - 0.1

Solvent system	Dimension	
1	1	chloroform, ethanol, acetic acid 9:1:1
	2	chloroform, ethanol, acetone 9:2:1
2	1	ether, ethanol, acetone, acetic acid 9:2:1 + 0.1%
	2	chloroform, ethanol, acetic acid 9:2:1

All ratios as v:v

in this species so that the greatest percentage of the metabolites (some 40%) was contributed by N-didesmethylated diuron (XXIII). Two unidentified radioactive compounds occurred in all plants treated with diuron; their Rf values in the various solvent systems are presented in Table 17. They did not co-chromatograph with any of the authentic standards available, and were present in both the polar and non-polar metabolite fractions. Further identification of these two compounds was not attempted.

In agreement with earlier reports (Smith and Sheets, 1967; Onley, Yip and Aldridge, 1965; Swanson and Swanson, 1968a) the degradation of diuron was dependent on N-demethylation, as was primarily the case for CGA 43057. For these herbicides this degradation in the grass species did not proceed to any major extent beyond the stage of monodesmethylation, a fact which probably accounts for the higher sensitivity of wheat and barley to diuron and CGA 43057 and for the lack of selectivity of these herbicides in cereals. The more complete N-demethylation observed in cotton, of all the three herbicides may well have a direct bearing not only on the existence of a tolerance to chlortoluron in cotton comparable to that of the wheat and barley varieties examined but also on the enhanced resistance of cotton to CGA 43057 and diuron. The results clearly illustrate the drastic effect small variations in structure may have on susceptibility to metabolic attack and thus on performance in the field.

Time course study of the degradation of chlortoluron in whole plants

Having demonstrated a significant difference in the degradation of chlortoluron by resistant and susceptible plants over a period of ten days following uptake of the ^{14}C-labelled herbicide, it was considered of particular interest to determine if the observed differences in both type and rate of degradation were apparent at all times after the uptake of the herbicide. In addition, the sequence of appearance of the metabolites could be examined.

In the present study plants of both resistant and susceptible varieties were treated with (carbonyl-^{14}C)chlortoluron in the nutrient medium for 48 hours only so as to avoid any possible complication in interpretation resulting from the continued uptake of unchanged herbicide that might otherwise occur throughout the subsequent duration of the study. After treatment the roots were rinsed and the plants were transferred to culture containers with fresh nutrient solution without added herbicide, samples being removed for extraction and analysis of radioactive metabolites after various time intervals. Data of the recovery of radioactivity from the various varieties and cultivar at each harvest time are presented in Table 19. It is apparent from these data, that the total uptake of radioactivity in the 48 hour period of treatment is very similar for the four cereal varieties. However, the wild oat cultivar absorbed significantly more radioactivity from the nutrient solution than any of the varieties. Reference to the data also reveals that the total amount of radioactivity, and amounts of extractable and non-extractable radioactivity, remained relatively constant at each harvest for individual varieties and the cultivar. This would indicate that no

Table 19: Recovery of radioactivity

Species	Variety/ Cultivar	Harvest (days from cessation of treatment) 0	1	2	3	7
T. aestivum L.	Atou (R)	8.68 (0.21)	8.75 (0.32)	8.32 (0.25)	8.41 (0.31)	8.59 (0.22)
	M. Huntsman (S)	8.12 (0.19)	7.95 (0.27)	7.77 (0.18)	8.01 (0.22)	7.91 (0.24)
H. vulgare L.	Sonja (R)	7.52 (0.26)	7.63 (0.22)	7.49 (0.33)	7.62 (0.25)	7.55 (0.29)
	Katy (S)	7.61 (0.29)	7.73 (0.26)	7.62 (0.35)	7.59 (0.29)	7.78 (0.16)
A. fatua L.	USA (Si)	13.46 (0.21)	12.93 (0.31)	13.25 (0.29)	13.16 (0.16)	12.92 (0.37)

Results expressed as dpm x 10^{-5}/g fresh weight of plant material; data for non-extractable radioactivity extracted as presented in parenthesis. R, S and Si are as described in Table 3.

Nutrient solution grown plants were treated with ^{14}C-chlortoluron for 48 hours, after which the plants were transferred to nutrient culture solution not containing herbicide. Plants were harvested at intervals and extracted with methanol:water (8:2, v/v). The extracted radioactivity was characterised and quantified by thin layer chromatography and liquid scintillation counting.

metabolite of any significance evaded detection, and that the formation of a non-radioactive metabolite such as the aniline derivative (N-(3-chloro-4-methylphenyl)aniline) of chlortoluron did not occur to any appreciable extent. Increased binding of the herbicide or its radio-labelled metabolites did not occur up to seven days after cessation of treatment and there was no loss of radioactivity as $^{14}CO_2$ and no exudation of radioactivity by the roots. Data for the characterisation of the extractable radioactivity is presented in Tables 20 (wheat), 21 (barley) and 22 (wild oat). Throughout the duration of the experiment the amount of parent herbicide in extracts from both resistant and susceptible plants can be seen to decrease, this being accompanied by a concomitant increase in the proportion of radioactive metabolites.

Reference to the tables shows that in this experiment at the end of the treatment period the amount of unchanged chlortoluron was between 63% and 35% for the resistant cereals, 65% and 61% for the sensitive cereals and 62% for the sensitive wild oat. The corresponding values at the end of the experiment are respectively, 7% and 5%, 22% and 12% and 20%. These data thus support the results of a previous study by clearly indicating a more rapid degradation of chlortoluron in the resistant varieties.

Degradation by the resistant varieties (Tables 20, 21) was manifested by a rapid rise in the amount of conjugated ring-methyl oxidation derivatives (mainly IV) paralleled by a decline in the initially high amounts of non-conjugated IV. The changes in the levels of the monomethyl derivative of chlortoluron II, follow the same general pattern

Table 20: Quantitative metabolism of chlortoluron at various times after treatment - wheat

Variety	Time from cessation of treatment (days)	1	Metabolites						Conjugates of					
			11	111	1V	V	V1	V11	11	111	1V	V	V1	V11
Atou (R)	0	63.0	4.9	nd	5.2	0.4	0.8	0.8	8.3	nd	14.8	nd	nd	nd
	1	29.5	3.6	nd	3.1	0.4	1.3	1.7	24.1	nd	33.1	nd	nd	nd
	2	10.2	2.1	nd	1.7	1.4	1.2	1.9	25.1	nd	50.7	1.2	1.5	0.7
	3	10.8	1.8	nd	1.4	0.4	2.5	1.9	18.5	nd	49.2	5.6	4.8	1.5
	7	7.5	1.4	nd	1.4	1.1	0.7	1.6	18.9	nd	45.4	7.2	9.8	3.1
M. Huntsman (S)	0	65.4	12.3	nd	4.9	0.5	0.4	nd	8.9	nd	7.5	nd	nd	nd
	1	37.7	8.0	nd	4.1	2.8	0.7	nd	25.3	nd	20.3	0.9	nd	nd
	2	34.4	5.9	nd	4.8	3.0	1.0	nd	26.2	nd	21.2	1.0	nd	nd
	3	24.5	5.6	nd	7.4	3.8	1.8	nd	29.4	nd	25.3	1.2	0.5	0.3
	7	22.6	5.6	nd	8.5	3.3	nd	0.4	27.8	nd	25.0	4.0	1.5	1.1

Results are expressed as the percentage of total radioactivity detected. nd = non-detectable

Experimental conditions were as described for Table 19. Structure of compounds 1 - V11 are given in attachment 1.

Table 21: Quantitative metabolism of chlortoluron at various times after treatment - barley

Variety	Time from cessation of treatment (days)	1	Metabolites						Conjugates of					
			11	111	1V	V	V1	V11	11	111	1V	V	V1	V11
Sonja (R)	0	35.3	4.6	1.0	9.9	2.6	1.8	1.0	20.3	nd	22.9	0.4	0.1	nd
	1	20.9	5.5	0.7	6.6	3.0	1.0	3.6	28.4	nd	29.6	0.5	0.2	nd
	2	17.3	5.6	nd	3.2	2.4	1.0	1.0	28.2	nd	36.8	1.2	0.6	nd
	3	9.8	5.5	nd	0.4	0.5	0.9	0.5	29.0	nd	48.1	2.3	1.9	0.8
	7	5.0	2.9	nd	0.9	2.0	2.1	2.0	28.0	nd	35.3	9.9	6.5	5.3
Katy (S)	0	61.6	8.8	0.3	9.8	3.4	0.3	nd	9.7	nd	5.6	0.2	nd	nd
	1	36.6	3.3	1.1	9.8	nd	2.0	0.9	27.4	nd	16.0	1.8	0.4	0.6
	2	23.4	2.0	1.2	22.9	5.7	2.0	0.7	26.2	nd	13.2	1.5	0.5	0.5
	3	12.9	0.5	1.9	13.2	4.8	7.4	4.6	29.9	nd	24.2	6.4	3.2	2.7
	7	11.9	2.3	2.5	4.3	1.5	7.1	4.0	22.3	nd	23.4	9.4	6.7	4.3

Results are expressed as the percentage of total radioactivity detected. nd = non-detectable

Experimental conditions were as described for Table 19. Structures of compounds 1 - V11 are given in attachment 1.

