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The Mechanism of Selectivity and Phytotoxic Action for Picloram and Clopyralid

by

© John Christopher Hall

A THESIS

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Abstract

Sunflower (*Helianthus annuus* L.) and Canada thistle (*Cirsium arvense* L.) plants are susceptible to picloram (4-amino-3,5,6-trichloropicolinic acid) and clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) whereas rapeseed (*Brassica napus* L.) plants are susceptible to picloram only. This study was undertaken to elucidate whether intra- and/or inter-species differences in sensitivity of the three species to the two herbicides can be attributed to differences in absorption, translocation, and/or metabolism of the herbicides and/or the extent of ethylene biosynthesis after application of the herbicides.

Differences in sensitivity to picloram and clopyralid within and between a species were not attributable to differences in absorption, translocation, and metabolism.

Thin layer chromatography revealed that one water-soluble metabolite was formed from each of the two herbicides. In each instance, the metabolite was the same in all three plant species. The metabolite may be a disaccharide ester of picloram and clopyralid.

This is the first experimental work in which herbicide-induced ethylene production has been shown to occur in intact plants using a continuous flow system. Picloram caused an increase in ethylene production in rapeseed plants whereas clopyralid did not. Both herbicides caused an increase in ethylene production in sunflower plants. Treatment of susceptible species with

aminoethoxyvinylglycine (AVG) before picloram or clopyralid was applied prevented an increase in ethylene production. Pretreatment with AVG also delayed the development of morphological changes induced by picloram or clopyralid in susceptible species.

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1. Introduction

Plants in the Cruciferae family tend to be at least moderately resistant to picloram and clopyralid (6, 44). In particular, clopyralid, unlike picloram, can be used to selectively control many broadleaf weeds in rapeseed without causing damage to the crop. This makes clopyralid of particular interest in western Canada and Europe, because of its phytotoxic effect on weedy members of the Compositae, Leguminoseae, and Polygonaceae families which are a problem in rapeseed crops (17, 35, 79). Clopyralid has little herbicidal activity against many weed species in the Cruciferae, Amaranthaceae, and Chenopodiaceae families.

With this in mind, the primary objective of this study was to elucidate the difference in selectivity between the two herbicide in susceptible and resistant species. This objective was achieved by examining three major areas. First, it was determined whether differences in sensitivity between picloram and clopyralid in rapeseed, sunflower, and Canada thistle plants could be attributed to differences in absorption, translocation, and the extent of metabolism of the two herbicides in these plant species. The second objective was to determine whether ethylene is involved in the herbicidal activity of the two herbicides in susceptible and resistant species. The third objective was to determine whether the two herbicides interact differently with some target site in the cells of resistant and susceptible species. Differences in the extent of ethylene biosynthesis

were used as a tool to determine whether there was difference in the way the herbicides interacted with a target site in resistant and susceptible species. Once these objectives had been achieved it was determined whether the metabolites produced in all three plant species were the same for each herbicide. Furthermore, the metabolite(s) were isolated and purified to determine their structure and physical properties.

All experiments were conducted under controlled environment conditions. Sunflower and rapeseed plants were chosen because both are annual species; sunflower plants are susceptible to both herbicides whereas rapeseed plants are moderately susceptible to picloram only. Therefore, intra- and/or inter-species comparisons of the absorption and translocation patterns and of the extent of metabolism were made to determine if these factors were important in contributing to sensitivity differences within a species and between plant species.

Because Canada thistle is a severe weed problem in western Canada in many crops including rapeseed, it was included in some experiments. Information on the absorption, translocation and extent of metabolism of the two herbicides may explain some of the differences in response to clopyralid and picloram by field-grown Canada thitle plants. Furthermore, this perennial species was included with a view to determining possible differences in absorption, translocation, and metabolism of the herbicides in annual

and perennial species.

2. LITERATURE REVIEW

2.1 Introduction

In order to review all the pertinent literature as efficiently and thoroughly as possible, the topics of interest are emphasized according to their importance to the thesis research. The topics deemed to be of greatest importance are reviewed in a general manner, followed by specific discussion of the synthetic pyridines. The topics of less importance are addressed with regard to the synthetic pyridines only.

2.2 Pyridines

2.2.1 General

It has been known for many years that naturally occurring pyridine compounds such as fusaric acid (5-butyl-2-pyridinecarboxylic acid), nicotinic acid (3-pyridinecarboxylic acid), nicotinamide, nicotine (3-(1-methyl-2-pyrrolidinyl)pyridine), NAD (nicotinamide adenine dinucleotide), trigonelline (N-methyl nicotinic acid), uracil, thymine, and cytosine exert biological activity in plant and/or animal systems (39, 44). Schreiner and Shorey (111, cited in 44) were the first to report on the plant growth-inhibitory action of a pyridine derivative after isolating α-picoline-γ-carboxylic acid from the soil of barren land. Yabuta et al. (126, cited in 44) found that

fusaric acid, isolated from *Gibberella fujikuroi* Wr., a pathogenic mold of rice plants, exerted growth-inhibitory action on rice. Gauman (48) also showed that when fusaric acid, isolated from *Fusarium lycopersici* Sacc., was applied to tomato plants they wilted.

The synthetic pyridines of interest to this discussion are picloram (4-amino-3,5,6-trichloropicolinic acid) and clopyralid (3,6-dichloro-2-pyridinecarboxylic acid). Their physicochemical properties are listed in Table 1 (125). Triclopyr ([(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid) will also be discussed, but in less detail. These three compounds, like the benzoic acid and phenoxyacetic acid derivatives that have herbicidal activity, are classified as auxin or auxinic-type herbicides (6, 44, 125). They will control many broadleaf herbaceous and woody plants, and may be significantly more toxic than 2,4-D or 2,4,5-T to some plant species. Other pyridine compounds shown to have plant growth-inhibitory action are 2,3,5-trichloro-4-pyridinol (DAXTRON), 6-chloropicolinic acid, and isonicotinic acid (44, 49).

2.2.2 Physiological Effects

2.2.2.1 Effects on Plant Growth and Anatomy

The effects of picloram and clopyralid on plant growth and anatomy of susceptible species are similar to those caused by IAA and auxinic-type herbicides such as 2,4-D or dicamba (24, 45, 52). Extension of the mid-rib and

Table 1

Nomenclature and Physicochemical Properties of Clopyralid

Common name:	3,6-dichloro-2-pyridinecarboxylic acid	
Product name:	LONTREL	formulated as monoethanolamine salt
Molecular formula:	$C_6H_3Cl_2NO_2$	
Molecular weight:	192	
Physical description:	White crystalline solid, odourless.	
Melting point:	151 - 152°C	
Vapour pressure:	1.3×10^{-8} mm Hg at 25°C.	
pKa	2.3	
Solubility:	water	0.1 g/100 ml at 25°C
	acetone	>25
	methanol	>15
	xylene	02.3

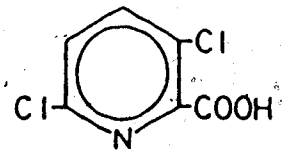
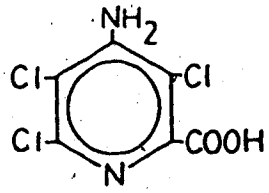


Table 1 (continued)

Nomenclature and Physicochemical Properties of Picloram

Common name:	4-amino-3,5,6-trichloropicolinic acid	
Product name:	TORDON	formulated as potassium salt
Molecular formula:	$C_6H_3Cl_2N_2O_2$	
Molecular weight:	241.5	
Physical description:	White powder. Chlorine like odour.	
Melting point:	Decomposes before melting.	
Vapour pressure:	6.16×10^{-7} mm Hg at 35°C.	
	1.07×10^{-6} mm Hg at 45°C.	
pKa:	3.6	
Solubility:	water	0.043 g/100 ml at 25°C
	acetone	1.98
	ethanol	1.05
	dichloromethane	0.06



thickening of the mesophyll may occur in leaf tips of susceptible plant species treated with picloram. With increased dosage, cupping and stunting of the leaves are observed (6). Terminal leaf growth ceases. Tissue proliferation along the stem may take place first at the stem tip, then at the nodes, and finally along the length of the entire stem. Meanwhile, epinasty, bending, and splitting of the stem occur. The roots become thickened and stunted. Adventitious root initials develop which crush the phloem and cortex, eventually resulting in rupturing of the epidermis of stem tissues (44). The plant soon dies.

Anatomical modifications in susceptible species following treatment with herbicidal concentrations of picloram or clopyralid have been described (6, 24, 44, 51, 85, 86, 101) and are essentially the same as the modifications caused by other auxinic-type herbicides (6). They include the disintegration of phloem and cortical cells (69), and reduction in periclinal cell division in the cambial region (6). Peterson et al. (101) noted xylem blockage, enlargement of cortical and pith cells, and necrosis of the apex, parenchymatous, and phloem tissues in shoots of red maple treated with picloram. Both picloram and clopyralid had similar effects on cell division and elongation in soybean and pea hypocotyls, respectively (24, 85, 86). After herbicide treatment, cell division decreased in the meristem but not in the elongation zone of the hypocotyls; cell elongation ceased in the meristem and

elongation zones, and radial enlargement of cells occurred in the elongation and mature zones of the hypocotyls of both species.

2.2.2.2 Effects on Oxidative Phosphorylation

Picloram has been shown to inhibit respiration in isolated cucumber mitochondria (46). Using succinate as a substrate, inhibition of the TCA cycle occurred with picloram concentrations in the range of 10^{-6} to 10^{-3} M. An α-ketoglutarate substrate system, however, was much less sensitive to similar picloram concentrations. Fusaric acid has been shown to depress respiration in isolated mitochondria of tomato and cauliflower (46).

Picloram at 10^{-3} M caused swelling of barley and safflower mitochondria that had previously contracted when ATP was added prior to the picloram treatment (22). Picloram at 10^{-3} M increased ATPase activity in safflower. This was consistent with results that showed picloram-induced swelling in mitochondria previously contracted by ATP treatment. The authors (22) suggest that the changes manifested after picloram treatment may be due primarily to conformational changes in the state of the mitochondrial inner membrane.

2.2.2.3 Metal Binding Activity

Several researchers have shown that auxins, phenoxyacetic acid herbicides, and some pyridine compounds form complexes with metal ions (22, 45, 107). For example,

fusaric acid chelated heavy metal ions involved in the Fe-porphyrin oxidase complex (44). This inhibitory effect was reversed by the addition of suitable metal ions. Enzymes that were not associated with heavy metals were not inhibited by fusaric acid. Therefore, it was postulated that picloram may form complexes with metal ions associated with the respiratory enzyme systems in mitochondria (22). This chelation of metal ions may lead to changes in membrane structures and/or protonic equilibrium of the mitochondria, thereby explaining the action of picloram on respiration in mitochondria.

Peroxidase, which is associated with the auxin oxidase system, requires Mn^{+2} as a cofactor. Foy (44) hypothesized that inhibition of the oxidase system through chelation of Mn^{+2} may result in excessive endogenous levels of auxin which result in many of the physiological abnormalities seen in susceptible species after picloram treatment. However, Foy and Chang (45) found no correlation between the metal complexing properties of the pyridine compounds and growth regulatory activity. Furthermore, recent studies involving *in vitro* oxidation of IAA by picloram and the comparison of the growth kinetics of IAA- and picloram-treated coleoptiles suggest that the growth-promoting action of the pyridine herbicides does not involve inhibition of auxin oxidase by metal chelation (20).

2.2.2.4 Nucleic Acid Metabolism and Protein Synthesis

Picloram has been reported to have a stimulative effect on mRNA and protein synthesis (23, 76). Chen et al. (23) reported increased levels of RNA, DNA, and protein in root tissues of cucumber seedlings treated with picloram. The greatest decrease in the protein/RNA ratio occurred when picloram concentrations were most stimulatory for RNA, DNA, and protein synthesis. Consequently, they concluded that picloram was making more DNA template available for transcription, thus increasing RNA levels. Chang and Foy (21) found that growth-promoting levels of picloram enhanced synthesis of RNAase, while the converse was shown when growth-inhibitory concentrations of the herbicide were applied. Picloram caused both qualitative and quantitative alterations in the band patterns of soluble proteins as determined by gel electrophoresis and isoelectric focusing techniques.

Malhotra and Hanson (76) indicated that total RNA and DNA content of tissues was correlated inversely with plant resistance to picloram; resistant species were low in nucleic acids, whereas sensitive species were high in nucleic acids. The presence of higher levels of native bound nucleases in the resistant species may prevent the accumulation of nucleic acids; these nucleic acids being degraded as soon as they are synthesized in resistant species.

Baur and Bowman (12) found that picloram had a stimulatory effect on ^{14}C-leucine uptake in the hook but not the hypocotyl tissues of bean. This would indicate that increased protein synthesis was related to the triggering of cell elongation. Inhibition of protein synthesis with cycloheximide and erythromycin indicated that picloram-stimulated protein synthesis was a function of 80S cytoplasmic ribosomes rather than 70S chloroplast or mitochondrial ribosomes.

Application of clopyralid to intact soybean hypocotyls resulted in dramatic nucleolar swelling (24). However, there was no significant alteration in the size of the nuclei. Relative DNA, RNA, and protein values were about 1:3:11 for untreated nuclei, whereas the nuclei isolated from clopyralid-treated tissues contained almost twice as much RNA and protein as untreated nuclei. DNA per nucleus was the same for treated and untreated tissues. Isolated nuclei from treated and untreated hypocotyl tissues contained nearly equivalent levels of RNA polymerase II, but the herbicide-treated nuclei had three to four fold higher levels of RNA polymerase I. The above results were consistent with those obtained from nuclei isolated from soybean seedlings treated with 2,4-D (25).

2.2.3 Edaphic Behaviour

Minimal soil adsorption of picloram and clopyralid occurs in neutral or alkaline sandy loam that is low in

organic matter content; it increases with decreasing pH, increasing organic matter, and increasing concentrations of hydrated iron and aluminium oxides (35, 44, 102). Leaching of picloram and clopyralid is greatest in light-textured low organic matter soils (60, 61, 62, 102). Generally, there is an increase in adsorption and a decrease in leaching of acid herbicides as soil organic matter increases or pH decreases (44). Furthermore, both picloram and clopyralid have a pKa of less than 4 (125). Since most agricultural soils have a pH that is greater than 4, these herbicides probably exist primarily in the anionic form (35, 102). Based on the above information, it has been suggested that adsorption of the non-ionic form of picloram and clopyralid by physical forces on hydrophobic adsorbents probably represents the major mechanism for sorption of these herbicides (44).

Microbial degradation is the principal mechanism for picloram and clopyralid reduction in the soil (102, 127). Meikle et al. (82) proposed a reaction sequence for the degradation of picloram by soil microbes. These authors determined that the rate-limiting step in picloram disappearance was the first step in microbial degradation, since the products of the reactions were more easily degraded than picloram itself. Although a degradation scheme has not yet been proposed for clopyralid, it does have a shorter half-life than picloram, and may be regarded as being considerably less recalcitrant than the latter herbicide under most soil conditions.

In general, the persistence of picloram and clopyralid is directly related to the depth of the herbicides in the soil profile, inversely related to organic matter content and temperature of the soil, and decreases with the presence of plant roots (44, 52, 102). Furthermore, Chen and Farrow (26) showed that degradation processes were affected adversely by drying and water-logging of the soil.