Table 22: Quantitative metabolism of chlortoluron at various times after treatment - wild oat

Time from cessation of treatment (days)	1	Metabolites						Conjugates of					
		11	111	1V	V	V1	V11	11	111	1V	V	V1	V11
0	62.2	14.7	nd	3.7	0.4	1.0	0.8	14.3	nd	3.0	nd	nd	nd
1	34.7	22.0	2.1	2.4	0.2	0.4	0.1	29.5	nd	8.3	nd	0.2	0.2
2	33.9	23.2	nd	2.1	0.4	nd	0.3	30.0	nd	8.0	nd	nd	nd
3	34.3	23.9	nd	1.3	0.6	nd	0.5	30.1	nd	8.9	nd	nd	nd
7	20.4	21.5	2.1	2.0	3.2	0.8	0.3	36.1	nd	11.9	0.3	0.2	0.2

Results are expressed as the percentage of total radioactivity detected. nd = non-detectable

Experimental conditions were as described for Table 19. Structures of compounds 1-V11 are given in attachment 1.

albeit at a lower level throughout. The other metabolites present (111, V, V1, V11) occur at low levels in the non-conjugated form, but with the exception of metabolite 111 reach relatively high levels as conjugates in the later stages of the experiment. Metabolite 111 occurred only in the non-conjugated form and only in low amounts, in addition it was detected at two harvest times in barley (Table 21) and only one in wheat (Table 20). The same general pattern of the degradation of chlortoluron was apparent in the sensitive cereals, with the important qualification that the products of ring-methyl oxidation were at a lower level than the products of N-demethylation. This is most apparent in the data presented for the wild oat (Table 22), where the products of N-demethylation were substantially in excess of the products of ring-methyl oxidation at all times. The highest quantities of metabolite 111 (the didesmethylated derivative of chlortoluron) were, as could be expected, detected in the varieties displaying high rates of N-demethylation. Paradoxically, however, in one of these sensitive varieties (M. Huntsman, Table 20) no metabolite 111 could be detected. As for the resistant varieties no metabolite 111 was detected in the conjugated form. As referred to earlier, since the chlortoluron used in the present study was labelled at the carbonyl group it was realised that degradation of chlortoluron to the aniline metabolite would go undetected. However, little formation of the aniline derivative has been noted in the past in studies of the metabolism of the related phenylurea herbicides monuron (Smith and Sheets, 1967; Swanson and Swanson, 1968), diuron (Onley et al, 1968; Osgood et al, 1972), fluometuron (Rogers and Funderburk, 1968; Neptune, 1970), linuron (Kuratle, 1968) and metoxuron (Emami-Saravi, 1979). An hypothesis

advanced by Rogers and Funderburk (1968) was the existence of an enzyme system with so low an affinity for the didesmethylated derivative as substrate, that the aniline derivative would be formed to only a very slight extent or not at all, as was the case in their study of the metabolism of fluometuron in cucumber.

It is possible to produce a scheme for the sequence of appearance of the metabolites of chlortoluron based on data generated by the present study. The earliest detectable metabolites are non-polar products of either a primary N-demethylation or ring-methyl oxidation. Their decline is paralleled by an increase in the occurrence of the polar form of these metabolites. After two or three days the increase in these compounds ceases as the secondary metabolites (primarily V, Vl, Vll) appear as water soluble conjugates. This would indicate N-demethylation or ring-methyl oxidation followed by a combination of these reactions. It may well be that as postulated by Gross et al (1979) the polar conjugates need not be regarded as stable, terminal products, but rather they may be cleaved leading to the further degradation and reconjugation of the pesticide moiety.

Time course study of the degradation of chlortoluron in leaf discs and root segments

A study made utilising root and leaf segments would clarify whether the photosynthetic tissue possessed an ability to degrade chlortoluron. The previous studies reported in the present thesis on the metabolism of chlortoluron employed whole plants, with the result that it could not be determined whether the metabolites found in the shoots were produced there, or in the roots and subsequently translocated to the leaves.

The present studies utilised leaf and root tissue segments of both resistant and sensitive plants. The prepared tissue was treated with ^{14}C-labelled chlortoluron by suspension for two hours in 5 ml of 0.05M phosphate buffer pH 7 containing 3μCi of ^{14}C-chlortoluron (sp. act. 6.502μCi/μmole) and 1% sucrose. Tissue was subsequently transferred to fresh buffer not containing herbicide, and tissue harvested at intervals for analysis. The Rf values for chlortoluron and the metabolites detected in the present study, in the various solvent systems employed, are presented in Table 23. Details of the recovery of radioactivity from treated tissue is presented in Table 24.. Data for non-extractable

Table 23: rf values for chlortoluron and metabolites

Compound	Solvent System 1	2	3
1	0.76	0.83	0.84
11	0.55	0.68	0.77
1V	0.34	0.51	0.50
Conjugated	0	0 - 0.05	0 - 0.1

1. chloroform, ethanol 1:1 (v/v)
2. chloroform, ethanol 9:2 (v/v)
3. chloroform, ethanol, acetic acid 9:1:1 (v/v/v) developed twice

or non-identified radioactivity are not presented as in all cases they amounted to less than 1% of the total radioactivity extracted. It can be seen that root tissue absorbed proportionally less radioactivity than did leaf tissue. However, there were no consistent differences

between the varieties as regards absorption and retention of radioactivity, similar amounts being present at all harvests.

The data for radioactivity which leaked from the treated tissue into the incubation buffer are not presented as in all cases this was between 3.3 to 4.7% of the extracted radioactivity. It was noted in a previous study (p. 92) of the recovery of oxygen evolution by chlortoluron treated leaf segments that leakage of chlortoluron from the tissue may have contributed to the recovery of oxygen evolution. It is apparent from this study that the amount of leakage of radioactivity is low and thus unlikely to contribute significantly to recovery from chlortoluron inhibition. The data for the metabolism of chlortoluron by root and leaf tissue are presented in Tables 25 and 26 (resistant and sensitive wheat), 27 and 28 (resistant and sensitive barley) and 29 (sensitive wild oat). The data presented clearly shows that the leaf tissue degraded chlortoluron more rapidly than the root tissue. After 24 hrs some 10% to 37% less chlortoluron remained in the leaf tissue than the root tissue. The quantitative and qualitative differences in the degradation of chlortoluron by resistant and sensitive noted in previous studies is apparent in the radioactive extracts from the leaf tissue. Thus the tissue for resistant varieties displayed a greater overall rate of degradation and utilised ring-methyl oxidation to a larger extent than tissue from sensitive varieties. The wild oat leaf tissue (Table 29) displayed the lowest rate of degradation and depended to the largest extent on N-demethylation. These differences were not as apparent in the root tissue, although differences in the amounts of the N-demethylated compound (11) and the product of ring-methyl oxidation (111) are readily

Table 24: Recovery of radioactivity

Species	Variety/ Cultivar		Time from cessation of treatment (hours) 0	4	8	12	24
T. aestivum L.	Atou (R)	Shoot	13.2	12.7	12.9	13.1	13.3
		Root	9.4	8.9	9.9	7.9	8.3
	M. Huntsman (S)	Shoot	12.4	12.9	13.4	12.6	13.1
		Root	9.2	8.5	7.9	8.2	8.7
H. vulgare L.	Sonja (R)	Shoot	11.9	12.8	12.4	13.1	12.6
		Root	8.9	8.3	8.7	7.9	9.2
	Katy (S)	Shoot	12.5	12.9	13.2	12.1	12.5
		Root	8.7	8.4	8.5	9.3	9.1
A. fatua L.	USA (Si)	Shoot	13.3	12.9	13.5	12.2	12.7
		Root	9.1	7.9	9.1	8.8	8.5

Results expressed as extracted dpm x 10^{-5}/g fresh weight of tissue.

R, S and Si are as described in Table 3.

Experimental conditions were as described for Table 26.

Table 25: Quantitative metabolism of chlortoluron at various times after treatment of root and shoot tissue - wheat

Atou

Time from cessation of treatment (hours)	Leaf Tissue			Root tissue		
	Compound			Compound		
	1	11	1V	1	11	1V
0	98.1	nd (nd)	1.0 (nd)	98.3	nd (nd)	0.3 (nd)
4	92.1	2.0 (0.6)	6.0 (2.0)	98.0	nd (nd)	1.1 (nd)
8	82.5	5.9 (2.1)	11.6 (4.1)	94.9	2.0 (nd)	2.7 (0.6)
12	65.8	8.8 (4.0)	25.3 (8.0)	89.5	4.3 (0.1)	5.2 (1.7)
24	52.6	12.2 (5.5)	34.9 (12.3)	89.1	4.9 (0.2)	5.7 (1.3)

Table 26: Maris Huntsman

Time from cessation of treatment (hours)	Leaf Tissue			Root tissue		
	Compound			Compound		
	1	11	1V	1	11	1V
0	96.9	1.7 (nd)	0.6 (nd)	98.2	0.6 (nd)	nd (nd)
4	91.8	4.2 (nd)	2.8 (nd)	98.1	0.9 (nd)	nd (nd)
8	85.3	9.0 (0.3)	5.0 (0.7)	96.9	2.1 (nd)	0.3 (nd)
12	81.2	11.5 (1.0)	6.7 (1.5)	91.0	5.9 (0.3)	2.0 (0.2)
24	69.2	20.6 (4.2)	9.8 (5.5)	88.9	6.3 (0.4)	3.2 (0.3)

Results are expressed as the percentage of each metabolite of the total radioactivity at each harvest. Data for conjugated material is additionally presented in parenthesis. nd = non-detectable. Plant tissue was incubated on 0.05M phosphate buffer pH 7.0 containing 3 μCi ^{14}C-labelled chlortoluron for two hours prior to transfer to nutrient solution not containing herbicide.

Table 27: Quantitative metabolism of chlortoluron at various times after treatment of root and shoot tissue - barley

Astrix

Time from; cessation of treatment (hours)	Leaf Tissue			Root Tissue		
	Compound			Compound		
	1	11	1V	1	11	1V
0	98.0	nd (nd)	0.8 (nd)	98.4	nd (nd)	0.4 (nd)
4	92.4	1.4 (0.3)	4 5 (1.4)	97.3	0.5 (nd)	1.2 (nd)
8	82.7	3.6 (1.2)	12.3 (3.4)	96.0	1.4 (nd)	2.1 (nd)
12	76.2	5.1 (2.2)	17.1 (5.7)	90.9	3.9 (0.2)	4.3 (1.2)
24	57.3	11.2 (5.3)	29.3 (11.4)	88.9	4.4 (0.3)	5.7 (1.4)

Table 28: Katy

Time from cessation of treatment (hours)	Leaf Tissue			Root Tissue		
	Compound			Compound		
	1	11	1V	1	11	1V
0	96.0	0.3 (nd)	0.5 (nd)	98.3	0.6 (nd)	0.3 (nd)
4	91.5	2.3 (nd)	2.2 (nd)	96.9	1.4 (nd)	0.6 (nd)
8	85.9	4.9 (0.3)	7.4 (1.1)	94.7	3.2 (nd)	1.0 (nd)
12	73.5	11.1 (4.0)	10.7 (2.1)	89.3	7.4 (0.4)	3.0 (0.3)
24	61.8	17.7 (6.7)	15.7 (3.4)	87.0	8.7 (0.6)	3.5 (0.4)

Results are expressed as the percentage of each metabolite of the total radioactivity at each harvest. Data for conjugated material is additionally given in parenthesis. nd = non-detectable.

Experimental conditions were as described for Table 26.

Table 29: Quantitative metabolism of chlortoluron at various times after treatment of root and shoot tissue - wild oat

Time from cessation of treatment (hours)	Leaf Tissue			Root Tissue		
	Compound			Compound		
	1	11	1V	1	11	1V
0	97.3	0.9 (nd)	nd (nd)	98.7	0.1 (nd)	nd (nd)
4	92.1	6.1 (nd)	0.5 (nd)	97.5	2.3 (nd)	nd (nd)
8	86.2	10.9 (0.7)	1.8 (0.5)	94.3	3.0 (1.1)	2.2 (0.5)
12	79.6	15.3 (1.3)	3.9 (1.0)	98.5	6.7 (1.7)	4.1 (0.7)
24	72.0	21.3 (2.6)	5.4 (1.2)	82.1	12.6 (1.4)	4.1 (0.7)

Results are expressed as the percentage of each metabolite of the total radioactivity at each harvest. Data for conjugated material is additionally presented in parenthesis. nd = non-detectable. Experimental conditions were as described for Table 26.

apparent 12 hr after treatment. In contrast to earlier investigations only two metabolites were identified in the present study, possibly due to the short term nature of the experiment. Both metabolites were present in polar and non-polar forms, however, the amount of polar material was low in all the varieties, indicating that initially at least the rate of conjugation is less than the rates of N-demethylation and ring-methyl oxidation.

It is apparent from this study that the differences in the degradation of chlortoluron noted in whole plants is significantly dependent on the degradation of the herbicide by photosynthetic tissue.

Chlortoluron degradation by subcellular organelle fractions

In a final series of experiments on the metabolism of chlortoluron, an attempt was made to locate the site(s) of chlortoluron metabolism within the plant cell. The existence of an *in vitro* cotton leaf microsomal oxidase system, which N-demethylated monuron to form the N-monodesmethylated derivative has been reported by Frear (1968). In further reports (Frear *et al*, 1969; Tanaka *et al*, 1972a, b) active enzyme preparations were also obtained from cotton, plantain, buckwheat, wild buckwheat and broadbean, which utilised monuron, monomethylmonuron, diuron and fluometuron as substrates. The enzyme was apparently specific for N-(phenyl)-N-methylureas and required molecular oxygen and either NADPH or NADH as co-factors. In addition, Emami-Saravi (1979) has more recently described an N-demethylase associated with the microsomal fraction extracted from wheat, which was capable of N-demethylating metoxuron.

These N-demethylation reactions have been ascribed to the action of a mixed function oxidase system similar to that of castor bean which catalyses the N-demethylation of p-chloro-N-methylaniline (Young and Beevers, 1976). The present study utilised plants of resistant and sensitive varieties, both light and dark grown, harvested at various growth stages. Subcellular organelle fractions were isolated by an adaptation of the procedure of Frear (1968). The brei obtained by the initial homogenisation of the tissue was subsequently fractionated by differential centrifugation at 300 x g, 1500 x g, 9000 x g and 100,000 x g. After washing individual fractions were assayed for chlortoluron ring-methyl oxidation and N-demethylation activity. Following termination of the reaction by rapid freezing and lyophilisation, the chlortoluron and metabolites were extracted and identified and quantified by thin layer chromatography and liquid scintillation counting.

Initially, however, an organelle characterisation study was carried out utilising NADPH-DCPIP oxidoreductase, NADPH-cytochrome C oxidoreductase and NADH-cytochrome C oxidoreductase as 'markers' for microsomes, succinate-cytochrome C oxidoreductase and succinate-DCPIP oxidoreductase as 'markers' for mitochondria, and chlorophyll as a 'marker' for chloroplasts. This study demonstrated that the organelle fractions obtained by sedimentation at 1500 x g, 9000 x g and 100,000 x g consisted predominantly of chloroplasts, mitochondria and microsomes respectively. Enzyme activity in the 100,000 x g supernatant was too low to be measured accurately though it would be expected that some microsomal particles such as endoplasmic reticulum stripped of ribosomes and smooth endoplasmic reticulum would be located in this fraction. This study also demonstrated

a low degree of cross contamination between fractions which was generally in the region of 5% to 10%, the major contaminant being chlorophyll.

The results of a number of experiments involving the incubation of carbonyl-^{14}C chlortoluron with various organelle fractions are presented in Table 30. These data clearly demonstrate the association of chlortoluron degradation predominantly with the post-mitochondrial fractions. Although every fraction displayed N-demethylation activity, the highest specific activity obtained was shown by the microsomal (100,000 x g) pellet and microsomal supernatant fractions, this being in agreement with results obtained by Emami-Saravi (1979) for the N-demethylation of metoxuron. Ring-methyl oxidation activity, however, was only detected in post-mitochondrial fractions, clearly linking this activity to microsomal particles. The highest specific activity can be seen to be associated with the microsomal pellet rather than being evenly distributed between the microsomal pellet and supernatant fractions as was the case for N-demethylation. As with ring-methyl oxidation the production of polar material was only associated with post-mitochondrial fractions, most notably in the microsomal pellet and supernatant, the amounts produced being usually slightly larger in the 100,000 x g supernatant.