2.3 Absorption and Translocation of Pesticides

2.3.1 General

The cuticle is the initial barrier to penetration of pesticides into the leaves and aerial parts of the plant and must be breached by the compound before a biological response can be induced (6, 18, 32, 37). The entire structure of the cuticle is non-living and exists on the outer wall of the epidermal cells which are in contact with the protoplasm (32). Cuticles are heterogeneous membranes, their main components being lipids. Based on chloroform solubility, two types of cuticular lipids exist; the insoluble polymer matrix and the soluble cuticular lipids known as the waxes (108). The former behaves as a porous membrane while the latter represents a solubility membrane. The soluble cuticular lipid layer has the greater effect of the two lipid components on the overall permeability of cuticular membranes to polar and non-polar solutes. A large number of pores in the polymer matrix are blocked or covered

by soluble cuticular lipids, thereby reducing permeability coefficients of polar solutes by two or three orders of magnitude. Schonner (108), therefore, partitioned the permeability coefficients for the transfer of solutes across cuticles into contributions from the polymer matrix and from the soluble cuticular lipids. Depending on the properties of the solute molecule, a cuticular membrane behaves as a porous membrane, a solubility membrane, or as a mixture of both. Penetration of water and solutes may also take place through ectodesmata and stomata. Indications are that stomatal entry is possible only for liquids with a contact angle of zero, and as long as the surface area is wet (109, 110).

Systemic pesticide transport is described as being apoplastic, symplastic or ambimobile (29, 37, 59, 106). Long distance transport of symplastic chemicals occurs in the phloem while for apoplastic chemicals it occurs in the xylem. The term ambimobile describes transport in both systems. Two subdivisions exist with the apoplastic group of pesticides; euapoplastic, and pseudoapoplastic (99). Those xenobiotics incapable of penetrating the cell plasmalemma and, therefore, moving only in the apoplast, are termed euapoplastic. Herbicides from the urea and triazine groups, largely inhibitors of photosynthesis, must penetrate the membranes of the cytoplasm and chloroplast. The term pseudoapoplastic is used to describe these chemicals because of their movement in both the apoplast and symplast (37). In

general, apoplastic chemicals are swept acropetally in the transpiration stream, accumulating in the tips and margins of leaves and other organs that possess physiologically active stomata (100).

As a young leaf matures, it changes from an importer to an exporter of photosynthetic assimilates (50, 124). Movement of assimilates within the plant symplast is from source to sink (6). The mass flow theory, first proposed by Münch, states that higher sugar concentrations in the phloem cause water to move from surrounding tissue by osmosis; the high turgor pressure then forces the contents of the sieve tubes to flow "en masse" (59). It has been assumed that herbicides and other exogenous compounds would be carried along this stream. Work done by Giaquinta (50) supports this theory. Theoretical mechanisms of phloem transport and loading are discussed by Baker (7) and Lüttge and Higgenbotham (75).

2.3.2 Pyridines

Picloram is readily taken up by leaves after foliage treatment (6, 15, 16, 44, 56, 57, 113, 115), by the roots from soil or nutrient solution (6, 15, 16, 44, 55, 66, 113), or by such tissue as potato tuber slices (11, 118).

On the basis of temperature coefficients and metabolism studies, picloram uptake by roots appears to be governed by both passive and active mechanisms (66, 92). The initial phase of entry is governed mainly by passive processes,

whereas the continuing uptake is controlled, at least in part, by active mechanisms (92). Evidence also exists that foliar uptake of picloram by leaf tissue involves an active component (115). The rate and extent of foliar uptake of picloram increase as relative humidity, temperature, and light intensity increase (93). Foliar absorption of picloram and triclopyr is greatest when leaf surfaces have small amounts of epicuticular wax, high stomatal density, a thin cuticular membrane, and high stellate trichome density (67, 115). These properties are generally associated with immature leaves and/or the abaxial surface of many plant species.

Picloram uptake by the roots of oat and soybean plants and potato tuber slices was markedly reduced as the pH of the nutrient solution was increased from 4 to 6 (11, 66, 118). Keeping in mind that the the pKa of picloram is approximately 4, the above observations are consistent with Crisp's weak acid hypothesis (31) in which he proposes that herbicides such as picloram may be taken up more readily in the undissociated acid form than in the ionic form. However, with foliarly applied picloram an increase in pH above that of the value of the pKa did not reduce herbicide uptake until pH levels exceeded 7. The reason for the discrepancy between foliar and root absorption at various pH levels is not understood.

Picloram is readily exported acropetally to the shoots after absorption by the roots from nutrient solution (55,

95, 112). For example, Hallmen (55) found that more than 50% of the radioactivity from root-applied ^{14}C-picloram was found in the shoots of resistant and susceptible species, 3 days after treatment. However, a difference between the distribution of radioactivity in the shoots of susceptible versus resistant species has been found following root uptake of picloram. In a resistant species, barley, radioactivity was distributed throughout the shoot, whereas in susceptible species radioactivity accumulated mainly in the shoot meristems (113). Similar distribution patterns of picloram, clopyralid, and triclopyr have been observed in susceptible species after these herbicides were absorbed from the soil by roots (15, 16, 35).

Following foliar absorption, picloram and clopyralid were readily transported out of treated leaves (96, 112, 119). Approximately 50 and 36% of the recovered radioactivity was exported from the treated leaf in rapeseed (119) and Canada thistle (96) plants, respectively, 24 hours after foliage was treated with ^{14}C-clopyralid. Over 70% of the exported picloram and clopyralid accumulated mainly in the shoot meristem in annual plants such as sunflower (56) and rapeseed (56, 119). In a perennial species, Canada thistle, the roots often act as a strong sink, depending on the plant's stage of development. For example, Turnbull (119) found that 65% of the radioactivity exported from foliage treated with ^{14}C-clopyralid was found in the roots 24 hours after treatment. Radosevich and Bayer (103)

examined the effect of temperature and photoperiod on picloram and triclopyr translocation in five plant species and found that, regardless of species or treatment, ^{14}C-movement from the treated foliage was greatest under conditions of long days and high temperature.

In general, the pyridines appear to be translocated in both the xylem and phloem following root or foliar absorption by resistant and susceptible species (6, 44, 66, 103, 112). O'Donovan (95) showed that radioactivity was associated mainly with the protoplasm of root cortical cells, the xylem and phloem of the stem, the xylem of older primary leaves, and the xylem and phloem of younger apical leaves of soybean plants following root-absorption of ^{14}C-picloram from nutrient solution.

Egress of picloram and clopyralid from plant roots following foliar or root absorption has been detected (56, 57, 66, 105, 112, 113, 119). In foliage-applied treatments the extent of root exudation depends on the portion of the total herbicide dose translocated to the roots. Hallmen (56) found 10% and less than 1% of the recovered radioactivity in the roots and nutrient solution, respectively, 9 days after a foliar application of ^{14}C-picloram to sunflower plants. In similar experiments, several authors showed that over a 9-day period no picloram or clopyralid was translocated to the roots of rapeseed plants (56, 119). Consequently, there was no herbicide available in the roots to exude into the nutrient solution. Conversely, 24 and 20% of the total

recovered radioactivity was found in the roots and nutrient solution, respectively, 9 days after foliar application of ^{14}C-clopyralid to Canada thistle plants (119). Considerably less clopyralid than 2,4-D was exuded from the roots following herbicide application to the foliage of Canada thistle plants.

2.4 Herbicide and Auxin Metabolism in Plants

2.4.1 General

The metabolism of herbicides has been thoroughly reviewed by Hatzios and Penner (58). These authors classify herbicides according to their type of metabolism, that is: stable herbicides, those which are not metabolized; herbicides deactivated by metabolism; herbicides deactivated by metabolism at rates dependent upon the plant species; and finally, herbicides activated by the process of metabolism. The major mechanisms active in the plant to transform herbicides are oxidation, reduction, hydrolysis, dehydration, dehalogenation, exchange reactions, isomerization, and conjugation, of which the first three and the last one are the most common and important reaction mechanisms.

Hatzios and Penner (58) suggest that xenobiotics undergo a two-phase process in metabolism. The primary phase includes reactions such as oxidations, reductions, and hydrolysis, which may alter herbicide selectivity and

phytotoxicity. The secondary phase generally involves conjugation which results in metabolites with little or no phytotoxicity, higher water solubility, and limited movement in the plant because of compartmentalization. Herbicides have been shown to conjugate with glutathione, cysteine, coenzyme A, amino acids, glucose, or other sugars (6, 58).

One of the most common detoxification processes involving the auxinic-type herbicides such as 2,4-D, 2,4,5-T, MCPA, and picloram is conjugation of these compounds with amino acids and sugars. Several plant species are known to form amino acid conjugates with 2,4-D, MCPA, and 2,4,5-T (6, 40, 41, 42, 58, 129). Seven ether-soluble amino acid conjugates of 2,4-D have been isolated from soybean cotyledon callus cultures (42). These conjugates are linked through the alpha amide bond of the amino acid. Amino acids that have been shown to be conjugated to 2,4-D, in soybean callus tissue, include aspartate, glutamate, alanine, valine, leucine, phenylalanine, and tryptophan (41). The amino acid conjugates of 2,4-D appear to have biological activity but limited mobility within the plant (58).

The conjugation of IAA with sugars, such as glucose and myo-inositol, by means of an ester linkage, has been reported (8, 38, 68, 73, 83, 94, 98, 121, 122, 123). Furthermore, the organic chemical synthesis of ^{14}C-indole-3-acetyl-myo-inositol has been successful (94). It has also been demonstrated that the crude enzyme systems

responsible for the biosynthetic conjugation of myo-inositol and glucose with IAA can be isolated (68, 83). These glucose and myo-inositol esters of IAA may act to homeostatically control hormone concentrations, to facilitate the transport of IAA from seed to shoot, and to protect IAA against oxidation by peroxidases (58).

The type of glycoside conjugates formed when various herbicides are metabolized in plants includes: O-glucosides, N-glucosides, glucose esters, disaccharides, gentiobiosides, and glucosylarabinoses, of which the O-glucosides appear to be most common type of conjugates of herbicides. The structures of these glycoside conjugates of herbicides have been described (6, 27, 47, 58, 72).

2.4.2 Pyridines

Early workers reported that picloram was extremely stable in treated plants, with few metabolites recovered (6, 44, 78). Concentrations of these metabolites were very low. Meikle et al. (81) reported that picloram was decarboxylated very slowly in cotton, yielding $^{14}CO_2$ from ^{14}C-carboxyl picloram in cotton. The herbicide was associated with insoluble protein at a level representing only 3% of total radioactivity. After acid hydrolysis, the herbicide was liberated from the protein. Sharma and Vanden Born (113) reported slow decarboxylation of picloram in Canada thistle, soybean, and barley. In spring wheat, picloram was degraded to a small extent (104). Metabolites included

4-amino-3,5,6-trichloro-6-hydroxypicolinic acid, oxalic acid, and 4-amino-2,3,5-trichloropyridine, but 83% of the activity was still present as picloram.

In recent years, researchers have found that picloram is metabolized to a greater extent in many plant species than was reported previously (55, 56, 57, 70, 71, 77, 78, 114). Maroder and Prego (78) found that *Prosopis ruscifolia* actively transformed picloram to a conjugated derivative of the herbicide. The quantity of the water-soluble conjugate ranged from 1.3 to 3.2 times that of picloram. Similarly, Sharma and Vanden Born (114) showed that conjugated picloram accounted for 40% and 25% of the total radioactivity in excised barley and Canada thistle leaf tissue, respectively, 3 days after treatment. Using root applications of ^{14}C-picloram, Hallmen (55) showed that little of the herbicide was metabolized in susceptible sunflower and spruce plants, while in picloram-tolerant wheat and rapeseed plants most of the herbicide was changed to water-soluble conjugates that increased in concentration with time. Similarly, after leaf applications of ^{14}C-picloram, approximately 20% and 70% of the recovered radioactivity was found as water-soluble conjugates in susceptible sunflower and resistant rapeseed plants, respectively (56). Hallmen (56) hypothesized that as the extent of picloram metabolism increased the amount of herbicide translocated out of the treated leaf would decrease. He concluded that this hypothesis may explain the susceptibility differences

between rapeseed and sunflower plants to picloram. In contrast, Turnbull (119) found no clopyralid metabolites in rapeseed plants.

After isolation and acid hydrolysis of picloram conjugates, several authors found derivatives that had the same Rf value as picloram, as determined by TLC (55, 56, 57, 77, 78, 114). Sharma and Vanden Born (114) found that conjugates gave a positive reaction for sugars, a negative reaction for amino acids, and that less than 1% of radioactivity was associated with protein and nucleic acid fractions. Kudaikina et al. (70, 71) have since isolated and identified two water-soluble sugar conjugates of picloram, 1-O-(4-amino-3,5,6-trichloropicolyl)-D-glucopyranoside and N-(2-carboxy-3,5,6-trichloropyridine-4-yl)-glucosamine, from corn and sunflower plants, respectively.

2.5 Ethylene and Herbicide Action

2.5.1 General

Herbicides have been reported to stimulate ethylene production in a number of plant species (2, 3, 53, 64, 74, 89, 90, 97, 117). In particular, the auxinic-type herbicides such as 2,4-D, 2,4,5-T, picloram, 2,5-dichlorophenoxyacetic acid, and dicamba promote ethylene biosynthesis (13, 80, 89, 90, 91, 117). A controversy exists among researchers as to whether there is an association between plant sensitivity to auxinic-type herbicides and the increase in ethylene

production brought about by this herbicide treatment (88). Ethylene has been shown to induce epinasty, enhance senescence, reduce the amount of chlorophyll, and cause abscission of leaves and other organs (53). Many of these responses are similar to those induced by the auxinic herbicides. However, the separation of some of the effects of auxinic herbicides from those of ethylene has been shown (2, 5, 6, 88). There is some evidence, nonetheless, to indicate that the auxinic herbicide-induced responses that precede death, such as leaf and stem epinasty and leaf abscission, may be accounted for by ethylene (6, 88). Auxinic herbicides have been shown to promote ethylene biosynthesis more in susceptible than in resistant species (88, 90). Furthermore, analogs of auxinic herbicides that are inactive as synthetic auxins are also inactive as ethylene biosynthesis promoters. For example, phenoxyacetic acid, the chloro-substituted phenoxyacetic acids, and 3,4,5-trichlorophenoxyisobutyric acid, are all known analogs of auxinic herbicides but have little auxin activity and do not markedly increase ethylene biosynthesis.

There are five criteria for investigating whether the action of an auxin, which is mimicked by ethylene, is due to auxin-induced ethylene synthesis (88). These criteria, which also may be applied to the auxinic-type herbicides, are: the kinetics of auxin-induced ethylene synthesis should show that timing and amount of ethylene synthesized allow a cause and effect relationship to occur; ethylene should produce

the same effect more rapidly than auxin; saturating levels of ethylene should mask the response to auxin and the additional effects of auxin must be due to it rather than to ethylene; reduction of internal concentrations of ethylene in auxin-treated tissue by vacuum or by flushing with air should reduce or delay the response; CO_2 should reduce or delay the effects of auxin since CO_2 is usually a competitive inhibitor of ethylene action.