With the exception of organelle fractions isolated from etiolated tissue, chlortoluron degradation activity was very similar regardless of whether the organelle fraction originated from resistant or sensitive plants, or from root or shoot tissue. This is in contrast to the results of *in vivo* studies reported earlier, which demonstrated differences in chlortoluron degradation between resistant and sensitive plants and root

Table 30: Chlortoluron degradation by subcellular organelle fractions

Fraction	Product	Atou (R)			A. fatua (Si)		USA (S)	M. Huntsman (R)
		leaf	root	etiolated	leaf	root	leaf	leaf
Cell free extract	11	306	497	262	428	395	377	419
	1V							
	conjugate							
1500 x g pellet	11	479	532	202	506	441	435	404
	1V							
	conjugate							
9000 x g pellet	11	554	568	221	763	582	499	523
	1V							
	conjugate							
9000 x g supernatant	11	1876	1993	850	2012	1932	1980	1950
	1V	295	210	106	265	392	342	320
	conjugate	421	209	136	230	290	274	345
100,000 x g pellet	11	2350	2105	1016	2194	2107	2242	2134
	1V	579	830	421	772	634	710	729
	conjugate	402	506	123	479	593	482	584
100,000 x g supernatant	11	2439	2219	1209	2032	1976	2134	2243
	1V	321	401	276	302	367	429	427
	conjugate	505	576	116	499	601	550	554

Results are expressed as dpm product formed/mg protein/hr. Plants were at growth stage 11 at harvest, the etiolated tissue (leaf) was taken from plants five days after germination. Each reaction mixture contained 6.99 x 10^{-3} μmole chlortoluron (sp. act. 6.502 μCi/μmole), 0.05 μmole NaCN, 50 μmole K_2HPO_4 pH 7.5, 1-3 mg protein and an NADPH generating system.

and shoot tissue. This may indicate that the true activity of the enzyme system(s) responsible for chlortoluron degradation were not expressed in the present study and that further investigation of isolation and incubation conditions may elicit greater activity and reveal varietal and tissue differences. Alternatively, the data produced by the present study may indeed represent the true rate of chlortoluron degradation by *in vitro* systems. The *in vivo* rates being due to differential compartmentalisation of the enzyme system(s) or altered substrate affinity in the whole cell, or the presence of inhibitory factors lost during organelle isolation.

Organelle fractions isolated from dark grown plants proved to possess a considerably reduced rate of chlortoluron degradation activity. This result is in contrast to the data of Frear *et al* (1969), which demonstrated that the N-demethylase activity of etiolated cotton tissue was greater than that of light grown tissue. However, the present study raises the possibility that the degradation of chlortoluron is partially light activated. It is interesting to note that the rates of formation of the N-demethylated product (11) and the product of ring-methyl oxidation (1V) by the 100,000 x g pellet were in the region of 0.7 and 0.2 nmoles/mg protein/30 minutes respectively. These data compare favourably with the N-demethylase activity of the 78,000 x g pellet isolated from cotton leaf (Frear *et al*, 1969) which was 0.08 nmoles product formed/mg protein/30 minutes. However, the comparable preparation from etiolated hypocotyl yielded N-demethylation activity of 2.16 nmoles/mg protein/30 minutes. Thus it may be that the degradation of phenyl-ureas occurs, as suggested by Frear *et al* (1969) and Young and Beevers

(1976) due to the action of cytochrome P_{450} dependant mixed function oxidase system with a limited substrate specificity and activity dependant on tissue type.

The nature of the polar conjugates of metabolites of chlortoluron

The degradation of the substituted phenylureas by plants can be classified into two distinct phases (Parke, 1968; Williams, 1971). 'Phase one' reactions involve primarily demethylation, demethoxylation, hydroxylation and oxidation, and 'phase two' reactions involving principally conjugation. Though pesticide metabolism studies have in the past tended to emphasise 'phase one' reactions, evidence which suggests that the rate and extent of glycoside formation is a significant factor in regulating the biological activity and selectivity of pesticides and their metabolites is increasing (Frear, 1976). Where the conjugated or polar metabolites of the phenylureas have been fully investigated as in the studies of Frear and Swanson (1972) and Lee, Griffin and Fang (1973) of monuron metabolism by cotton, and Geissbuhler and Voss (1971) of the metabolism of fluometuron and metabromuron by cotton and tobacco respectively, they have invariably been found to consist of a pesticide metabolite conjugated as a β-glucoside.

In the present study chlortoluron treated and control plants of wheat variety Atou were exhaustively extracted with methanol:water (8:2) and the polar compounds separated from contaminating material by partitioning, ultrafiltration, gel filtration and preparative paper chromatography.

Table 31: Rg values of various sugars and the radioactive conjugates

Compound	Rg value
radioactive conjugates	500 - 627
glucuronic acid	360 - 366
rhamnose	270 - 274
ribose	225 - 229
xylose	171 - 173
fucose	156 - 158
arabinose	143 - 146
mannose	116 - 118
fructose	111 - 114
glucose	100 - 103
galactose	87 - 90
sucrose	70 - 74
raffinose	50 - 52

$$Rg = \frac{\text{distance of the compound from the origin}}{\text{distance of glucose from the origin}} \times 100$$

The solvent system consisted of ethyl acetate/acetic acid/water 9:2:2 v/v/v irrigating Whatman No. 3 chromatography paper.

A considerable difficulty associated with studies of the identification of the glycone moiety of pesticide conjugates is contamination with endogenous plant sugars. To remove such sugars the aqueous fraction was submitted to preparative paper chromatography utilising a comprehensive selection of sugars as standards. The Rg values for these sugar standards and the radioactive conjugates are presented in Table 31. After hydrolysis of the aqueous extract from both treated and control plants the aglycone moiety was characterised and quantitated by thin

layer chromatography and liquid scintillation counting, and the glycone moiety by the quantitative chromatography method of Menzies et al (1979) and the glucose oxidase assay.

The results of the identification and quantitation of the aglycone moiety are presented in Table 32.

Table 32: Identification and quantitation of the aglycone moiety of the conjugates of chlortoluron

	Metabolite fraction				
	11	1V	V	V1	V11
percentage	1.9	78.1	17.2	1.3	1.5

Results are expressed as the percentage of the total conjugated radioactivity present (722,666 dpm)

Calculation from the individual molecular weights of metabolites 11, 1V, V, V1 and V11, and their percentage occurrence, reveals the presence of 0.123μ moles of chlortoluron metabolites after hydrolysis of the conjugated radioactivity.

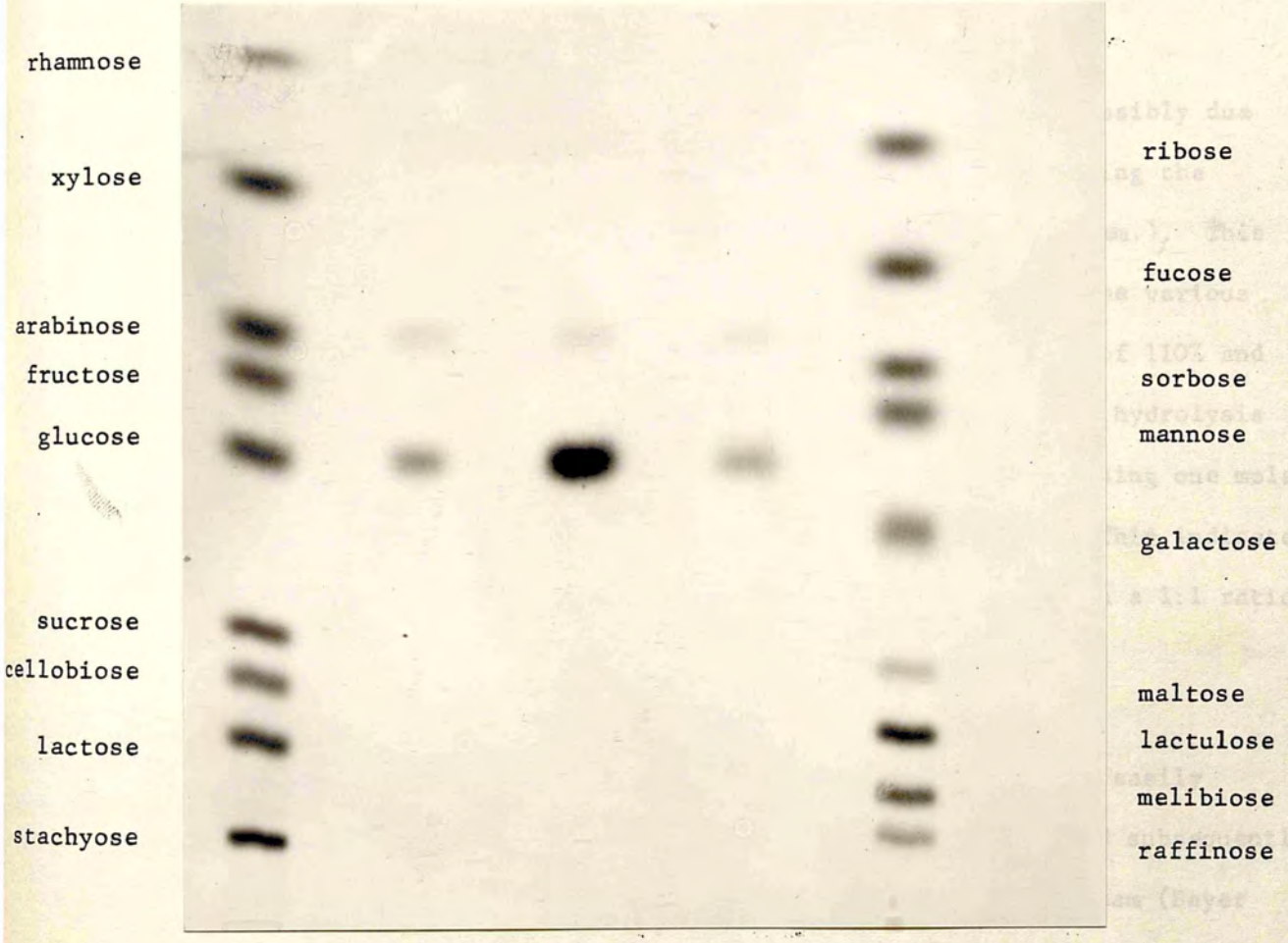
The results of the thin layer chromatography analysis of the glycone moiety are presented in Table 33 and Fig. 17.

Table 33: Quantitation of the glycone moiety of the conjugates of chlortoluron

	hydrolysed (μg)	unhydrolysed (μg)	control (μg)
glucose	28.51	3.46	3.51
arabinose	2.26	2.31	2.27

The results presented are the mean for extracts hydrolysed using β-glucosidase and 0.1M trifluoracetic acid.

Fig. 17: Identification and quantitation of the glycone moiety by the method of Menzies et al (1979)



The sugar standards, unhydrolysed extract and 5-10 μl of the hydrolysed samples were applied to silica gel F1800 plastic backed thin layer chromatography plates, layer thickness 0.25 mm and developed three times with ethyl acetate, pyridine, acetic acid, water (6:3:1:1 v/v/v/v).

The total amount of glucose released from conjugation after allowance has been made for that present in the control assays is 0.139 μmoles. Determination by the glucose oxidase assay which is specific for β-D-glucose yielded 0.136 μmoles. The identity of the glucose was subsequently confirmed in several thin layer chromatography systems and in all cases co-chromatographed with an authentic standard.

The small amount of arabinose present in all extracts is possibly due to the production of contaminating amounts of arabinose during the manufacture of chromatography paper (J.B. Pridham, pers. comm.), this arabinose being eluted from the chromatography paper with the various samples. The two assays for glucose indicate the recovery of 110% and 113% of the theoretical amount of glucose expected from the hydrolysis of conjugates of metabolites II, IV, V, VI and VII containing one mole of glucose for each mole of the chlortoluron metabolites. This indicates the conjugation of the pesticide metabolites with glucose in a 1:1 ratio.

Studies on the Uptake and Translocation of Chlortoluron

It is generally agreed that the substituted phenylureas are easily absorbed from nutrient solution and soil by root systems and subsequently translocated into stems and leaves by the transpiration stream (Bayer and Yamaguchi, 1965). Translocation of the phenylureas has in the past been shown to be confined to the apoplast; however recent evidence (Emami-Saravi, 1979) indicates that they may also move in the symplast.

The present study was undertaken to examine the contribution of absorption and distribution to the selectivity of chlortoluron in cereals and cereal

weeds. The varieties of resistant and sensitive wheat and barley, and sensitive wild oat were grown in nutrient solution culture containing 3.1 ppm (carbonyl-^{14}C)chlortoluron. Plants were harvested at intervals and the amount and distribution of the absorbed radioactivity determined by liquid scintillation counting of the culture solution and of the shoots after NCS digestion as described in the relevant section of the preceeding chapter.

The data of the uptake and distribution of chlortoluron in resistant and sensitive plants are presented in Table 34. The data demonstrates a progressive absorption of (carbonyl-^{14}C)chlortoluron throughout the duration of the experiment. All plants showed increasing amounts of radioactivity associated with the shoots as the time of root contact with the nutrient solution increased. Thus at the final harvest all the plants examined contained in excess of 90% of the absorbed radioactivity in the shoots. There appears to be no difference amongst the wheat varieties as regards the amount of radioactivity absorbed, or the rate at which this absorption took place. There were, however, differences amongst the barley varieties in the total amount of radioactivity absorbed, reflected in differences in the rate of absorption during the latter part of the experiment. It is apparent, however, that these observed differences do not correlate with the cultivar specific response, thus Katy, a sensitive variety, absorbed substantially less chlortoluron than did resistant Astrix. There are, however, major differences between the wild oat and the cereals with a substantially greater amount of radioactivity present in the wild oat than the cereal at all harvest times (Table 34). This result may partially explain the

Table 34: Absorption and distribution of radioactivity in cereals and a cereal weed after treatment with (carbonyl-^{14}C)chlortoluron

TIME days	µg Chlortoluron absorbed/g fresh weight								
	Triticum aestivum L.				*Hordeum vulgare* L.				*Avena fatua* L.
	Atou (R)	Bouquet (R)	Maris Huntsman (S)	Maris Kinsman (S)	Astrix (R)	Sonja (R)	Katy (S)	Maris Otter (MR)	USA (S)
0.5	5.12 (51.1)	1.93 (48.3)	4.68 (53.7)	4.69 (55.2)	4.21 (57.6)	4.65 (49.3)	5.66 (51.9)	5.02 (55.6)	19.68 (51.2)
1	10.65 (68.4)	3.63 (63.4)	10.61 (63.6)	9.83 (62.2)	9.14 (68.7)	9.08 (59.5)	7.24 (59.1)	10.52 (63.6)	21.48 (63.2)
2	14.1 (75.3)	11.94 (72.3)	13.07 (75.2)	14.3 (74.9)	11.44 (78.2)	14.69 (71.7)	6.66 (74.9)	10.61 (77.6)	33.27 (72.4)
3	22.45 (85.8)	12.0 (83.7)	20.64 (81.1)	20.24 (86.6)	11.42 (84.1)	14.54 (85.8)	14.99 (89.6)	16.42 (81.7)	38.82 (84.7)
3.5	19.04 (89.7)	24.6 (91.4)	26.2 (89.8)	25.5 (90.7)	21.4 (91.3)	19.06 (92.4)	16.39 (91.7)	25.9 (88.9)	38.7 (90.6)
6	23.03 (92.6)	26.6 (90.2)	26.9 (90.1)	26.1 (91.3)	26.7 (93.5)	22.3 (90.5)	18.3 (91.6)	31.3 (90.3)	51.45 (91.1)

The percentage of the absorbed radioactivity present in the shoots is presented in parenthesis. Nutrient solution grown plants were treated with 3.1 ppm chlortoluron and harvested at intervals. At each harvest the amount of radioactivity present in the culture solution and the shoots was determined.

low level of DCPIP photoreduction of chloroplasts isolated from chlortoluron treated wild oat plants (p. 83) and the observation that the initial oxygen evolution of leaf segments of similarly treated wild oat plants is significantly less than that of comparable cereal plants (p. 90)

Radioautographs prepared from plants at each harvest are not presented as they mirror those prepared from plants grown in soil treated with (carbonyl-^{14}C)chlortoluron (p.99), indicating translocation of radioactivity to all parts of the plants. Radioautography, as noted by Hays (1976) cannot be regarded as a quantitative technique, especially for compounds such as the phenylureas which undergo extensive degradation by plants.

Previous studies have shown that differences in susceptibility of several plants to monuron and diuron appear to be related to differential absorption (Smith and Sheets, 1967). Similar conclusions were arrived at by Neptune (1970) following a study of the selective action of fluometuron on maize (resistant) and wheat (susceptible), and Feeny et al (1974) of the selective action of chloroxuron on soybean and tall morning glory. The accumulation of approximately two fold greater amounts of radioactivity in the leaves of the wild oat compared to the cereals noted in the present study, may be responsible in part for the different reactions of the wild oats and cereals to chlortoluron. Differences in absorption, however, do not appear to contribute to the varietal response of the cereals.

Intracellular distribution of chlortoluron and its metabolites in wheat and wild oat

In the previous study a distinction was demonstrated between the cereals and the wild oat with respect to the uptake of chlortoluron, such a result, however, is only significant if it can be shown to be reflected in similar differences in respect to the amounts of chlortoluron associated with the chloroplast fraction. It was also of interest to determine if any organelle fractions contained the major proportion of any of the metabolites of chlortoluron. Such a determination would aid in the definition of the organelle fraction(s) responsible for chlortoluron degradation.