Yu and Yang (128) have determined that aminoethoxyvinylglycine (AVG) is a potent inhibitor of ethylene biosynthesis. Their results suggest that in the ethylene biosynthetic pathway (methionine → s-adenosyl-methionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene), IAA stimulates ethylene production by inducing the activation of ACC synthetase, which catalyzes the conversion of SAM to ACC. Aminoethoxyvinylglycine (AVG) acts to inhibit the conversion of methionine to ACC, thus preventing ethylene biosynthesis. Paradies et al. (97) have shown that AVG can be used with the non-auxinic herbicide metolachlor to determine whether symptoms expressed by treated sorghum seedlings can be attributed to increased ethylene production brought about by the herbicide treatment. Aminoethoxyvinylglycine decreased ethylene formation by metolachlor-treated sorghum seedlings, but the authors observed that deformation in the seedlings was the same as in metolachlor-treated controls. They concluded that the herbicide-induced ethylene production is a symptom and

not the inducer of the morphological effects visible after metolachlor treatment of sorghum seedlings.

3. MATERIALS AND METHODS

3.1 Herbicides

The radioactive herbicides used were: clopyralid (2,6-^{14}C; sp. act. 429 MBq·mmole^{-1}, 11.6 mCi·mmole^{-1}; radiochemical purity >99%) and picloram (2,6-^{14}C; sp. act. 370 MBq·mmole^{-1}; 10.0 mCi·mmole^{-1}; radiochemical purity >99%). Analytical grade standards (5 g) of clopyralid and picloram were >99% pure. Commercial herbicide formulations used were clopyralid (360 g acid equivalent (ae)/L) and picloram (240 g ae/L as K$^+$ salt). Radioactive, commercial, and analytical grade herbicides were obtained from Dow[1].

3.2 Plant Material

The plants used were rapeseed (*Brassica napus* L. cv. Altex), sunflower (*Helianthus annuus* L. cv. Mammoth Grey Stripe), and Canada thistle (*Cirsium arvense* L.). Regardless of the media or the environment in which the plants were grown, six rapeseed or sunflower seeds were planted per pot. After 10 days, plants were thinned to three plants per pot. At the required stage of growth the plant number per pot was reduced to one. Canada thistle plants were started from 4- to 6-cm root cuttings. These cuttings were planted in 54 x 28 x 6 cm flats containing perlite and, 14 to 21 days later, after sprouting, were transplanted to pots (one plant/pot) containing the appropriate growth medium. Until the time of

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[1]Dow Chemical Co., Midland, MI.

transplanting, the cuttings were grown in a greenhouse maintained at a temperature of 20/16°C day/night. Sunlight was supplemented with light from high pressure sodium lamps to provide a 16-hour photoperiod.

3.3 Absorption and Translocation

3.3.1 Absorption and Translocation of Picloram and Clopyralid

The absorption and translocation patterns of ^{14}C-picloram and ^{14}C-clopyralid were investigated in all three plant species. Canada thistle plants were transplanted into 9-cm diam plastic cups containing 350 ml of silica sand. Rapeseed or sunflower seeds were sown into plastic cups containing the same medium. Each cup had three holes in the bottom and was set inside a second cup that acted as a reservoir for the nutrient solution. Plants were watered with half-strength Hoagland's solution as required.

Plants were grown in a controlled environment growth cabinet maintained at 20/16±1°C day/night with a 16-hour photoperiod and relative humidity of 65%. Light intensity[2] (400 to 700 nm) was constant throughout at 250 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Herbicides were dissolved in ethanol:water (1:9 v/v) containing 0.5% (v/v) oxysorbic (20 POE)[3] (polyoxyethylene sorbitan monolaurate) to a concentration with approximate

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[2]Measured with a Li-cor Quantum Meter, model LI-185; Lambda Instr. Corp., Lincoln, NE.
[3]Tween 20; Matheson, Coleman and Bell, Manuf. Chemists, Norwood, OH.

radioactivity of 3300 Bq (2.0 X 10^5 dpm) per 10 µl, which is equivalent to 780 and 900 µM of ^{14}C-picloram and ^{14}C-clopyralid, respectively. The quantity of radiolabeled herbicide in the 10 µl of solution applied to the plants was 7.8 X 10^{-3} or 9.0 X 10^{-3} µmol of ^{14}C-picloram and ^{14}C-clopyralid, respectively. Cotyledons were removed from sunflower and rapeseed plants prior to treatment. A micropipette[4] was used to apply a total of 10 µl of a herbicide solution as eight to ten drops across the midsection (perpendicular to the mid-vein) of a leaf. Herbicide treatments were applied to the third leaf of rapeseed plants at the five-leaf stage, one leaf of the second leaf-pair of sunflower plants at the three-leaf stage, and the fourth leaf from the apex of Canada thistle plants at the pre-bud stage (8- to 10-leaf stage) of development. The leaf that received a treatment was fully expanded in all experiments except one.

Plants were dissected 24, 72, or 144 hours after treatment. Rapeseed plants were dissected into the treated leaf, first leaf, second leaf, fourth leaf, fifth leaf, growing point (all tissue above the fifth leaf), stem, and roots. Sunflower plants were dissected into the treated leaf, the leaf opposite the treated leaf, the leaf directly below the treated leaf, the leaf below and opposite the treated leaf, the growing point (all tissue above the treated leaf), the stem between the treated leaf and the

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[4]Wiretrol, Drummond Scientific Co., Broomall, PA.

cotyledons, the stem below the cotyledons, and the roots. Canada thistle plants were dissected into the treated leaf, the apex (including the growing point and the newest expanding leaves), the leaves and stem in the region between the apex and treated leaf, all leaves below the treated leaf, the stem tissue below the treated leaf, and the roots, which were sub-divided into the original root cutting, the newly developed tap root, and the fibrous roots.

The amount of herbicide present on the treated leaf surface 24, 72, or 144 hours after application was determined by means of a leaf rinse technique (33). The technique consisted of holding the treated leaf in a plastic funnel, and directing 10 ml ethanol:water (1:9 v/v) over the treated area of the leaf. The rinse solution was collected in a 22-ml scintillation vial, from which a 5-ml aliquot was taken and added to a similar vial containing 10 ml of scintillation liquid[5]. Radioactivity was quantified by standard liquid scintillation spectrometry (LSS)[6].

In order to estimate the extent of the radiolabelled herbicide exuding from the roots of treated plants, the nutrient solution was assayed for radioactivity. At the time of harvest the nutrient solution was drained from the plastic cup that was used as a reservoir. The solution was poured over the silica sand in the plastic cup in which the plant was growing, and allowed to drain into a beaker. This

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[5]Aquasol-2; New England Nuclear, Boston, MA.
[6]Model-Tri-Carb 460 CD; Packard Instr. Co. Inc., Downers Grove, IL.

process was repeated twice. After the final rinsing of the silica sand, the nutrient solution was collected and its volume was measured. A 5-ml aliquot of the nutrient solution was transferred to a 22-ml scintillation vial containing 10 ml scintillation fluid[3]. Radioactivity was quantified by LSS.

Plant tissue was dried for 48 hours at 40°C, stored in a freezer (-20°C) and later combusted in a biological sample oxidizer[1]. Standards were prepared by combusting a known weight of dried plant tissue or filter paper with a ^{14}C-methyl methacrylate disc[2] that had a total radioactivity of 595 Bq (3.56 X 10^4 dpm). Plant tissue and filter paper that was combusted weighed 50, 100, 200, 300, 400, and 500 mg. More than 90% of the $^{14}CO_2$ generated by combustion of samples weighing 100 mg or less could be collected in a vial containing 15 ml scintillation fluid[3]. For samples that weighed more than 100 mg the oxidizer had to be modified to achieve the same CO_2-trapping efficiency. This was done by arranging two vials in series, each with 15 ml scintillation fluid. Any $^{14}CO_2$ not trapped in the first vial was collected in the second vial. In this manner, samples weighing as much as 400 mg could be combusted while maintaining a $^{14}CO_2$-trapping effiency greater than 90%.

All experiments were of a randomized design with four plants per treatment. Experiments were conducted twice and

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[1]Model OX300; R. J. Harvey Instr. Corp., Hillsdale, NJ.
[2]New England Nuclear, 549 Albany St., Boston, MA 02118
[3]Carbon 14 Cocktail (CO_2 trapping); R. J. Harvey Instr. Corp., Hillsdale, NJ.

data from a typical experiment were analyzed by analysis of variance. Data representing distribution of a ^{14}C-labeled herbicide were expressed as a percentage of total radioactivity recovered in the entire plant (including the leaf wash), or of the data for the entire plant less the leaf wash and treated leaf.

3.3.2 Translocation of the Acid Amide of Clopyralid

The carboxylic acid amide of ^{14}C-clopyralid, a suspected metabolite of clopyralid, was synthesized (section 3.8.1) and applied to rapeseed plants to determine how alteration of the carboxylic acid moiety would affect absorption and translocation. The ^{14}C-radiolabeled acid amide was dissolved in ethanol:water (1:9 v/v) containing 0.5% Tween 20, to a concentration with approximate radioactivity of 670 Bq (4.0×10^4 dpm) in 10 µl and was applied to the third leaf of rapeseed plants in the four-leaf stage. After 72 h, plants were harvested, dissected, and combusted as described in Section 3.3.1.

3.4 Quantification of Water-Soluble Conjugates of Herbicides

In a preliminary experiment, the effects of pH on the efficiency of ^{14}C-picloram or ^{14}C-clopyralid transfer from buffered aqueous solutions into dichloromethane were determined. Nine sodium phosphate/citrate buffer solutions

were prepared, ranging in pH from 1.6 to 5.0. Each solution contained ^{14}C-picloram or ^{14}C-clopyralid at a concentration of 3300 Bq,(2.0 X 10^5 dpm) per 5 ml. An aliquot (5 ml) of the buffered solution was partitioned three times against dichloromethane (5 ml). The quantity of herbicide in the aqueous and each organic phase was determined by LSS.

The extent of conversion of ^{14}C-picloram and ^{14}C-clopyralid to water-soluble derivatives was quantified in all three plant species. A method described by Hallmen and Eliasson (57) was modified and used to determine the quantity of recovered radioactivity present as herbicide and herbicide conjugates. Plants were treated with ^{14}C-picloram or ^{14}C-clopyralid and harvested 24, 72, and 144 hours after treatment. The silica sand was rinsed from the roots of each plant with water and the entire plant was wrapped in aluminum-foil and immediately frozen (-20°C). After freezing, each plant was cut into small pieces and homogenized in cold 85% acetone (10 ml/g fresh weight of tissue) with a Sorval Omni-mixer (3 minutes) and a Polytron sonic mixer (1 minute). The homogenate was filtered in a Buchner funnel through a Whatman #1 filter paper. The filter cake was rinsed once with 85% acetone (25-50 ml) and then repeatedly with methanol until the residue was white in colour. The residue was frozen and later combusted to determine the amount of ^{14}C-herbicide present.

The filtrate was reduced in volume under a stream of air (40°C) or by vacuum rotary evaporation (35°C) to

approximately 5 to 10 ml. The aqueous solution remaining in the beaker was removed. The beaker was rinsed with water (7 ml) and scoured with a rubber policeman in order to solubilize any residue. The rinsing and scouring procedures were repeated. The aqueous solution and washings were combined and centrifuged (10^4 rpm for 10 minutes). The supernatant was decanted. The pellet was dissolved in dichloromethane, transferred to a combustion vessel, reduced to dryness, and the residue was combusted and assayed for radioactivity.

The volume of the aqueous supernatant was measured and a 1-ml aliquot was assayed for radioactivity as previously described for the leaf rinse solution (Section 3.3.1). The quantity of radioactivity recovered in the supernatant was expressed as a percentage of the total herbicide recovered in the supernatant, pellet, and filter cake. A 5-ml aliquot of the supernatant was adjusted to pH 1.6 with HCl and partitioned four times against dichloromethane (5 ml). After each partition the organic phase was transferred to a 22-ml scintillation vial containing 10 ml scintillation liquid and assayed for radioactivity. The remaining aqueous phase contained the water-soluble derivative of the herbicide. Most of the radioactivity in the aqueous phase partitioned into dichloromethane after hydrolysis with 1 M NaOH for 1 hour at 100°C and acidifying the solution to pH 1.6, or after hydrolysis with 1 M HCl for 1 hour at 100°C.

In a another experiment, ^{14}C-picloram or ^{14}C-clopyralid was applied to rapeseed plants that were homogenized and extracted immediately. The aqueous residue was partitioned with dichloromethane as described above. This was done to determine if water-soluble conjugates were produced as a result of the extraction procedure.

3.5 Purification of the Naturally Produced Herbicide Metabolite

Analytical grade picloram or clopyralid was dissolved in ethanol:water (1:9 v/v) containing 0.5% Tween 20 to a concentration of 3.1 and 3.9 mM, respectively. The radiolabelled herbicide was dissolved in the corresponding solution to a concentration with approximate radioactivity of 330 Bq (2.0 X 10^4 dpm) per 10 μl[10]. A micropipette was used to disperse 50-100 μl of the herbicide solution, as 2- to 10-μl drops, over the entire surface of fully expanded rapeseed leaves. A total of 1.5 ml of the treatment solution was applied to 15 to 20 leaves. After 4 hours, the treated leaves were removed from the plant and the leaf petiole was immersed in 1.5 ml water in a vial. The leaf and vial were enclosed in an 8-cm diam jar containing 50 ml of water and placed in an incubator at 23°C ± 2 with a 24-hour photoperiod and a light intensity (400 to 700 nm) of 125 $\mu E \cdot m^2 \cdot s^{-1}$. After 72 hours the leaves were harvested and

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[10]The concentrations of ^{14}C-picloram and ^{14}C-clopyralid were 89.8 and 78.1 μM, respectively. The quantity of radiolabeled herbicide in the 10 μl of solution was 77.7 X 10^{-5} or 90.1 X 10^{-5} μmol of ^{14}C-picloram and ^{14}C-clopyralid, respectively.

homogenized as described previously (see Section 3.4), in order to isolate the aqueous extract containing the herbicide and herbicide conjugate.

3.5.1 Purification of the Derivatized Plant Metabolite

The aqueous extract (26 ml) described in section 3.5 was diluted with an equal volume of 28% NH_4OH and incubated at 30°C for 1 hour in order to derivatize the herbicide metabolite. The mixture was vacuum-evaporated (50°C) to remove NH_3, adjusted to pH 1.6 with HCl, and extracted five times with equal volumes of dichloromethane. The dichloromethane fractions were pooled and dried over anhydrous Na_2SO_4, and reduced to dryness under a stream of nitrogen gas. The residue was dissolved in 10 ml anhydrous acetone, reduced in volume to 1 ml, and streaked across a silica gel TLC plate[11]. The plate was developed in benzene:methanol (9:1 v/v). After development, the amide derivative of picloram or clopyralid was located by scanning the plate with a Geiger-Mueller TLC-plate scanner[12] and by visualization under UV (254 nm) radiation. The zone of interest was scraped from the plate and the silica gel was eluted with 20 ml ethyl acetate. The eluant was reduced in

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[11]Plate type: Linear-K Preadsorbent (LK5), 250 μm thickness, 20 X 20 cm; Whatman Chemical Separation Inc., 9 Bridewell Place, Clifton, NJ 07014.

[12]Berthold LB2760; scanning parameters: voltage - 1695 V; time constant - 10 sec; ratemeter - 1K; slit: number LB6292-1, width 2 mm, length 20 mm; counting wire length - 25 mm; scaling factor - 8; gas - P-10 (10% argon, balance mathane); gas flow setting - 6 to 8.

volume to 800 µl under a stream of nitrogen gas and re-chromatographed using the same procedure. The final ethyl acetate eluant was concentrated to 800 µl under nitrogen and 0.2 µl was analyzed by GC-MS.

3.5.2 Purification of Non-Derivatized Plant Metabolite

The aqueous extract (5 ml) from section 3.5 was added to 10 g silica gel[13] (40 - 140 mesh), mixed thoroughly, and dried at 40°C for 2 hours. The mixture was stirred and allowed to cool before adding absolute ethanol (50 ml). The ethanol-silica gel mixture was stirred for several minutes and then separated by filtration. The extraction of the silica gel with the ethanol was repeated three times. Ethanol fractions were combined and concentrated to 1.5 ml in vacuo, streaked on a silica gel TLC plate[14], and chromatographed in ethanol:dichloromethane (1:1 v/v). A broad zone containing ^{14}C (Rf 0.35 - 0.70) was scraped from the plate and the silica gel was eluted with absolute ethanol. The eluate was concentrated and streaked on a silica gel TLC plate[15], developed with acetone:water (9:1 v/v), the ^{14}C zone was removed and the silica gel was eluted with ethanol. The ethanol solution was reduced to dryness in vacuo and dried over P_2O_5 for 24 hours, in preparation for acetylation or silylation of the metabolite.

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[13]J.T. Baker Chemical Co., Phillipsburg, NJ 08865.
[14]Plate type: Linear-K Preadsorbent Preparative (PLK5), 1000 µm thickness, 20 X 20 cm; Whatman Chemical Separations Inc., 9 Bridewell Place, Clifton, NJ 07014.
[15]Plate type: 250 µm, 20 X20 cm; J.T. Baker Chemical Co., Phillipsburg, NJ 08865.

An equally effective but less timeconsuming method for metabolite purification was developed. With this method, the aqueous plant extract (5 ml) was passed through a C_{18} Sep-Pak[16] cartridge. The cartridge was flushed with water (5 ml) to remove all sugars and salts, and then rinsed with methanol (5 ml) to elute all radioactivity. The methanol eluate was reduced in volume to 0.5 ml, applied to a silica Sep-Pak[17] cartridge, and rinsed with tetrahydrofuran (10 ml). The eluate was reduced in volume to 0.75 ml, applied to a second silica Sep-Pak, and rinsed with tetrahydrofuran (15 ml). The eluate was concentrated, streaked on a silica gel TLC plate[18], and developed with acetone:water (9:1 v/v).

Acetylation of the herbicide metabolite was performed at 40°C for 4 hours with an excess of acetic anhydride:pyridine (2:1 v/v). Excess reagents were removed in vacuo (50°C) and by TLC with diethyl ether. The acetylated products were spotted on a silica gel TLC plate and developed in benzene:methanol (9:1 v/v) or dichloromethane:methanol (9:1 v/v). Silylation was conducted by dissolving the herbicide metabolites in Tri-Sil Z[19] (1 ml) at 30°C. After 30 minutes an aliquot (0.5 µl) was analyzed by GC-MS.

Combined GC-MS was performed on a Hewlett Packard 5985 GC-MS equipped with a fused silica capillary column[20] (i.d.

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[16]Part No. 51910; Waters Associates, Maple Street, Milford, MA 01757.
[17]Part No. 51900; Waters Associates, Ibid.
[18]Pierce Chemical Co., Rockford, IL.
[19]J&W Scientific Inc., 3871 Security Park Dr., Rancho Cordova, CA 95670.

0.32 mm; 15 m length) coated with SE-54 stationary phase. Helium was used as carrier gas at a flow rate of 50 cm/minute. The temperatures of the injector and GC-MS transfer line were 280 and 275°C, respectively. The MS had an ionizing energy of 70 eV, EM potential of 2200 volts, and an ion-source temperature of 200°C. The GC was temperature-programmed from 90 to 275°C at 30°C/minute.

3.6 Effect of pH on the Stability of the Herbicide Metabolite

The isolated ^{14}C-clopyralid metabolite (Section 3.5.2) was dissolved in ethanol to a concentration with approximate radioactivity of 660 Bq (4.0 X 10^4 dpm) per 100 µl. An aliquot (100 µl) of the metabolite was transferred to each of several test tubes, reduced to dryness, and the residue was taken up in a 0.1 M buffered solution (5 ml). Buffered solutions ranged in pH from 3 to 10, increasing by 1 pH unit. The buffers used were: sodium citrate/citrate, pH 3 - 6; potassium phosphate monobasic/KOH, pH 7 - 8; potassium carbonate/potassium borate/KOH, pH 10. The test tubes were incubated for 3 hours in a water bath at 37°C, cooled, and the solution was adjusted to pH 1.6 before being partitioned three times with dichloromethane. The aqueous and dichloromethane fractions were assayed for radioactivity as described previously. The experiment consisted of three replicates per treatment.

3.7 *In Vitro* Synthesis of the Herbicide Metabolite

Leaves (25-35 g fresh weight) from 30-day-old rapeseed plants were homogenized in a cold mortar for 5 minutes with 10 g sea sand and 0.1 M sodium citrate buffer (pH 5.2) containing 0.01 M cysteine. The homogenate was strained through cheesecloth and centrifuged at 1500 G (5 minutes; 3°C). Enzymes were isolated from the supernatant using two modified procedures reported by Kopcewicz et al. (68) and Frear et al. (47), respectively.

Method 1.

The supernatant was centrifuged at 10,000 G (5 minutes; 3°C). Two incubation mixtures were prepared, each containing, in μmoles, CoA, 1; ATP, 10; glucose, 80; $MgCl_2$, 80; UDP-glucose, 7; ^{14}C-clopyralid, 1.56×10^{-3} (4.0×10^5 dpm; 6600 Bq) in 4 ml sodium citrate buffer (pH 5.2). To tube A, 4 ml of fresh enzyme-supernatant were added, and to tube B, 4 ml of boiled enzyme-supernatant.

Method 2.

The supernatant was centrifuged at 70,000 G (30 minutes; 3°C). The resulting solution was precipitated with $(NH_4)_2SO_4$ (30-60% saturation), centrifuged at 10,000 G (10 minutes; 3°C), and the pellet was dissolved in 5 to 10 ml of 0.05 M sodium citrate buffer (pH 5,2). An aliquot (2.5 ml) of the solution was placed on a PD-10 column[20] and the enzymes were eluted with 3.5 ml of sodium citrate buffer (pH 5.2). Two incubation mixtures were prepared, each

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[20]Columns pre-packed with Sephadex G-25 M; bed volume 9 ml; Pharmacia Fine Chemicals AB, Uppsala, Sweden.

containing, in μmoles, UDP-glucose, 0.9; ^{14}C-clopyralid, 1.56 X 10^{-3}, in 1 ml of 0.1 M sodium citrate buffer (pH 5.2). To test tube A, 1 ml of fresh enzyme solution was added, and to tube B, 1 ml of boiled enzyme solution.

Both methods were repeated at pH 7.5 using a 0.1 M potassium phosphate buffer.

Regardless of the method used, the mixture was incubated for 4 or 24 hours at 30°C and the reaction was stopped by adding two volumes of cold acetone. The protein precipitate was removed by centrifugation, the supernatant was concentrated, and the remaining aqueous phase was adjusted to pH 1.6 and extracted three times with dichloromethane as described previously. The aqueous and dichloromethane fractions were asayed for radioactivity by LSS to determine the amount of water-soluble herbicide metabolite synthesized after incubation in the enzyme mixture.

3.8 Chemical Synthesis of the Possible Herbicide Metabolites

3.8.1 Synthesis of Ester and Amide of Picloram and Clopyralid

A diazomethane-ether solution was made by placing a solution of 6 g KOH dissolved in 10 ml water, 35 ml of 2-(2-ethoxyethoxy)-ethanol and 20 ml of ethyl ether into a 100 ml long-necked distilling flask containing a teflon stirring bar. A dropping funnel was attached to the

distilling flask. A solution of 25.1 g (0.1 mole) of p-tolylsulfonylmethylnitrosamide in 125 ml of ether was placed in the dropping funnel. The distilling flask was heated to 70-75°C and the solution from the dropping funnel was added slowly, over 15-20 minutes. When the dropping funnel was empty, more ether was added to the distillation flask at a rate equivalent to the rate of distillation. The ether was added until the distillate was colourless.

A solution (1 ml) of ^{14}C-picloram or ^{14}C-clopyralid in methanol (156 μM; 30 μg/ml) was placed in a 25-ml round-bottomed flask and 5 ml of the diazomethane-ether solution was added. The mixture was warmed, allowed to react for 5-10 minutes, placed in a warm water bath, and the diazomethane and ether were allowed to evaporate under a stream of nitrogen gas. The residue, the methyl ester of picloram or clopyralid, was taken up in 1 ml of methanol.

In some instances, ammonia gas was bubbled through the methanol solution containing the methyl ester of the herbicide in order to produce the carboxylic acid amide of picloram or clopyralid. The solution was allowed to stand at 25°C for 24 hours before TLC.

The above procedure was repeated with analytical grade picloram and clopyralid to produce the methyl esters and carboxylic acid amides of the herbicides. The methyl ester and amide of the herbicides were purified by chromatography on silica gel TLC plates[5] using the solvent systems iso-propanol:NH_3OH:water 8:1:1 v/v/v), CH_2Cl_2:methanol (9:1

v/v), or benzene:methanol (9:1 v/v).

3.8.2 Synthesis of Glucose and Sucrose Conjugates of Clopyralid

A 100-µl aliquot of an ethanol solution of ^{14}C-clopyralid (3.1 mM) was transferred to a 20-ml round-bottomed flask and reduced to dryness under a stream of nitrogen gas. Analytical grade clopyralid (100 mg; 520 µmoles; dried over P_2O_5) was added to the flask, followed by 10 ml of thionyl chloride ($SOCl_2$). The mixture was refluxed (85°C) for 2.5 hours after which the excess thionyl chloride was evaporated under vacuum at 60°C. The residue was dissolved in 5 ml of anhydrous tetrahydrofuran and slowly added to a solution consisting of anhydrous pyridine (10 ml) and 200 mg (1.1 mmoles) of powdered glucose which had been dried over P_2O_5. The reaction mixture was stirred vigorously for 12 h at 25°C and then evaporated to dryness under vacuum (60°C). The residue was taken up in water (5 ml), reduced to dryness, redissolved in water (3 ml), and then partitioned against ethyl acetate (10 ml). The sucrose conjugate of clopyralid was synthesized using the same method, except that glucose was replaced by sucrose (300 mg; 0.88 mmoles; dried over P_2O_5).

After partitioning, the aqueous phase was streaked (5 µl/cm) along the origin on a silica gel plate (250 µm thickness) and developed in iso-propanol:acetic acid:water (18:1:1 v/v/v) in order to separate clopyralid, glucose, and

pyridine from the glucose-clopyralid conjugate (Rf=0.62). The sucrose-clopyralid conjugate was separated (Rf=0.02) from some components of the reaction mixture on silica gel TLC plates[15] that were developed in dichloromethane:acetic acid:acetone:methanol (8:1:1:1 v/v/v/v). The glucose- or sucrose-herbicide conjugate was located, scraped from the TLC plate, and eluted from the silica gel with ethanol (5 ml). The ethanol solution was reduced in volume to 0.5 to 1 ml under a stream of nitrogen gas.

The glucose or sucrose conjugates of clopyralid were co-chromatographed on silica gel TLC plates[15] with the carboxylic acid amide of clopyralid, the methyl ester of clopyralid, the natural metabolite of clopyralid isolated from rapeseed plants, glucose, sucrose, and clopyralid. The solvent system used was either dichloromethane:acetic acid:acetone:methanol (8:1:1:1 v/v/v/v) or iso-propanol:acetic acid:water (18:1:1 v/v/v). In some cases, the compounds were spotted on the TLC plate and then exposed to NH_3 vapours for 2 hours before development. Clopyralid and its sugar conjugates were visualized on the TLC plates by exposure to UV (254 nm) light. Glucose, sucrose, and sugar conjugates of clopyralid were detected by spraying the plates with a mixture of 0.5 g thymol dissolved in 95 ml ethanol to which 5 ml of concentrated H_2SO_4 was added. Sugars and sugar-clopyralid conjugates appeared as pink spots after the plate was heated for 15-20 minutes at 120°C.

3.9 Enzymatic Hydrolysis the of Water-Soluble Herbicide Metabolite

The water-soluble herbicide metabolite of clopyralid was purified to remove all free soluble sugars by employing the purification procedure described in Section 3.5.2. which utilizes the C_{18} and silica Sep-Paks. An aliquot (0.5 ml) of the solution containing 1660 Bq (1.0×10^5 dpm) of the water-soluble herbicide metabolite was transferred to a test tube and reduced to dryness under a stream of nitrogen gas. The herbicide metabolite was then incubated for 4 or 24 hours at 36°C in 5 ml of 0.1 M sodium citrate buffer (pH 4.8) containing 2 mg/ml of β-glucosidase from almonds (emulsin)[21], 16 mg/ml of hesperidinase from *Penicillium* spp.[22], or 0.4 mg/ml of α-glucosidase from yeast[23]. Controls were established in which the corresponding enzyme was boiled for 10 minutes in 5 ml of 0.1 M sodium citrate buffer (pH 4.8), cooled, and an aliquot of the water-soluble herbicide metabolite was transferred to this incubation mixture.

The enzymatic hydrolysis reactions were stopped by adding cold acetone (-10°C; 7 ml) to the test tubes. The mixture was then centrifuged to remove the denatured enzyme,

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[21] β-glucosidase (EC 3.2.1.21), activity 1010 units/mg powder; Calbiochem-Behring Corp., La Jolla, CA 92037.

[22] Hesperidinase (EC 3.2.1.40), activity 0.27 units/mg powder and also containing β-glucosidase (EC 3.2.1.21), activity of 0.003 units/mg powder; Sigma Chemical Co., St. Louis, MO 63178.

[23] α-glucosidase (EC 3.2.1.20), activity 100 units/mg protein and also containing amylase (EC 3.2.1.1), activity 0.5 units/mg protein; Sigma Chemical Co., St. Louis, MO 63178.

and the acetone was removed by vacuum evaporation (40°C). The pH of the remaining aqueous solution was adjusted to 1.6 before three extractions with equal volumes of dichloromethane. The aqueous and three dichloromethane fractions were each assayed for radioactivity by LSS.

In another experiment, an aliquot (0.5 ml) of the purified water-soluble metabolite was reduced to dryness and incubated in 0.5 ml of 0.1 M sodium citrate buffer (pH 4.8) which contained 8 mg/ml of β-glucosidase, 16 mg/ml of hesperidinase, or 0.4 mg/ml of α-glucosidase. These solutions were incubated at 36°C and after 4 and 24 hours an aliquot (20 μl) was spotted on a TLC plate'' that was developed with dichloromethane:methanol:acetone:acetic acid (8:1:1:1 v/v/v/v). A series of controls was also established for this experiment, using boiled enzyme preparations.

Prior to incubation with the water-soluble metabolite, the enzymatic activity of β-glucosidase, α-glucosidase, and hesperidinase were determined by the enzymatic liberation of glucose from arbutin (hydroquinone-β-D-glucopyranoside), p-nitrophenyl-α-D-glucopyranoside, and hesperidin (hesperidin 7-rhamnoglucoside), respectively. Solutions containing 2 mg/ml of β-glucosidase with arbutin, 1 mg/ml of α-glucosidase with *p*-nitrophenyl-α-D-glucopyranoside, or 16 mg/ml of hesperidinase with hesperidin were incubated at 36°C for 4 hours in 0.1 M sodium citrate buffer (pH 5.0). The concentration of arbutin, *p*-nitrophenyl-α-D-glucopyranoside, or hesperidin that was

incubated with the appropriate enzyme ranged from 0 to 1 mM. A set of controls was established by adding boiled enzyme preparation to the incubation solution containing the appropriate substrate.