In the present study the amount of radioactivity associated with several organelle fractions, following uptake of ^{14}C-labelled chlortoluron by whole plants was quantitated. For this purpose plants were grown in soil treated with 36.72 μCi (wheat, variety Atou) or 18.36 μCi (A. fatua L., USA) of (carbonyl-^{14}C)chlortoluron (sp. act. 6.502 μCi/μmole). All plants were harvested at growth stage 12, some 10 days after sowing. After homogenisation the organelle fractions were obtained by differential centrifugation at 300 x g, 1500 x g, 9000 x g and 100,000 x g. The radioactivity was characterised and quantified as described previously (p. 64). The results of a preliminary organelle characterisation experiment have been presented previously (p. 138). This study indicated that the pellets obtained by differential centrifugation at 1500 x g, 9000 x g and 100,000 x g consisted predominantly of chloroplasts, mitochondria and microsomes respectively.

The distribution of radioactivity amongst organelle fractions prepared from chlortoluron resistant and sensitive plants are presented in Table 35. From these data it is apparent that the chlortoluron sensitive wild oat absorbs a proportionally greater amount of the available radioactivity than does the chlortoluron resistant wheat. Examination of the data presented for the 300 x g and 1500 x g pellets reveals that these fractions from the wild oat contain greater absolute amounts of radioactivity than do the equivalent fractions from wheat. The approximately two fold greater concentration of radioactivity associated with the chloroplast fraction of the wild oat compared to wheat is of significance as the mode of action of chlortoluron is through an inhibition of photosynthesis. The true significance of this difference becomes apparent after examination of Tables 36 and 37 which contain data on the identity of the radioactivity associated with the organelle fractions. It is apparent that there is significantly more chlortoluron and the toxic metabolite 11 associated with the chloroplast fraction from wild oat than that from the wheat. These data clearly show that the lesser ability of the wild oat to degrade chlortoluron results in greater quantities of toxic material associated with the chloroplast fraction, than in the same fraction of the wheat variety. Kuratle (1968) in a study of the subcellular localisation of absorbed linuron found the greatest amounts associated with the chloroplast fraction of sensitive ragweed than resistant carrot and concluded that this was a significant factor in the differential response of these two species.

Table 35: Intracellular distribution of absorbed radioactivity in wheat and wild oat

Fraction	DPM x 10^4/fraction/gram tissue	
	T. aestivum L. Atou	*A. fatua* L. USA
Debris	5.06	2.74
300 x g pellet	1.02	1.67
1500 x g pellet	1.66	1.92
9000 x g pellet	2.47	2.60
100,000 x g pellet	0.89	0.51
100,000 x g supernatant	16.67	7.54

Soil grown plants were treated with 36.72 μCi or 18.36 μCi (Atou and USA respectively) of (carbonyl-^{14}C)chlortoluron. Plants were harvested, homogenised and subcellular fractions obtained by differential centrifugation. Radioactivity was quantified by liquid scintillation counting.

Table 36: Intracellular distribution of absorbed radioactivity - identification and quantitation of the metabolites of chlortoluron in the resistant wheat variety Atou

Fraction	1	Metabolites					Conjugates of	
		11	1V	V	V1	V11	11	1V
Debris	5.91	0.56	1.24	0.2	0.43	0.61	2.02	10.05
300 x g pellet	3.37	0.03	0.04	0.01	nd	0.01	0.48	0.8
1500 x g pellet	2.98	0.11	0.13	0.05	0.1	0.13	1.25	2.72
9000 x g pellet	4.65	0.23	0.35	0.13	0.2	0.26	2.24	3.15
100,000 x g pellet	0.78	0.11	0.26	0.04	0.08	0.11	0.89	1.67
100,000 x g supernatant	12.89	1.27	2.14	0.4	0.5	0.45	5.02	28.4

Results are expressed as each metabolite in each fraction as a percentage of the total identified extractable material/gram tissue.

The identified material amounted to 2.7×10^5 dpm/g and unidentified/non-extractable material to 5.6×10^3 dpm/g.

The experimental conditions were as described for Table 35.

Table 37: Intracellular distribution of absorbed radioactivity - identification and quantitation of the metabolites of chlortoluron in the sensitive wild oat cultivar USA

Fraction	1	Metabolites					Conjugates of		
		11	111	1V	V	V1	11	111	1V
Debris	8.75	3.32	0.21	0.32	0.37	0.21	2.0	nd	0.96
300 x g pellet	7.27	0.66	nd	0.20	0.21	0.13	0.90	nd	0.42
1500 x g pellet	9.83	0.92	nd	0.12	0.16	nd	0.38	nd	0.13
9000 x g pellet	10.74	1.68	nd	0.40	0.30	nd	1.77	nd	0.42
100,000 x g pellet	1.28	0.86	nd	0.09	0.06	nd	0.47	0.04	0.21
100,000 x g supernatant	27.78	10.59	nd	0.83	1.13	0.54	2.39	nd	0.95

Results are expressed as each metabolite in each fraction as a percentage of the total identified extractable material/gram tissue.

The identified material amounted to 1.6×10^5 dpm/g and unidentified/non-extractable material to 3.7×10^3 dpm/g.

The experimental conditions were as described for Table 35.

In the present study the highest concentration of metabolites occurred in the post-mitochondrial preparations indicating that these fractions have a significant role in the degradation of chlortoluron. Care must be taken in the interpretation of the data, however, as considerable relocation of radioactivity may have occurred during the extraction procedure. It is also apparent that any chlortoluron or its metabolites present in the apoplast at the time of harvest would be detected in the 100,000 x g supernatant after fractionation.

DISCUSSION

In this section it is intended to summarise the experiments presented and discussed separately in the preceeding section. In addition suggestions for areas of future research arising from conclusions made as a result of the present investigations will be discussed.

The studies on the Hill activities of chloroplasts incubated with chlortoluron or isoproturon in vitro demonstrated a similar inhibition of DCPIP photoreduction by chloroplasts prepared from either resistant or sensitive plants. In previous studies, the I50 of pI50 values determined for Hill reaction inhibitors (Wessels and Van Der Veen, 1956; Moreland and Hill, 1959; Good, 1961; Moreland, 1969) have been taken to indicate the toxicity of the herbicides under study and the inherent sensitivity of the chloroplasts to these herbicides. It is unusual for herbicide selectivity to be due to inherent differences at the chloroplast level (Moreland and Hill, 1962), though recently (Machado, Bandeen, Stephenson and Jensen, 1977) such differences have been noted for the triazine group of herbicides which, like the substituted phenylureas, are inhibitors of photosynthetic electron transport. Further investigations (Arntzen, Ditto and Brewer, 1979) led to the conclusion that a component of the PS II complex was altered in resistant chloroplasts which reduced the binding of the herbicide. The same investigation, however, failed to reveal any such response to diuron.

Studies on the Hill reaction of chloroplasts isolated from chlortoluron-treated plants demonstrated a greater reduction of DCPIP photoreduction by chloroplasts from sensitive than from resistant plants (Table 4).

The only significant reduction in activity of chloroplasts from isoproturon-treated plants, however, occurred in the weed species. These results correlate well with data obtained in field trial studies where isoproturon has been found to be non-toxic to cereals but highly toxic to monocotyledonous weeds. Chlortoluron, however, has been demonstrated to be toxic to certain varieties of cereals in addition to weed species. As the in vitro Hill reaction studies demonstrated that there was no inherent differences in chloroplast sensitivity, the results obtained in this latter study indicate that there is more phytotoxic material in the chloroplasts of sensitive compared to resistant plants. As distinct from the in vitro Hill reaction studies in which the herbicides arrived at the site(s) of inhibition within the chloroplast by mainly physical means. Phytotoxic material present in chloroplasts isolated from treated plants had been affected by the physiological phenomena of absorption, distribution and degradation.

The above phenomena were subsequently investigated with the exception of that of possible differential penetration of the herbicide or its metabolites into the chloroplast. A major difficulty of this type of study is the preparation of intact functional chloroplasts for in vitro studies. Although there are many references in the literature to the preparation of whole chloroplasts (Leech, 1964; Walker, 1965; Karlstam and Albertsson, 1969; Lilley, Fitzgerald, Rienits and Walker, 1975) they are most usually prepared from soft tissue from which, unlike cereal leaves, the chloroplasts can be released by gentle homogenisation. However, Emami-Saravi (1979) using an isolation procedure adapted from Miyachi and Hogatsu (1970) has isolated intact functional chloroplasts from wheat to study the penetration

of the structurally-related phenylurea metoxuron into chloroplasts. His data indicated a ready penetration of metoxuron into the chloroplasts of varieties designated resistant or sensitive, and concluded that impeded access to the sites of inhibition within the chloroplast was unlikely to contribute to metoxuron resistance. Though chlortoluron is somewhat more hydrophobic than metoxuron due to the replacement of the p-methoxy group of metoxuron with a methyl group, the results obtained by Emami-Saravi would suggest that differential penetration of chlortoluron across the chloroplast envelope is unlikely to be a contributory factor to the varietal response of cereals to the herbicide. This view could, however, be verified using a technique refined by Heldt (Heldt and Rapley, 1970a, b; Gaenssleu and McCarty, 1972; Gimmler, Schafer and Heber, 1974; Fliege, Flugge, Werden and Heldt, 1978) which would enable the quantitation of the amount of herbicide entering intact chloroplasts. Such a study would provide information on chloroplast membrane permeability and would also enable calculation of the amount of herbicide giving rise to known levels of inhibition of the Hill reaction. This technique, used initially in work with mitochondria (Klingenberg and Pfaff, 1967) would involve the incubation of the radioactive herbicide with intact organelles subsequently rapidly centrifuged through a layer of silicone oil to remove the unabsorbed tracer.