The glucose liberated by each enzyme was assayed spectrophotometrically at 540 nm using the Somogyi method (116). The results were compared to the control containing the corresponding boiled enzyme. In addition, a standard curve of glucose, ranging in concentration from 0 to 200 μg, was prepared and the relative enzymatic release of glucose from the respective substrate was determined by comparison to the glucose standard curve.

3.10 Ethylene Biosynthesis and Herbicide Action

Seeds of sunflower and rapeseed were planted in individual pots containing sand, soil, and peat-moss (1:1:1). After 10 days, plants were thinned to three plants per pot. Plants were grown in a growth cabinet maintained at 20/16±1 °C day/night with a 16-hour photoperiod and relative humidity of 80%. The light intensity (400 to 700 nm) was constant at 450 $\mu E \cdot m^{-2} \cdot s^{-1}$.

In all experiments, commercial formulations of picloram and clopyralid were used. Initial experiments on the selectivity of picloram and clopyralid in sunflower and rapeseed were done in a growth cabinet. Picloram and clopyralid were applied with a motorized laboratory sprayer at rates of 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, and 5.0

kg/ha. Plant response was monitored for 18 days for herbicide symptoms. In all other experiments, herbicides were applied as 10-µl drops with an automatic pipet, to the third leaf of rapeseed or to one leaf of the second leaf pair of sunflower. Concentrations of the herbicide solutions used were 0.52, 2.6, 13, or 25 mM, and 0.04, 2.1, 10.4, or 19.9 mM for clopyralid and picloram, respectively. These concentrations are equivalent to 0.1, 0.5, 2.5, or 4.8 g ai/L for both herbicides. Depending on the experiment, the quantity of herbicide applied to a plant was either 5.2×10^{-2}, 2.6×10^{-1}, 13×10^{-1} µmol in 100 µl or 5 µmol in 200 µl of solution for clopyralid, and either 4.0×10^{-2}, 2.1×10^{-1}, 10.4×10^{-1} µmol in 100 µl or 39.8×10^{-1} µmol in 200 µl of solution for picloram. These quantities are equivalent to 10, 50, 250 µg in 100 µl or 960 µg in 200 µl of solution for both herbicides. In some experiments a pre-treatment of a 31 or 125 µM solution of aminoethoxyvinylglycine[24] (AVG) was applied with an atomizer by spraying the plants until run-off. Tween 20 (0.05% v/v) was added to the AVG solution applied to rapeseed, in order to achieve adequate wetting of the waxy leaves.

For each experiment on the measurement of the rate of ethylene production, one plant in the 4-leaf stage was selected for similar morphological characteristics. Cotyledons were removed and the plant was sealed around the stem in a glass cuvette described previously (10). The

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[24]Fluka Chemical Corp.; 255 Oser Ave., Hauppauge, NY 11788.

system was allowed to equilibrate for several hours in a stream of air to avoid problems arising from ethylene production resulting from mechanical stimulation during insertion of the plant into the cuvette. To remove hydrocarbon contaminants, air containing 500 µl/L of CO_2 was passed through a stainless steel tube (2.5 cm diam) packed with platinized asbestos maintained at 700°C (36). The purified cool air was passed through the cuvette at a flow rate of 200 ml/minute. The total volume of the system was 3.8 L, requiring approximately 20 minutes for equilibration and complete gas turnover. Temperature of the cuvette was maintained at 27±1 °C by water from a constant temperature bath, circulating within a jacket enclosing the cuvette. Light (400 to 700 nm) intensity was constant at 125 $\mu E \cdot m^{-2} \cdot s^{-1}$, provided by six 150 Watt incandescent bulbs. The light was passed through a Plexiglas filter containing a 12-mm depth of $CuSO_4 \cdot 5H_2O$ (2% w/v) solution with a cut-off point at 730 nm.

Air leaving the cuvette was bubbled through 15 ml ice-cooled, saturated solution of KOH contained in a gas washing bottle, to remove CO_2 and water vapour. To collect the ethylene, the air was then passed through a trap containing silica gel (0.5 g, 60 to 80 mesh) kept at -86 °C in a dry ice-acetone bath (10). Ethylene concentrations were determined on a gas chromatograph (Hewlett Packard 5830A) equipped with a Porapak Q column[25] (80 to 100 mesh) and a

[25]Chromatographic Specialties Ltd., 300 Laurier Blvd., Brockville, Ont.

flame ionization detector. The helium carrier gas flow rate was 60 ml/minute. The gas chromatograph oven temperature was maintained at a constant 60 °C. Concentrations of CO_2 were determined on a gas chromatograph (Hewlett Packard 5880) equipped with the same column, and a thermal conductivity detector. Helium carrier gas flow was 60 ml/minute, while the gas chromatograph oven temperature was maintained at a constant 50 °C.

A study of changes in morphology and leaf axil angle after treatment with AVG, herbicide, or AVG plus herbicide, was conducted with sunflower plants. The plants were transferred to a growth cabinet maintained at 27±1 °C and relative humidity of 90%, 24 hours prior to treatment. The light intensity (400 to 700 nm) was constant at 250 $\mu E \cdot m^{-2} \cdot s^{-1}$. Details of the chemical treatments are described with the results. Morphological changes were assessed by observation at 0, 4, 8, 12, and 24 hours after treatment.

Each experiment was repeated at least three times and the same trend was obtained consistently in all experiments. Data presented are from one of the three experiments.

4. Results and Discussion

4.1 Absorption and Translocation

4.1.1 Herbicide Loss Due to Environmental Conditions

Prior to beginning the experiments on the absorption and translocation of ^{14}C-picloram and ^{14}C-clopyralid, an experiment was conducted to determine if any herbicide was lost due to the environmental conditions under which the plants were grown, dried, and stored. Radiolabeled picloram or clopyralid was applied to detached leaves of rapeseed, sunflower, and Canada thistle plants, as well as filter paper disks and glass microscope cover slips, before being placed in a growth chamber for 8, 24, 72, and 144 hours. The herbicides were also applied to filter paper disks that were placed in an oven at 50°C for 48 hours or in a freezer at -15°C for one month. After completion of each treatment, the fraction of radiolabeled picloram and clopyralid remaining was determined by combustion of the leaves and filter paper disks, or by inserting the glass cover slips directly into scintillation vials containing scintillation fluid. Results were compared statistically, by means of a paired t-test, to a set of controls that were established by placing a measured amount of ^{14}C-picloram or ^{14}C-clopyralid directly into a scintillation vial. None of the results differed significantly from the respective controls.

4.1.2 Absorption and Translocation in Rapeseed and Sunflower Plants

The absorption and translocation pattern of ^{14}C, after application of radiolabeled picloram or clopyralid to rapeseed and sunflower plants, is shown in Table 2. Data for each plant part are expressed as a percentage of the sum of radioactivity recovered in the leaf wash and all plant parts.

The type of herbicide, the time of harvest, and the plant species treated had no major effect on the amount of ^{14}C washed off the treated leaf, except in the case when picloram was applied to rapeseed plants that were harvested 24 hours after treatment (Table 2). In most cases, more than 95% of the radioactivity from ^{14}C-picloram and ^{14}C-clopyralid treatments was absorbed by the rapeseed and sunflower plants.

Within a plant species, there was no difference between the amounts of ^{14}C-picloram and ^{14}C-clopyralid recovered in the treated leaf, regardless of the harvest time. However, a comparison between the plant species showed that, as the time between treatment and harvest increased, less picloram and clopyralid remained in the treated leaf of sunflower than of rapeseed plants. Approximately 25% less picloram or clopyralid remained in the treated leaf of sunflower than of rapeseed plants 144 hours after treatment.

Less than 5% of the total radioactivity recovered in both plant species was transported basipetally, regardless

Table 2. Distribution of ^{14}C as a percentage of total radioactivity recovered 24, 72, and 144 hours after application of ^{14}C-picloram and ^{14}C-clopyralid to the third leaf of rapeseed and one leaf of the second leaf pair of sunflower plants. Rapeseed and sunflower plants were in the five-leaf and three-leaf stage, respectively. Treated leaves were fully expanded[1].

Time (h)	Plant part	Rapeseed[1]		Plant part	Sunflower[1]	
		^{14}C-picloram	^{14}C-clopyralid		^{14}C-picloram	^{14}C-clopyralid
		------(% of recovered)------			------(% of recovered)------	
24	Leaf wash	14.5 (3.3)	1.4 (0.6)	Leaf wash	5.7 (3.0)	1.3 (0.5)
72		1.7 (0.6)	0.4 (0.2)		1.3 (0.6)	1.3 (0.9)
144		0.6 (0.3)	0.4 (0.2)		1.5 (0.8)	0.2 (0.0)
24	Treated leaf	58.8 (3.1)	55.4 (2.9)	Treated leaf	40.6 (3.6)	53.3 (9.9)
72		44.4 (1.0)	40.0 (3.7)		31.3 (6.1)	17.8 (6.6)
144		42.2 (6.6)	35.2 (0.8)		18.7 (6.9)	16.4 (4.7)
24	Growing point	7.5 (1.7)	9.5 (1.0)	Growing point	42.5 (4.0)	33.6 (7.7)
72		19.4 (4.7)	16.0 (4.7)		54.8 (5.1)	75.3 (6.3)
144		17.4 (5.1)	16.9 (2.3)		69.4 (6.9)	75.7 (5.8)
24	Fifth leaf	4.6 (1.3)	19.0 (2.9)	Stem tissue above	9.9 (1.3)	10.0 (2.2)
72		20.9 (4.3)	32.0 (2.2)	treated leaf and	9.0 (1.8)	3.5 (0.3)
144		25.3 (4.9)	32.8 (2.1)	growing point	7.3 (1.6)	4.0 (0.5)
24	Fourth leaf	3.9 (0.8)	12.2 (1.2)	Leaf opposite the	0.3 (0.0)	1.6 (0.5)
72		10.0 (2.5)	10.2 (1.8)	treated leaf	0.9 (0.2)	1.5 (0.3)
144		11.2 (3.8)	13.3 (1.9)		0.5 (0.1)	3.6 (0.9)
24	Tissue below treated	10.6 (2.0)	2.6 (0.3)	Tissue below treated	1.0 (0.3)	0.3 (0.1)
72	leaf including the	3.8 (0.5)	1.5 (0.2)	leaf including the	2.7 (0.5)	2.8 (0.8)
144	roots	3.4 (0.5)	1.5 (0.2)	roots	2.7 (0.5)	3.6 (0.9)

[1]Standard errors of the means are in parentheses.
[2]The percentage of total applied radioactivity that was recovered in rapeseed plants 24, 72, 144 hours after treatment was 105.7 (3.4), 86.6 (6.9), and 93.3 (3.5) for picloram, respectively, and 95.0 (6.2), 92.1 (3.0), and 82.1 (1.7) for clopyralid, respectively.
[3]The percentage of total applied radioactivity that was recovered in sunflower plants 24, 72, 144 hours after treatment was 100.1 (1.4), 93.4 (0.9), and 84.2 (1.5) for picloram, respectively, and 100.3 (2.0), 98.1 (2.7), and 94.6 (2.5) for clopyralid, respectively.

of the herbicide applied or the time of harvest (Table 2). The one exception occurred when picloram was applied to rapeseed plants that were harvested 24 hours later. Less than 0.5% of the radioactivity recovered in rapeseed and sunflower plants was found in the roots 24, 72, and 144 hours after treatment (data not shown). Little radioactivity (less than 0.2%) was found in the nutrient solution of both plant species at any time interval after treatment.

A comparison between the acropetal transport of radioactivity from ^{14}C-picloram and ^{14}C-clopyralid treatments was made at each time interval (Table 3). No difference was found at any time interval between the acropetal movement of the two herbicides within a plant species, except in one case. The exception occurred with rapeseed plants harvested 24 hours after herbicide application, and reflects the fact that a large portion of the recovered picloram dose moved basipetally.

The strongest sink for the radioactivity that moved acropetally in rapeseed plants appears to be the growing point and the fifth leaf. Both these plant parts were very small and undergoing rapid growth and development. However, the fourth leaf of the rapeseed plants, which was regarded as being fully expanded, also acted as a strong sink. In sunflower plants the rapidly expanding leaves above the treated leaf acted as the primary sink. Less than 10% of the radioactivity recovered in the plant was found in the stem tissue between the treated leaf and the growing point of

Table 3. Comparison at each time interval of the recovered radioactivity transported acropetally out of the treated leaf after application of ^{14}C-picloram or ^{14}C-clopyralid to rapeseed and sunflower plants. Data were obtained from Table 2 by summing the amount of radioactivity found in the plant parts situated above the treated leaf[1].

Time (h)	Plant species	^{14}C-picloram	^{14}C-clopyralid	LSD[2] (0.01)
		----------(% of recovered)----------		
24	Rapeseed	16.0 (2.6)	40.6 (2.6)	14.1
72		50.3 (1.7)	58.2 (3.8)	NS
144		53.9 (6.3)	63.0 (2.0)	NS
24	Sunflower	52.7 (5.0)	45.1 (9.9)	NS
72		64.7 (6.3)	80.7 (6.2)	NS
144		77.2 (6.7)	83.3 (4.7)	NS

[1]Standard errors of the means are in parentheses.
[2]NS = not significant as determined by an analysis of variance.

sunflower plants. Moreover, little radioactivity moved into the leaf opposite the treated leaf in sunflower plants.

The acropetal translocation data representing each plant species in Table 3 were analyzed by means of a two-factor analysis of variance. The analysis included the main effects of time (24, 72, and 144 hours after treatment) and herbicide treatment (picloram and clopyralid), as well as a time x herbicide interaction term. The interaction term was not significant ($P \leq 0.05$) for either rapeseed or sunflower. In rapeseed, the main effect of herbicide treatment was significant ($P \leq 0.05$), indicating that regardless of the harvest time, the acropetal movement of

picloram was less than that of clopyralid. In sunflower, there was no difference between the acropetal movement of the herbicides, regardless of the harvest time. The main effect of time was significant ($P \leq 0.05$) for both plant species. Therefore, regardless of the ^{14}C-herbicide used within a plant species, more radioactivity moved acropetally when the plants were harvested 72 or 144 hours after treatment than 24 hours after treatment.

In most instances, the recovery of picloram and clopyralid exceeded 90% when results were expressed as a percentage of the total herbicide dose applied to rapeseed and sunflower plants (Table 2). In those cases where the total herbicide recovery was less than 90%, there appears to be no pattern of loss based on the type of herbicide applied, the plant species treated, or the time between treatment and harvest. Loss of radioactivity following ^{14}C-herbicide application could not be explained on the basis of root exudation, or environmental conditions under which the plants were grown, dried, and harvested (see Section 4.1.1).