Differences in the absorption and subsequent distribution of herbicides by resistant and sensitive plants have in previous studies been shown to contribute to herbicide selectivity. In the present study with chlortoluron, however, no differences were noted between cereal varieties which could be related to cultivar response, though such a relationship was seen to exist

in the case of the cereal weed Avena fatua L. which absorbed considerably greater amounts of radioactivity. From these results it was earlier concluded that the greater rate of uptake of the herbicide by the wild oat contributes to the particularly sensitive response of this species to chlortoluron. In a study of the uptake of metoxuron by six wheat cultivars Muller and Sanad (1975) were unable to demonstrate any correlation between herbicide uptake and varietal response, a result in agreement with these obtained in the present thesis. The results obtained by these latter authors, however, appear to contrast with data obtained by Emami-Saravi (1979) who described differences in absorption of metoxuron between a resistant and a sensitive wheat cultivar. However, as the study of Muller and Sanad (1975) employed soil-grown plants, whereas that of Emami-Saravi (1979) used nutrient culture-grown plants, the results are probably not directly comparable especially as Emami-Saravi (1979) tested only two cultivars. These studies emphasise, however, that care must be taken in extrapolating data obtained from nutrient solution grown plants to situations existing under field conditions.

The results of the study on the subcellular distribution of absorbed ^{14}C-chlortoluron indicated that more phytotoxic material was associated with the chloroplast fraction of the sensitive weed species (Avena fatua L. USA) than with that isolated from the resistant wheat (Atou), a significant observation in view of the fact that phytotoxicity is primarily mediated through an inhibition of photosynthesis. A degree of caution, however, is necessary in interpreting results obtained in such subcellular organelle distribution studies made using whole plants, since it is difficult to assess the redistribution of radioactivity caused during organelle isolation.

Similar difficulties have been encountered in studies of the distribution of 2,4,5-T (Bretherton and Hallam, 1979) and atrazine (Shimabukuro and Swanson, 1969). In all cases studied previously, however, useful information on the intracellular distribution of the herbicide under investigation was obtained. The high levels of radioactivity in the 100,000 x g supernatant fractions of leaf tissue of both plant species used in the study reported in this thesis, could be due to redistribution of intracellular radioactivity in addition to the radioactivity associated with the apoplast. Alternatively it could indicate that this is the fraction most active in the degradation of chlortoluron. Data obtained by Lee and Fang (1972) suggest that the amount of radioactive material associated with the chloroplast fraction in vivo is likely to be somewhat higher than that indicated by the data presented in Table 35. Lee and Fang (1972) found that the absorption of monuron by isolated chloroplasts was a complex phenomenon. That proportion of the herbicide causing inhibition being reversibly bound, it proving possible to restore photochemical activity by washing the chloroplasts. The herbicide which was tightly bound within the chloroplast did not appear to have an inhibitory function, a result supported by the data of Izawa and Good (1965) on monuron and diuron absorption by isolated chloroplasts. Thus the data presented for the occurrence of ^{14}C-chlortoluron with the chloroplast fraction of wheat and wild oat does not include radioactivity removed from the chloroplasts during the isolation procedure.

Difficulties arising as a result of possible redistribution of radioactivity during organelle isolation might be at least partially overcome by studying

the uptake of ^{14}C-chlortoluron by protoplast preparations obtained from leaves of both resistant and sensitive plants. Procedures for preparing protoplasts from cereal leaves have been reported by Evans, Keates and Cocking (1972) and Miginiac-Maslow, Hoarau and Moyse (1979). The particular advantage that might be gained from the use of this technique arises from the fact that protoplast membranes are readily ruptured by gentle osmotic shock, thereby facilitating the release of structurally intact organelles which may then be separated by density gradient centrifugation employing media which retain structural integrity of the bounding membranes (Honda, Hongladarom and Laties, 1966). Such a study on the absorption of ^{14}C-labelled herbicides by protoplasts and isolated cells has been undertaken by Boulware and Camper (1973). This study indicated that there was no significant absorption of fluometuron and chlorbromuron; however, there was substantial absorption of trifluralin and fluorodifen. As the substituted phenylureas by necessity move in or are transported through the symplast to reach the chloroplast, this type of study could justifiably be extended to examine chlortoluron penetration into, and distribution within the cell.

Complications inherent in the isolation of cell organelles containing radioactivity may also be overcome by means of micro-autoradiography, a technique used to accurately locate radioactivity within cells (Fong, 1972). This latter technique was employed particularly successfully by Strang and Rogers (1971a, b) in a study of the absorption of ^{14}C-diuron and ^{14}C-trifluralin by resistant and sensitive plants. The results of a number of studies (Pickering, 1964; Fong, 1972; Moss, 1977) served to demonstrate that if adequate care is taken in the handling

of samples the relocation of radioactivity observed in some early micro-autoradiographic studies caused during slicing and the application of the film emulsion can be avoided. By use of this technique it may well be possible to qualitatively demonstrate the movement of substituted phenylureas in the symplast of plants.

Initial studies on the relative rates of detoxification of chlortoluron in resistant and sensitive species and cultivars were based on measurements of the recovery of photosynthetic activities following treatment. This type of approach has been employed by a number of other workers, but particularly by Van Oorschot (1969, 1970a,b) providing evidence for herbicide detoxification. In addition to these studies other related investigations on the photosynthesis inhibiting herbicides phenmedipham (Arndt and Kotter, 1968), diuron, fluometuron, norea and prometryne (Eshel, 1969), bentazone (Retzlaff and Hamm, 1976) and atrazine (Jensen, Stephenson and Hunt, 1977) have also suggested that the detoxification of many photosynthesis-inhibiting herbicides can be correlated with the recovery of photosynthetic activity. The results of studies utilising both treated whole plants and leaf segments demonstrated a more marked recovery from inhibition of photosynthesis, measured as a decrease in the rate of oxygen evolution, by chlortoluron-resistant compared to sensitive cereals. Less initial inhibition and greater recovery of oxygen evolution by all cereals was noted after isoproturon treatment. The particularly sensitive species Avena fatua L., however, was apparently unable to render either chlortoluron or isoproturon non-toxic to any significant extent.

The results reported in this thesis are supported by the data of Muller, Frahm and Sanad (1979) in a study of the influence of chlortoluron, isoproturon and metoxuron on photosynthetic oxygen evolution in two winter wheat cultivars. Although oxygen evolution in herbicide-treated susceptible plants remained at some 10% of the untreated control rates throughout the duration of the experiment, photosynthesis in the resistant cultivar recovered to 86% of the control rates six days after cessation of treatment. The studies detailed above, however, do not allow any conclusions concerning the metabolic fate of the herbicides under investigation.

Studies reported in the present thesis on the degradation of chlortoluron by resistant and sensitive plants were made using (carbonyl-^{14}C)-chlortoluron. The results obtained indicated that the selective properties of this herbicide are regulated at least in part not only by the rate and extent of the degradation, but also by the nature of the degradation. The data presented clearly demonstrated that ring-methyl oxidation and conjugation confer resistance to chlortoluron, this pathway being favoured to a much greater extent by the chlortoluron resistant plants. Sensitive plants displayed a greater preference for N-demethylation which gives rise to an initial product retaining a high degree of phytotoxicity as indicated in studies on the effectiveness of chlortoluron metabolites as Hill reaction inhibitors. The results obtained in the studies of chlortoluron degradation correlated well with the observed field behaviour of chlortoluron, where wheat was found to provide a wider spectrum of response than barley. The barley cultivar Maris Otter has been shown to be somewhat intermediate in its response

to chlortoluron, whilst the weed species are regarded as being particularly sensitive (Tottman et al. 1975). In the present study these characteristics could be related to the extent, rate and type of degradation of chlortoluron recorded for a particular plant.