4.1.3 Effect of the Maturity of the Treated Leaf on Absorption and Translocation

The degree of maturity of the treated leaf of rapeseed and sunflower plants had a marked affect on the absorption and translocation pattern of ^{14}C after application of ^{14}C-picloram and ^{14}C-clopyralid (Table 4). Data representing

Table 4. Distribution of ^{14}C as a percentage of total radioactivity recovered 24, 72, and 144 hours after application of ^{14}C-picloram or ^{14}C-clopyralid to the third leaf of rapeseed plants and to one leaf of the second leaf pair of sunflower plants. In one group of plants the treated leaf was not fully expanded, whereas in the other group of plants the treated leaf was fully expanded.[1]

Time (h)	Plant part	Rapeseed		Sunflower	
		Not fully expanded	Fully expanded	Not fully expanded	Fully expanded
		(% of recovered)		(% of recovered)	
			Picloram		
24	Leaf wash	98.0 (1.4)	73.3 (2.5)	93.2 (3.9)	46.3 (5.3)
72	plus	97.4 (1.4)	46.1 (1.4)	89.0 (2.4)	32.6 (6.6)
144	treated leaf	93.9 (2.9)	42.8 (5.9)	77.3 (2.4)	20.1 (6.8)
24	All plant	1.6 (1.3)	16.0 (2.6)	5.2 (3.0)	52.7 (5.0)
72	parts above	1.9 (1.9)	50.3 (1.7)	10.6 (4.5)	64.7 (6.3)
144	treated leaf	5.2 (1.2)	53.9 (6.3)	22.2 (5.6)	77.2 (6.7)
24	All plant	0.3 (0.0)	10.6 (2.0)	1.6 (0.5)	1.0 (0.3)
72	parts below	0.8 (0.0)	3.8 (0.5)	0.3 (0.0)	2.7 (0.5)
144	treated leaf	0.9 (0.2)	3.4 (0.5)	0.4 (0.2)	2.7 (0.5)
			Clopyralid		
24	Leaf wash	86.2 (4.9)	56.8 (2.7)	90.4 (4.4)	54.6 (9.8)
72	plus	63.7 (2.6)	40.3 (3.8)	63.7 (9.0)	19.1 (6.2)
144	treated leaf	54.1 (5.4)	35.6 (1.0)	48.9 (9.8)	16.6 (4.7)
24	All plant	11.6 (4.7)	40.6 (2.6)	8.0 (3.9)	45.1 (9.9)
72	parts above	33.9 (2.7)	58.2 (3.8)	33.5 (6.0)	80.7 (6.2)
144	treated leaf	43.8 (5.4)	63.0 (1.0)	47.6 (9.5)	83.3 (4.7)
24	All plant	2.3 (0.8)	2.6 (0.3)	0.3 (0.1)	1.6 (0.5)
72	parts below	2.4 (0.7)	1.5 (0.2)	0.3 (0.1)	2.8 (0.8)
144	treated leaf	2.1 (0.3)	1.5 (0.2)	0.1 (0.0)	3.6 (0.9)

[1]Standard errors of the means are in paretheses

absorption and translocation in plants that were considered to have fully expanded leaves are the same as those presented in Table 2. The absorption and translocation patterns in plants without fully expanded treated leaves were obtained by treating the plants 4-5 days earlier than the plants that had more mature treated leaves.

In both plant species the degree of maturity of the treated leaf did not affect the amount of herbicide that was absorbed. However, maturity of the treated leaf significantly affected the amount of radioactivity that moved out of the treated leaf following application of ^{14}C-picloram and ^{14}C-clopyralid to rapeseed and sunflower plants. Less mature leaves exported less radioactivity than more mature leaves. In both plant species, more than 80 and 50% of the recovered radioactivity remained in or on the less mature treated leaf 24, 72, and 144 hours after application of ^{14}C-picloram and ^{14}C-clopyralid, respectively.

4.1.4 Absorption and Translocation in Canada Thistle

More than 97% of the recovered radioactivity was absorbed by the treated leaf of Canada thistle plants 24, 72, and 144 hours after application of ^{14}C-picloram and ^{14}C-clopyralid (Table 5).

A two-factor analysis of variance that included the main effects of time and herbicide treatments was used to analyze the treated leaf data in Table [illegible] time x

Table 5. Distribution of ^{14}C as a percentage of total radioactivity recovered 24, 72, and 144 hours after application of ^{14}C-picloram or ^{14}C-clopyralid to the fourth leaf from the apex of Canada thistle plants in the 8- to 10-leaf stage[1].

Time (h)	Plant part	^{14}C-picloram[2]	^{14}C-clopyralid[2]
		--------(% of recovered)-------	
24	Leaf wash	0.0 (0.0)	0.6 (0.6)
72		2.8 (0.8)	0.4 (0.1)
144		2.0 (0.1)	1.6 (0.5)
24	Treated leaf	78.7 (3.8)	61.7 (4.2)
72		46.5 (9.8)	22.5 (4.3)
144		26.9 (3.7)	12.4 (1.2)
24	Apex: including	9.4 (0.7)	15.0 (1.6)
72	growing point and	32.0 (9.4)	38.2 (2.0)
144	expanding leaves	46.3 (3.5)	49.9 (2.4)
24	Tissue between	5.0 (1.7)	5.1 (0.8)
72	the treated leaf	5.3 (0.8)	7.1 (0.9)
144	and the apex	9.7 (1.1)	11.5 (1.2)
24	All leaves below	2.8 (0.5)	6.0 (1.1)
72	the treated leaf	4.3 (1.0)	9.4 (1.2)
144		6.5 (0.7)	7.9 (0.5)
24	Stem tissue below	2.0 (0.7)	3.8 (0.2)
72	the treated leaf	5.8 (1.4)	8.2 (1.1)
144		6.0 (0.7)	9.0 (1.1)
24	Roots	2.0 (0.9)	7.9 (2.4)
72		3.4 (1.4)	14.1 (3.6)
144		2.6 (0.4)	7.8 (1.1)

[1]Standard errors of the means are in parentheses.
[2]The percentage of total applied radioactivity that was recovered 24, 72, and 144 hours after treatment was 96.0 (4.5), 88.1 (6.8), and 81.2 (5.3) for picloram, respectively, and 98.5 (3.3), 96.5 (1.0), and 79.0 (4.0) for clopyralid, respectively.

herbicide interaction was not significant ($P \leq 0.05$). However, both main effects were significant ($P \leq 0.05$). Therefore, regardless of the harvest time, less clopyralid (32%) than picloram (51%) remained in the treated leaf. Furthermore, less radioactivity remained in the treated leaf 72 (34.5%) and 144 (19.7%) hours than 24 (70.2%) hours after treatment ($LSD_{.05}=16$).

More than 80% of the recovered radioactivity that was transported acropetally accumulated in the apex of Canada thistle plants 72 and 144 hours after application of ^{14}C-picloram or ^{14}C-clopyralid (Table 5). The stem and leaf tissue between the apex and treated leaf did not act as a strong sink for the herbicides. The amounts of radioactivity found in the plant parts located above the treated leaf (Table 5) were summed and are presented in Figure 1A. A two-factor analysis of variance was used to analyze the acropetal transport data shown in Figure 1A. The time x herbicide interaction and the main effect of herbicide treatment were not significant. However, the main effect of time was significant ($P \leq 0.05$). Therefore, regardless of the herbicide treatment, more radioactivity was transported acropetally at each successive time interval after treatment.

A substantial portion of the recovered radioactivity was transported basipetally after Canada thistle plants were treated with ^{14}C-picloram or ^{14}C-clopyralid (Table 5). A two-factor analysis of variance indicated that the time x

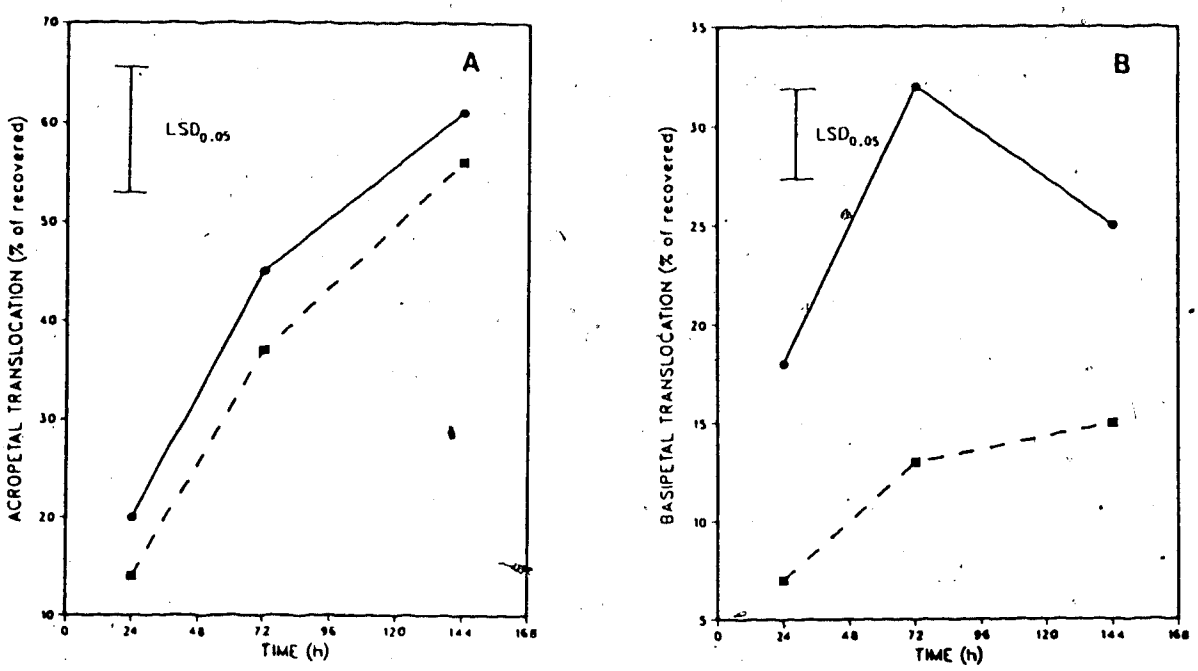


Figure 1. Percentage of total radioactivity recovered in the plant that was transported acropetally (A) and basipetally (B) out of the treated leaf 24, 72, 144 hours after application of ^{14}C-picloram (■) or ^{14}C-clopyralid (●) to Canada thistle plants.

herbicide interaction was not significant ($P \leq 0.05$). However, both the main effects of time and herbicide treatment were significant. Less of the total recovered radioactivity moved basipetally after Canada thitle plants were treated with picloram than with clopyralid, regardless of the harvest time. In addition, more of the recovered radioactivity was translocated basipetally 72 (22.6%) and 144 (19.8%) hours than 24 (12.3%) hours after treatment, regardless of the herbicide applied ($LSD_{.05}$=8.6%).

Table 5 and Figure 1B indicate that there was a decrease in the radioactivity found in the roots 144 hours after ^{14}C-clopyralid absorption. There was no apparent decrease in the radioactivity found in Canada thistle roots

144 hours after ^{14}C-picloram application (Table 5 and Figure 1B). Regardless of the herbicide applied, recovery of the total applied radioactivity was reduced from 96% to approximately 80%, respectively, 24 and 144 hours after treatment.

Since previous results indicate that little radioactivity was lost due to the environmental conditions under which Canada thistle plants were grown, dried, and stored, two additional experiments were conducted in order to determine how radioactivity was being lost in Canada thistle plants. In the first experiment, Canada thistle plants were treated with ^{14}C-clopyralid and isolated in a Bell jar. The amount of CO_2 entering the Bell jar was monitored by gas chromatographic techniques described in Section 3.10. CO_2 emanating from the chamber was bubbled slowly through CO_2-trapping scintillation fluid[2] that was changed as it approached CO_2-saturation. The experiment was repeated three times. Little $^{14}CO_2$ was trapped during the 144-hour period between treatment and harvest.

In the second experiment, Canada thistle plants were treated with ^{14}C-picloram and ^{14}C-clopyralid and harvested 144 hours later to determine the absorption and translocation pattern of the herbicides as well as the amount of ^{14}C exuded into the nutrient solution and growth medium (silica sand). Canada thistle plants were treated when they were at the same stage of development as the

[2] Ten ml of this scintillation fluid will absorb 0.6 g (14 mmols) of $^{14}CO_2$; R.J. Harvey Instr. Corp., Hillsdale, NJ.

plants used in earlier experiments. Roots were removed from the silica sand and the sand was rinsed repeatedly with methanol. The nutrient solution was assayed for radioactivity by LSS.

The absorption and acropetal translocation patterns of both herbicides were similar to that shown in Table 5. However, less than 15% of the total applied radioactivity moved basipetally and only 6% had moved into the roots 144 hours after application of ^{14}C-picloram or ^{14}C-clopyralid. Less than 1% of the total applied radioactivity was found in the nutrient solution plus growth medium after application of either herbicide. The recovery of total applied radioactivity was 102.2 ± 1.1% and 97.2 ± 4.1%, respectively, 144 hours after treatment with ^{14}C-picloram and ^{14}C-clopyralid.

4.1.5 Absorption and Translocation of the ^{14}C-amide of Clopyralid

The carboxylic acid amide of clopyralid was applied to the leaves of rapeseed and sunflower plants in order to determine what effect altering the carboxylic acid moiety of the herbicide would have on the uptake and translocation pattern of the compound.

Less than 4% of the applied radioactivity was washed off the treated leaf of rapeseed and sunflower plants 72 hours after treatment with the amide derivative (Table 6). However, more than 90% of the recovered radioactivity

Table 6. Distribution of ^{14}C as a percentage of total radioactivity recovered 72 hours after application of the ^{14}C-amide of clopyralid to the third leaf of rapeseed and to one leaf of the second leaf pair of sunflower plants[1].

Plant part	Rapeseed[2]	Sunflower[2]
	-------(% of recovered)--------	
Treated leaf and leaf wash	98.2 (0.1)	98.2 (1.9)
Tissue above treated leaf	1.4 (0.1)	1.4 (0.3)
Tissue below treated leaf	0.4 (0.0)	0.2 (0.0)

[1]Standard errors of the means are in parentheses.
[2]The percentage of total applied radioactivity that was recovered in rapeseed and sunflower plants was 89.0 (4.2) and 82.6 (1.3), respectively.

remained in the treated leaf, with less than 2% being transported out of the treated leaf of both species.

In another experiment, application of 10 mg and 100 mg of the acid amide of clopyralid caused no herbicidal symptoms in sunflower plants 18 days after treatment. The same doses of clopyralid resulted in herbicidal symptoms within 24 hours of treatment.

4.1.6 Discussion

The absorption and translocation patterns of picloram and clopyralid in rapeseed and sunflower plants were investigated to obtain a possible explanation for the

sensitivity differences manifested within rapeseed plants and between the two annual plant species to the herbicides. There was no difference between ^{14}C-picloram and ^{14}C-clopyralid absorption and translocation in sunflower plants. In rapeseed plants, more ^{14}C moved acropetally after ^{14}C-clopyralid than after ^{14}C-picloram application. This difference results from the poor acropetal movement of radioactivity 24 hours after application of ^{14}C-picloram. This anomaly cannot be explained. These results indicate that differences in the absorption and translocation patterns of ^{14}C after application of picloram or clopyralid to rapeseed plants, do not account for the difference in sensitivity within this plant species to the two herbicides.

Approximately 20% more picloram and clopyralid moved acropetally in sunflower than in rapeseed plants, 144 hours after treatment. One may argue that this could account for the extreme sensitivity difference that exists between rapeseed and sunflower plants to these herbicides. However, more than 60% of the recovered picloram and clopyralid moved acropetally in both plant species, 144 hours after treatment. With such large quantities of picloram and clopyralid moving acropetally in both plant species, it is unlikely that sensitivity differences between rapeseed and sunflower plants to these herbicides can be explained solely on the basis of absorption and translocation differences.