In previous studies differential rates of degradation have been shown to be the mechanism of selectivity; chlortoluron appears to be unique amongst the substituted phenylureas studied to date in that both the rate and type of degradation control selectivity. As referred to previously, a study of the toxicity of characterised chlortoluron metabolites to the Hill activity of isolated chloroplasts demonstrated that all degradation products were essentially non-inhibitory with the exception of the N-monodesmethylated derivative (11). This result is partially supported by the work of Good (1961) who commented that the N-demethylated derivatives of diuron were likely to contribute to the phytotoxic action of this closely related phenylurea. In addition, several other workers (Hansch and Deutsch, 1966; Geissbuhler et al. 1975; Dicks, 1978) have suggested that the effectiveness of the substituted phenylureas as inhibitors of photosynthesis can be related to a particular balance between the lipophilic and hydrophilic properties of the inhibitory molecule. As described by Shipman (1981) the primary function of the lipophilic components is to increase the lipid solubility of the entire herbicide molecule. It would be of particular interest to determine the pI50 values of such closely related compounds as chlortoluron and its degradation products and to examine any correlation with lipid or water solubility.

The study on the degradation of chlortoluron was subsequently extended to include the closely related phenylureas diuron and CGA 43057 in order to investigate the effect of variations in the nature and positioning of ring substituents on degradation. The results obtained clearly indicate that where the ring-methyl group is in the meta rather than the para position it is much less susceptible to oxidative attack. The replacement of the methyl group with chlorine as in diuron totally supresses herbicide degradation via alternation of the ring substituents. Where ring-methyl oxidation is reduced there appears to be increased N-demethylation as in the cases of Atou and Astrix (Tables 6 and 16, and 7 and 16). The data from these serve to emphasise the importance of the ring-methyl group with respect to the selectivity of chlortoluron.

It would be of considerable interest to extend the present study to include the related phenylurea isoproturon which has also been shown to exhibit some selectivity between cereal varieties (Tottman et al. 1975). Studies on the breakdown of isoproturon in soil (Fournier, Soulas, Catroux and Salle, 1975; Fournier, Reudet and Soulas, 1975) have indicated that degradation proceeded largely by N-demethylation. However, the only plant degradation study published to date is of a limited nature involving wheat (resistant) and wild oat and blackgrass (sensitive) (McIntosh et al. 1981) in which no degradation products were identified. It would be of considerable interest to study, in detail, the degradation of isoproturon by wheat and barley varieties, with particular reference to the fate of the isopropyl group. During the present study on the degradation of chlortoluron, diuron and CGA 43057 in resistant and sensitive species, a number of minor metabolites

were detected which remain unidentified. There are a number of reports in the literature on the characterisation of minor metabolites of monuron and diuron. It is possible that the unidentified metabolites in the present study are similar to the hydroxymethyl intermediates noted by Frear and Swanson (1972) or the ring-hydroxylated intermediates noted by Lee et al. (1973). However, as these compounds appear to be of little importance with respect to the selective action of the three herbicides studied their true identity is of minor significance in the present context.

The results of time course studies on the degradation of chlortoluron by nutrient solution grown plants and root and leaf segments revealed that the majority of the degradation occurring took place in the leaves. In addition, this was also the site of the varietal differences noted in the degradation of chlortoluron. The decrease in the amount of extracted parent compound at each harvest time was accompanied by increases in the N-monodesmethylated metabolite (11) and the initial product of ring-methyl oxidation (1V). This was followed and to some extent paralleled by the appearance as conjugates of these compounds, and the production of further degradation products. These studies indicated that conjugates need not be regarded as stable end products of chlortoluron degradation, but rather can be cleaved and the aglycone moiety degraded further and quite possibly reconjugated. This was indicated by the initial increase and subsequent decrease of conjugated metabolite 1V in extracts of the resistant wheat variety Atou (Table 20). It would be of interest to continue this type of study over a considerably longer time scale so as to enable the identification of the end products

of chlortoluron degradation, such as those metabolites which may become incorporated into plant material.

An interesting extension of the degradation studies reported in the present thesis is to the use of cell tissue culture techniques. This approach offers many advantages in the study of the metabolism of, and effects of xenobiotics, namely, sterility, reproducibility and relative homogenity of the plant material, reduced problems of uptake, ease of manipulation of the physiological environment and the large number of totipotent plants in each suspension culture considerably reduce the time required to select herbicide resistant phenotypes. The first study of xenobiotic metabolism by plant cell cultures were concerned with the fate of 2,4-dichlorophenoxyacetic acid (2,4-D) (Ojima and Gamborg, 1968). Since that time several studies have been carried out on the metabolism of pesticides by plant cell cultures, these include cisanilide (cis-2,5-dimethyl-1-pyrrolidinecarboxanilide) in carrot and cotton (Frear and Swanson, 1975), propanil (3,4-dichloropropionanilide) in rice (Ray and Still, 1975) and metribuzin (4-amino-6-t-butyl,3-(methylthio)-as-triazin-5(4H)-one) in soybean (Oswald, Smith and Phillips, 1978). In all cases extensive information on the degradation of the administered pesticide was obtained. It is a relatively simple matter to produce cell suspension cultures of many plant species (Gamborg, Miller and Ojima, 1968) including wheat and barley (Gamborg and Eveleigh, 1967; Schenk and Hildebrandt, 1971; Cheng and Smith, 1975; Jacobsen, 1976; Scheunert, Shamina and Koblitz, 1977; Bayliss and Dunn, 1979). It would thus appear in principle to be quite possible to study the degradation of the substituted phenylureas in these important field

crops, using cell culture techniques, with a view to easier and more rapid isolation of the degradation products and identification of unstable intermediates especially those degradation products formed after long term exposure to the pesticide. Additionally, valuable information could be obtained on the kinetics and enzymology of pesticide degradation.

The series of experiments investigating the intracellular distribution of absorbed chlortoluron demonstrated an association of chlortoluron degradation products with post-mitochondrial fractions. In a final series of experiments on the degradation of chlortoluron an attempt was made to locate the site(s) of chlortoluron degradation within the cell. The existence of an _in vitro_ cotton leaf microsomal mixed function oxidase system which N-demethylated a number of substituted phenylureas has been reported by Frear (1968). The results reported in the present thesis indicate the association of N-demethylase and ring-methyl oxidase activities with post-mitochondrial fractions from both resistant and sensitive plants. The differences noted _in vivo_ of the degradation of chlortoluron by resistant and sensitive plants were not apparent in these _in vitro_ studies, as it was not possible to detect differences between resistant and sensitive species and/or varieties with respect to _in vitro_ N-demethylase and ring-methyl oxidase activities. The manner of degradation noted _in vivo_ by sensitive plants were similar _in vitro_ in as much as a substantial rate of N-demethylase activity was apparent. Resistant plants, however, _in vitro_ did not demonstrate preferential ring-methyl oxidation shown in the _in vivo_ studies. It may well be that the enzyme activities demonstrated during the _in vitro_

studies are the true rates of chlortoluron N-demethylation and ring-methyl oxidation. The differences noted in vivo being due to differences in substrate availability or enzyme activity in whole plants.

It would be particularly interesting to know whether the enzyme system responsible for metabolism of chlortoluron (and other substituted phenylureas) is a novel one or whether the observed activities can be attributed to enzymes of known metabolic pathways in plants. An attempt could also be made to obtain more information on the specific activities of the detoxifying enzyme systems in susceptible and resistant plants, so as to correlate enzyme activity with susceptibility or resistance to phenylureas in the field. Inheritance studies (Tottman et al. 1975) have demonstrated a distinct segregation in the progeny of crosses between resistant and susceptible plants, implying that herbicide tolerance is simply inherited and that it should be possible to include selection for tolerance to chlortoluron in cereal breeding programmes. A rapid test for tolerance based on enzyme levels in particular cereal cultivars would be of considerable assistance in chlortoluron screening programmes.

The final series of experiments on the degradation of chlortoluron were concerned with the identification of the glycone moiety associated with the polar conjugated metabolites of chlortoluron. The results of this study indicated that β-D-glucose occurred in a 1:1 molar ratio with the aglycone chlortoluron metabolites. It would be of interest to extend this study over a considerably longer time scale to determine if glucose is indeed the only compound with which chlortoluron metabolites may be

conjugated. This may be of particular importance as regards the occurrence of chlortoluron residues in plant material at the time of harvest and consumption.

In conclusion the experiments embodied in the present thesis have indicated that the varietal differences in response to chlortoluron observed amongst cereals in field trials can be attributed to differences in the rate and type of degradation of the herbicide occurring in resistant and sensitive plants. The enhanced sensitivity of the monocotyledonous cereal weeds apparently results from a greater rate of uptake of the herbicide in addition to a lower rate of detoxification and a preference for the N-demethylation pathway.

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