Turnbull (119), studying the absorption and translocation of ^{14}C-clopyralid in rapeseed plants, found

results similar to those presented in Tables 2 and 3. He found 44, 35, and 29% of ^{14}C present in the treated leaf of rapeseed plants, 1, 3, and 9 days after ^{14}C-clopyralid application, respectively. Less than 3% of the radioactivity was present in the leaf wash, regardless of the time of harvest. The amount of ^{14}C in the acropetal foliage was 48, 56, and 53%, respectively, 1, 3, and 9 days after treatment. Less than 1.5 and 1% of the radioactivity was found in the roots and nutrient solution, respectively, regardless of the harvest time.

Hallmen's (56) results are markedly different from those presented in Tables 2 and 3. He found that approximately 24, 46, and 48% of the recovered radioactivity moved out of the treated leaf of sunflower plants, 1, 3, and 9 days after treatment with ^{14}C-picloram, respectively. In rapeseed plants, more than 85% of the radioactivity remained in the treated leaf 9 days after application of ^{14}C-picloram.

Hallmen (56) speculated that the selectivity difference between rapeseed and sunflower plants to picloram was accounted for, at least in part, by differences in acropetal translocation of the herbicide. Hallmen's results may be misleading since picloram was not applied to the same plant parts in the two species. Picloram was applied to the cotyledons of sunflower plants and to the first true leaf of rapeseed plants that were 15-20 days old. Results in Table 4 show that the extent of translocation in rapeseed and

sunflower plants is affected by the maturity of the treated leaf. It is possible that the treated leaf of the rapeseed plants used in the experiment conducted by Hallmen were not fully expanded. This may explain why Hallmen found little translocation of picloram in rapeseed plants.

Canada thistle plants were included in this study in order to determine whether the translocation pattern of the herbicides in this species was markedly different from that in the two annual species, and whether there was a difference between the absorption and translocation of picloram and clopyralid within this perennial species.

Radioactivity moved both basipetally and acropetally in Canada thistle plants treated with ^{14}C-picloram or ^{14}C-clopyralid (Table 5). A larger portion of the ^{14}C moved acropetally than basipetally. The acropetal movement was similar to that in the annual plant species; most radioactivity collected in the apical meristem. The pattern of acropetal translocation of picloram and clopyralid shown in Table 5 is similar to the pattern found by several other researchers (34, 98, 112, 113). These researchers found that more than half of the radioactivity that was translocated out of the treated leaf after application of picloram and clopyralid moved acropetally, regardless of harvest time.

Within Canada thistle plants, more radioactivity moved basipetally following ^{14}C-clopyralid than ^{14}C-picloram treatment (Table 5). Bovey and Mayeux (16) reported that more clopyralid than picloram moved into the roots of

Prosopis juliflora plants that had received foliar applications of these herbicides. The difference in basipetal movement of picloram and clopyralid may explain why the latter herbicide is more effective in controlling *P. juliflora*. Furthermore, it has been shown that Canada thistle regrowth was controlled more effectively with clopyralid than with picloram under greenhouse conditions (54).

Several researchers have shown that picloram (43, 65, 112) and clopyralid (119, 120) can be lost through root exudation in various plant species. More specifically, the loss of picloram (112) and clopyralid (120) by exudation from the roots of Canada thistle plants has been reported. Turnbull and Stephenson (120) found 4, 16, and 21% of the foliar applied ^{14}C-clopyralid dose unaccounted for 1, 3, and 9 days after treatment. Approximately 10 and 15% of this missing radioactivity was found in the nutrient solution of hydroponically grown Canada thistle plants.

Indirect evidence also exists for the loss of clopyralid by root exudation. For example, several researchers (34, 96) found that clopyralid moved into the roots of Canada thistle plants. These researchers found that more than 12% of the applied radioactivity was not recovered, several days after treatment. However, Devine and Vanden Born (34) found that little radioactivity was translocated to the roots of perennial sowthistle after application of ^{14}C-clopyralid. Furthermore, none of the

radioactivity from ^{14}C-clopyralid that was applied to perennial sowthitle was lost 144 hours after treatment. Devine and Vanden Born (34) suggested that the radioactivity not recovered from Canada thistle plants was not lost from leaf surfaces, since such losses presumably would have occurred in both species, but rather was lost once the herbicide was in the Canada thistle plant. It also has been shown that radioactivity from Canada thistle plants treated with ^{14}C-picloram (113) or ^{14}C-clopyralid (Section 4.1.3) was not lost as $^{14}CO_2$, or due to the environmental conditions under which the plants are grown, dried, and stored (Section 4.1.1).

The previously cited direct and indirect evidence that indicates exudation of picloram and clopyralid from the roots of Canada thistle, may explain why recovery of the applied herbicides was low in Table 5. However, in the experiment that was conducted to specifically determine the amount of picloram and clopyralid that was exuded into the nutrient solution, little of the applied radioactivity moved to the roots or was exuded into the nutrient solution 144 hours after treatment. Therefore, on the basis of this experiment it was not possible to determine if the two herbicides were exuded into the nutrient solution. These results indicate that when there is little translocation to the roots there is no opportunity for herbicide exudation. However, if there is adequate translocation of these pyridine herbicides to the roots, as in the case of Canada

thistle treated with clopyralid (Table 5), exudation may occur from the roots.

On the basis of the previous discussion, it was estimated that approximately 15 and 19% of the applied radioactivity may have been exuded from Canada thistle roots 144 hours after treatment with ^{14}C-picloram and ^{14}C-clopyralid, respectively. These estimates of exudation were obtained by subtracting the percentage of total applied radioactivity that was recovered in Canada thistle plants 144 hours after ^{14}C-picloram and ^{14}C-clopyralid application from the corresponding amount of herbicide recovered 24 hours after treatment (Table 5). Using these estimates of exudation, the data presented in Table 5 were modified. Consequently, it is estimated that 18 and 27% of the radioactivity moving basipetally was localized in the roots, while 30 and 40% was localized in the tissue below the treated leaf 144 hours after treatment with picloram and clopyralid, respectively.

Alteration of the carboxylic acid moiety of clopyralid by replacement with a less polar carboxylic acid amide group does not affect absorption but has a marked effect on translocation (Table 6). Crisp showed that chlorophenoxyacetic acid was readily loaded into the phloem. However, phloem loading of chlorophenoxy acetamide was minimal. The phloem loading that did occur with this amide compound was not dependent upon metabolic processes since loading was not stimulated by ATP or inhibited by KCN,

thereby indicating passive uptake. On the basis of these results, Crisp (31) hypothesized that the carboxyl group or any substituent group that can be converted by the plant into a carboxyl group is a prerequisite for phloem loading and entry of xenobiotic into the sieve tube-companion cell complex.

The portion of the recovered carboxylic acid amide of clopyralid that was exported out of the treated leaf (Table 6) probably represents a small amount of the applied compound that was not converted to amide before application to the plant or was somehow converted back to the carboxylic acid herbicide after application to the plant.

4.2 Quantification of the Water-Soluble Metabolite(s) of Picloram and Clopyralid

4.2.1 Results

The extent of metabolism of ^{14}C-picloram and ^{14}C-clopyralid in rapeseed, sunflower, and Canada thistle plants was investigated as a possible explanation for the observed differences in selectivity within and between plant species to the herbicides.

In a preliminary experiment the three plant species were treated with ^{14}C-picloram and ^{14}C-clopyralid and harvested 24, 72, and 144 hours later. Plants were extracted as described in Section 3.4 to obtain an aqueous extract that contained more than 90% of the total radioactivity

recovered in the plants. When this aqueous extract was adjusted to pH 1.6 and repeatedly partitioned with dichloromethane, a portion of the radioactivity from ^{14}C-picloram and ^{14}C-clopyralid treatments remained in the aqueous fraction of all three plant species. The pKa values of picloram and clopyralid are 3.6 and 2.3, respectively. Therefore, it was hypothesized that the herbicides were altered by the plants since the parent acid herbicides should have partitioned completely into dichloromethane at pH 1.6. Furthermore, an unknown radioactive compound was detected by thin layer chromatography (TLC) of the aqueous extract. All of the radioactivity in the dichloromethane fraction was found to be picloram or clopyralid as determined by TLC. There appeared to be no formation of any dichloromethane-soluble (non-polar) herbicide metabolites.

The solvent-partitioning and TLC methods were used to determine the amount of ^{14}C-herbicide that was metabolized. Both methods yielded the same results. The solvent-partitioning procedure provided the easiest and most reproducible of the two methods used to quantify the portion of applied ^{14}C-picloram and ^{14}C-clopyralid that was metabolized 24, 72, and 144 hours after treatment of sunflower, rapeseed, and Canada thistle plants. However, to ensure that the solvent-partitioning method was accurate, it was necessary to determine whether all the unmetabolized picloram and clopyralid could be extracted from the aqueous phase with dichloromethane and, if so, at what pH the

extraction was most efficient.

Buffered solutions, ranging in pH from 1.6 to 5.0, were prepared. Each solution was fortified with ''C-picloram and ''C-clopyralid and partitioned three times with equal volumes of dichloromethane. It was found that the optimal pH for extraction of more than 95% of ''C-picloram and ''C-clopyralid ranged from pH 1.6 to 2.0 (Figure 2). Aqueous extracts were obtained from untreated rapeseed, sunflower, and Canada thistle plants. The aqueous extracts were fortified with radioactive picloram or clopyralid, the pH was adjusted to 1.6, and they were extracted with dichloromethane. More than 95% of the applied radioactivity was recovered.

In a another experiment, plants of the three species were treated with ''C-picloram or ''C-clopyralid, immediately harvested, and subjected to the metabolite extraction procedure to determine if the water-soluble metabolite was an artifact of the extraction procedure. More than 97% of the radioactivity applied to the plants was recovered in the dichloromethane fractions and was found to be unaltered ''C-picloram or ''C-clopyralid, as determined by TLC.

Tables 7 and 8 show the amounts of ''C-picloram and ''C-clopyralid that were converted to water-soluble metabolite(s) 24, 72, and 144 hours after treatment. Recovery of herbicide in the aqueous extract after solvent extraction, hydrolysis, and further solvent extraction was

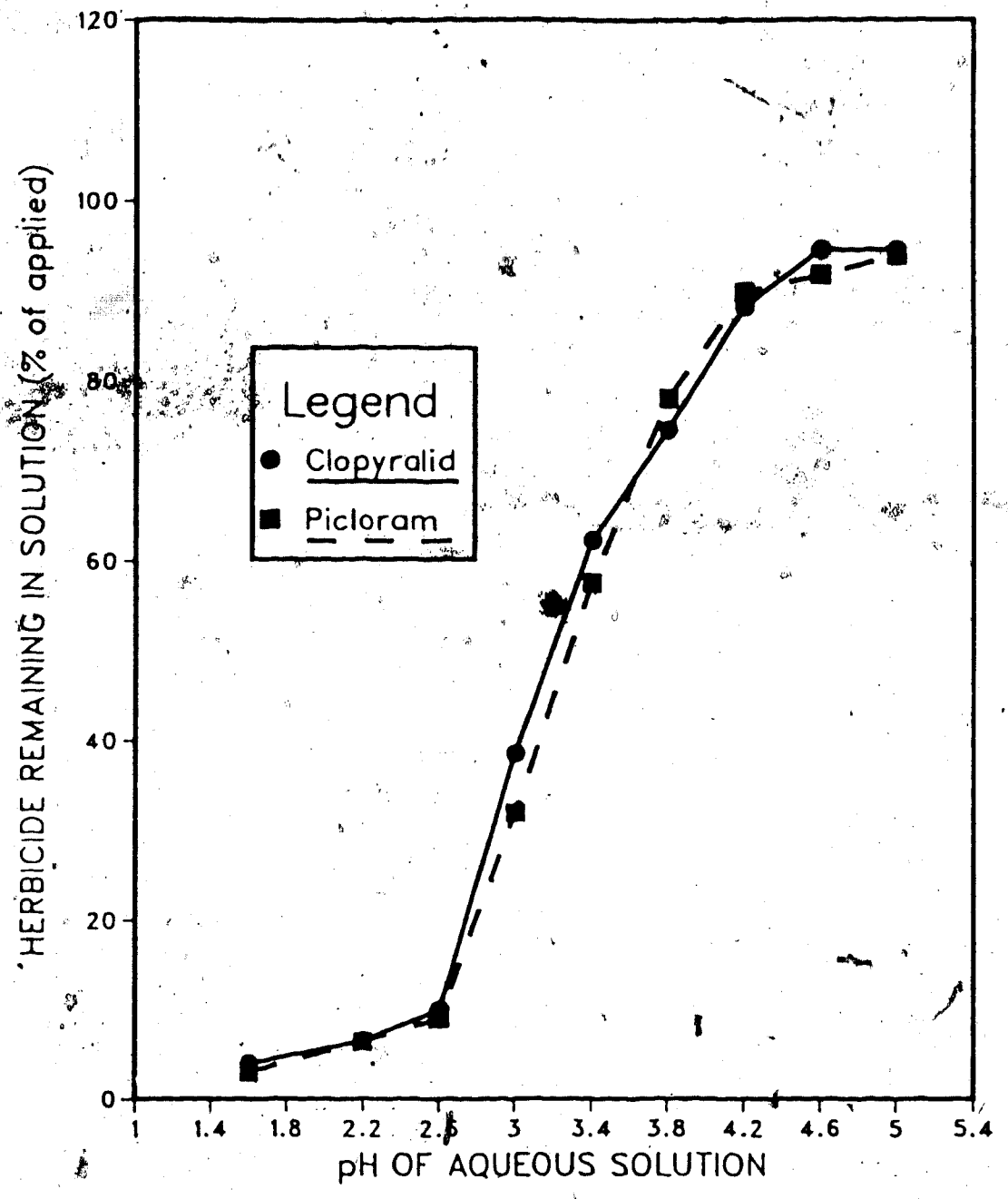


Figure 2. Effect of pH on the amount of ^{14}C-picloram and ^{14}C-clopyralid remaining in aqueous solution after three partitions with dichloromethane. Standard errors of the means were less than 3.5%.

Table 7. Fractions of ^{14}C-picloram and ^{14}C-clopyralid converted to water-soluble metabolite(s) 24, 72, and 144 hours after application of a herbicide treatment to the leaves of rapessed, sunflower, and Canada thistle plants

Time (h)	Description[1]	Rapeseed		Sunflower		Canada thistle	
		^{14}C-picloram	^{14}C-clopyralid	^{14}C-picloram	^{14}C-clopyralid	^{14}C-picloram	^{14}C-clopyralid
		(% of recovered)					
24	Metabolized	27.3 (1.8)	37.6 (1.9)	15.0 (2.0)	6.0 (0.4)	13.3 (4.5)	6.4 (2.8)
24	Recovered	90.4 (4.1)	88.8 (9.2)	104.4 (6.0)	94.3 (4.3)	96.3 (3.1)	92.0 (9.0)
72	Metabolized	44.0 (0.5)	61.2 (2.0)	25.5 (1.8)	8.0 (0.9)	21.4 (1.8)	17.0 (3.8)
72	Recovered	101.5 (1.3)	101.4 (1.3)	103.0 (1.8)	102.2 (1.2)	89.3 (3.1)	92.0 (0.9)
144	Metabolized	64.4 (4.2)	70.1 (1.6)	54.3 (7.8)	21.9 (0.9)	24.2 (4.2)	16.1 (0.9)
144	Recovered	91.8 (2.5)	89.1 (4.7)	90.6 (1.7)	92.5 (1.8)	95.2 (2.4)	93.2 (5.7)

[1]Standard errors of the means are in parentheses

[2]Metabolized: represents the percentage of the total herbicide dose recovered in the aqueous extract that was converted to the metabolite.

[3] Recovered: represents the percentage of the total herbicide dose found in the aqueous extract that was recovered after solvent partitioning of the non-metabolized herbicide and the hydrolyzed herbicide metabolite from the aqueous phase.

Table 8. Comparison, within a plant species and time, of the fractions of ^{14}C-picloram and ^{14}C-clopyralid converted to water-soluble metabolite(s).

Time (h)	Plant	Water-soluble metabolite ^{14}C-picloram	Water-soluble metabolite ^{14}C-clopyralid	LSD[1] (0.05)	LSD[1] (0.01)
		(% of recovered)			
24	Rapeseed	27.4	37.6	8.4	NS
72		44.1	61.3	7.7	14.1
144		64.4	70.1	NS	NS
24	Sunflower	14.9	6.0	3.9	7.2
72		25.5	8.0	3.8	7.0
144		54.3	21.9	9.4	NS
24	C. thistle	13.3	6.4	NS	NS
72		21.4	17.0	NS	NS
144		24.2	16.1	NS	NS

[1]NS = not significant as determined by an analysis of variance.

greater than 89% of the applied dose, regardless of the herbicide treatment or time of harvest (Table 7).

The extent of ^{14}C-picloram and ^{14}C-clopyralid metabolism was compared at each time interval within a plant species (Table 8). In rapeseed plants there was more clopyralid than picloram metabolized 24 and 72 hours after treatment. However, 144 hours after treatment there was no difference between the amount of picloram and clopyralid that was metabolized. A factorial analysis of the rapeseed data in Table 8 showed that the time x herbicide interaction was not significant ($P \leq 0.05$). The main effects of herbicide

treatment and harvest time were significant. Therefore, regardless of the time of harvest, more clopyralid was metabolized than picloram. Furthermore, there was more metabolism of the herbicides at each subsequent harvest time, regardless of the herbicide treatment (32, 53, and 67%; $LSD_{.05}$=6.7).

In sunflower plants, there was more metabolism of ^{14}C-picloram than ^{14}C-clopyralid at each time interval. A factorial analysis showed that the time x herbicide interaction and the two main effects were significant ($P \leq 0.05$). Therefore, there was an increase in metabolism as time progressed, regardless of the herbicide treatment. Furthermore, more picloram than clopyralid was metabolized, regardless of the time of harvest. However, the significant two-factor interaction indicates that as time progressed there was significantly more metabolism of picloram per unit time than of clopyralid (Figure 3).

In Canada thistle there was no difference between the metabolism of ^{14}C-picloram and ^{14}C-clopyralid at each harvest time (Table 8). Furthermore, a two-factor analysis of variance indicated that the time x herbicide interaction and the main effects of time and herbicide treatment were not significant ($P \leq 0.05$).

During the previous experiment to quantify the amount of herbicide that was metabolized, a balance sheet was maintained to determine how much radioactivity was found in the aqueous extract, water-insoluble residue, and the

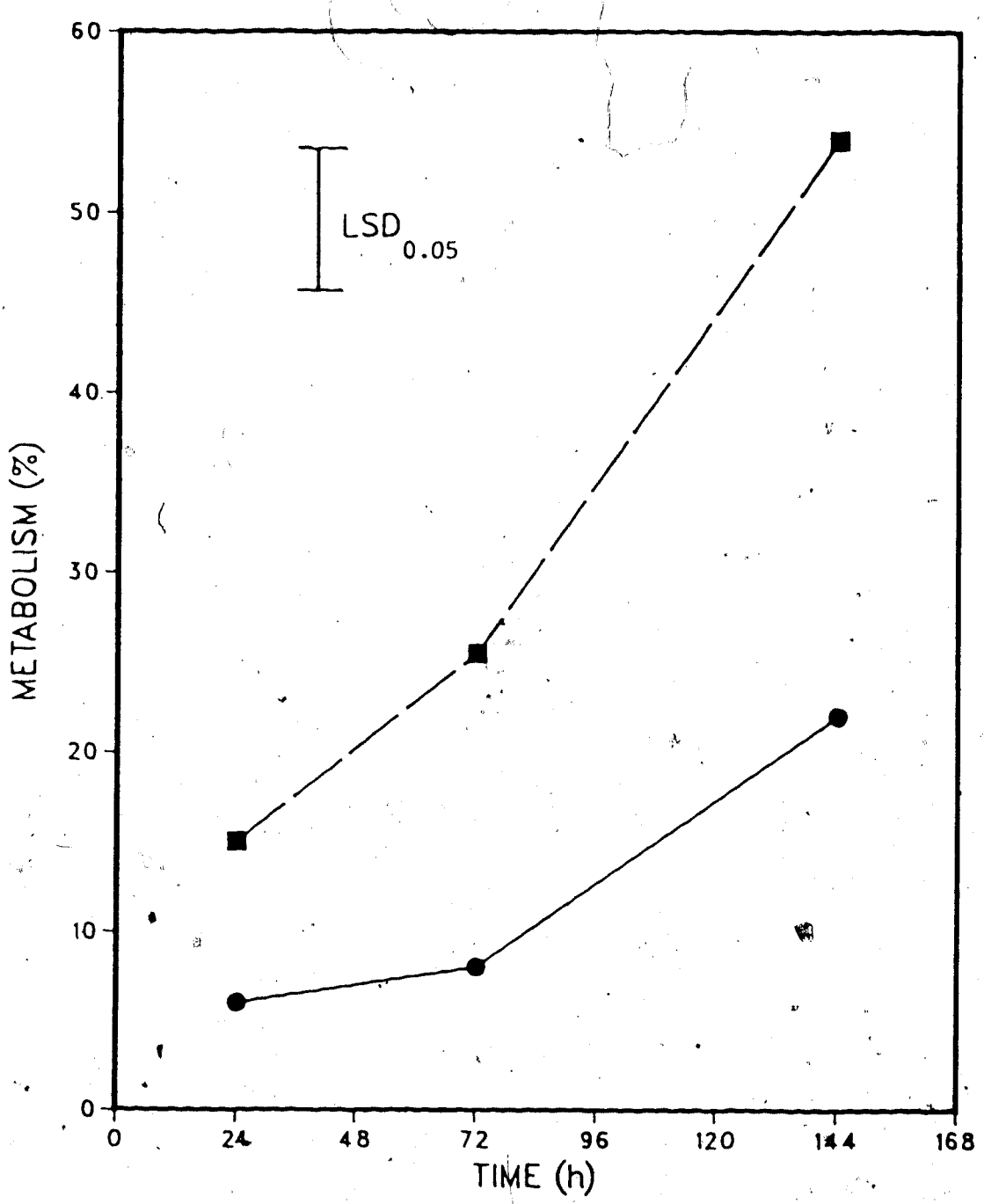


Figure 3. Metabolism of ^{14}C-picloram (■) and ^{14}C-clopyralid (●) in sunflower plants 24, 72, and 144 hours after treatment.

filtered residue. It was concluded that there was no difference in the distribution of radioactivity in the water-insoluble residue, the filtered residue, and the aqueous extract when the data were compared at each harvest time. Therefore, the data were averaged over time for each herbicide and the results are presented in Table 9. The amount of total applied radioactivity accounted for in the balance sheet exceeded 94% regardless of the time of harvest or the herbicide treatment. Approximately 1 and 5% of the radioactivity was recovered in the water-insoluble and the filtered residues of rapeseed and sunflower plants, respectively. More than 5% of the recovered radioactivity remained in the filtered residue of Canada thistle plants.

4.2.2 Discussion

The results indicate that the solvent-partitioning procedure was an effective method to completely separate the radioactive parent herbicides from the corresponding metabolites and to subsequently quantify both compounds. Furthermore, the water-soluble herbicide metabolites were not artifacts of the extraction procedure.

Most of the radioactivity from the ^{14}C-herbicide treatments was present in the aqueous extracts of the three plant species. Little radioactivity remained in the filtered residue and the water-insoluble residue isolated from rapeseed, sunflower, and Canada thistle plants. Because there was a larger volume of filtered residue in Canada

Table 9. Amount of radioactivity in the filtered residue, water-insoluble residue, and aqueous extract from plants used in herbicide metabolism experiments. Results are expressed as a percentage of total radioactivity recovered in rapeseed, sunflower, and Canada thistle plants, regardless of when the plants were harvested after application of ^{14}C-picloram or ^{14}C-clopyralid[1].

Herbicide	Plant species	Water-insoluble residue	Filtered residue	Aqueous extract
		----------(% of recovered)---------		
Picloram	Rapeseed	1.0 (0.3)	5.4 (1.2)	93.6 (4.0)
	Sunflower	0.9 (0.2)	3.7 (0.7)	95.4 (3.6)
	C. thistle	3.6 (1.5)	7.2 (2.4)	89.2 (4.5)
Clopyralid	Rapeseed	0.8 (0.3)	4.3 (1.1)	94.9 (7.6)
	Sunflower	1.3 (0.2)	3.5 (0.4)	95.2 (2.7)
	C. thistle	1.7 (1.2)	9.2 (5.4)	89.1 (7.8)

[1]Standard errors of means are in parentheses.

thistle than in the other two species, it was more difficult to thoroughly wash the filtered residue. This may explain why slightly more radioactivity was found in the filtered residue of Canada thistle plants than in the filtered residue of rapeseed and sunflower plants.

The water-insoluble residue (mainly chlorophyll) represents the material not soluble in the aqueous extract once the acetone was removed from the acetone:water solution in which the plants were macerated (Section 3.4). The

radioactivity associated with the water-insoluble residue may represent the herbicide that is soluble in the hydrophobic chlorophyll residue. Radioactivity associated with the filtered residue represents the herbicide associated with proteins and structural carbohydrates. Other researchers have used methods similar to the one described in Section 3.4 to extract ^{14}C-picloram and the metabolite of this herbicide from different plant species (19, 55, 56, 57). These authors reported that most of the radioactivity from the ^{14}C-picloram treatments was found in the aqueous plant extract. For example, Chaleff (19) recovered 90% of the radioactivity in the aqueous extracts taken from tobacco plants previously treated with ^{14}C-picloram. Approximately 10% of the radioactivity was associated with the water-insoluble residue that was trapped by filtration. Hallmen (55, 56) found that less than 5% of the recovered ^{14}C precipitated when trichloroacetic acid (8% w/v) was added to the aqueous extact taken from rapeseed and sunflower plants previously treated with ^{14}C-picloram. Furthermore, Chaleff (19) and Hallmen (55, 56) showed that partitioning the aqueous phase (pH 1.6) with dichloromethane was an effective procedure for completely separating and accurately quantifying ^{14}C-picloram and its water-soluble metabolite(s).

Other researchers (19, 55, 56, 57) have reported that picloram is metabolized in both resistant and susceptible species. Hallmen (56) found that 27, 46, and 48% of the

recovered ^{14}C-picloram was converted to water-soluble conjugates 1, 3, and 9 days after treatment of sunflower plants, respectively. In rapeseed plants, 39, 61, and 78%, respectively, of the picloram was converted to water-soluble metabolites after the same periods of time. Hallmen ([redacted]) hypothesized that the difference in rate of picloram metabolism between sunflower and rapeseed plants accounts for the difference in sensitivity between the two species.

Examination of the data (Tables 6 and 7) may lead one to conclude that the sensitivity of a plant to picloram or clopyralid is related to metabolism of the herbicides. However, the importance of picloram and clopyralid metabolism in conferring resistance within and between plant species can be questioned in light of the following facts. First, even though the herbicides have been converted to water-soluble metabolites, these metabolites may have herbicidal properties. Second, even if the water-soluble herbicide metabolites of picloram and clopyralid are not phytotoxic, the plants may be capable of converting the herbicide metabolites back to the parent herbicides. Therefore, the water-soluble herbicide metabolite could be sequestered within the plant and slowly converted to the herbicide to do damage to the plant at a later time. Third, the extent of metabolism 144 hours after treatment of sunflower and rapeseed plants with picloram was 54 and 64%, respectively. Nonetheless, sunflower plants are far more sensitive to picloram than rapeseed plants. Fourth, within

sunflower plants, significantly more picloram than clopyralid was metabolized 144 hours after treatment, and yet sunflower plants are no less sensitive to picloram than to clopyralid. Finally, the extent of picloram and clopyralid metabolism in rapeseed plants 144 hours after treatment was 64 and 70%, respectively (Table 7). Since not all of the picloram and clopyralid applied to rapeseed plants was converted to the apparently non-phytotoxic metabolite, one should be able to apply enough picloram and clopyralid to cause a phytotoxic effect in rapeseed plants. With this hypothesis in mind, rapeseed plants were treated with doses of picloram or clopyralid ranging from 0.1 to 5.0 kg/ha. A dose as low as 100 g/ha of picloram caused herbicidal symptoms in rapeseed plants 24 hours after treatment. However, clopyralid doses as high as 5 kg/ha had no herbicidal effect on the rapeseed plants. Taken together, these results indicate that differences in sensitivity within and between species cannot be attributed to differences in the extent of herbicide metabolism.

Chaleff (19) isolated picloram-tolerant *Nicotiana tabacum* mutants. He used a modified technique of Hallmen and Eliasson (57) to determine the amounts of picloram and of the water-soluble metabolite of picloram in resistant and susceptible seedlings of tobacco. Resistant seedlings incorporated more picloram than susceptible species. Therefore, resistance was not conferred by reducing the uptake of picloram. Furthermore, Chaleff (19) found no

difference in the amount of the water-soluble picloram metabolite formed in the resistant and susceptible tobacco plants. The ratio of the water-soluble metabolite of picloram to picloram exceeded 4:1 in both the resistant and susceptible seedlings. Mitchell et al. (87) found that the rate of metabolite formation and the quantity of picloram metabolized in tolerant white ash and resistant red maple were the same, 12 days after treatment.

Taken together, the results of these researchers and the results presented in this thesis imply that there may be a difference in how picloram or clopyralid interact with some target site within resistant and susceptible plant species. In fact, Chen et al. (23) and Chaleff (19) concluded that differential sensitivity at the site of picloram action rather than differential accumulation of the herbicide at the site of action or a differential rate of metabolism accounts for susceptibilty differences among different plant species to picloram.

4.3 Ethylene Synthesis and Herbicide Action

4.3.1 Results

The results in Sections 4.1 and 4.2 suggest that differences in the extent of absorption, translocation, and metabolism within rapeseed plants and between rapeseed plants and sunflower plants do not explain the differences in sensitivity within and among the plant species to

picloram and clopyralid. With this in mind, the generation of ethylene by rapeseed and sunflower plants, after application of picloram or clopyralid, was used as a tool to determine whether these herbicides interact differently with some target site in susceptible and resistant plant species.

Inasmuch as auxinic-type herbicides are known to increase ethylene production in susceptible plants, and in view of the fact that some of the symptoms induced by the two herbicides in susceptible species are typical of those induced by ethylene, it was of interest to determine the changes in rates of ethylene production following application of herbicide treatments to both plant species. Shortly after picloram application to the third leaf of a rapeseed plant, ethylene levels rose (Figure 4). Usually, morphological injury symptoms also became evident 5 to 10 hours after treatment. The same dose of clopyralid, on the other hand, did not elevate ethylene concentrations above basal values (Figure 4).

In another experiment, a rapeseed plant received a 960 µg/plant dose of clopyralid followed 23.5 hours later by a similar dose of picloram (Figure 5). Both treatments were applied to the third leaf. Clopyralid treatment had little effect on ethylene production. After application of picloram, however, ethylene production began to increase. This increased ethylene production occurred before any morphological changes became apparent. The rate of ethylene production was 4 to 5 times greater than the basal level, 24

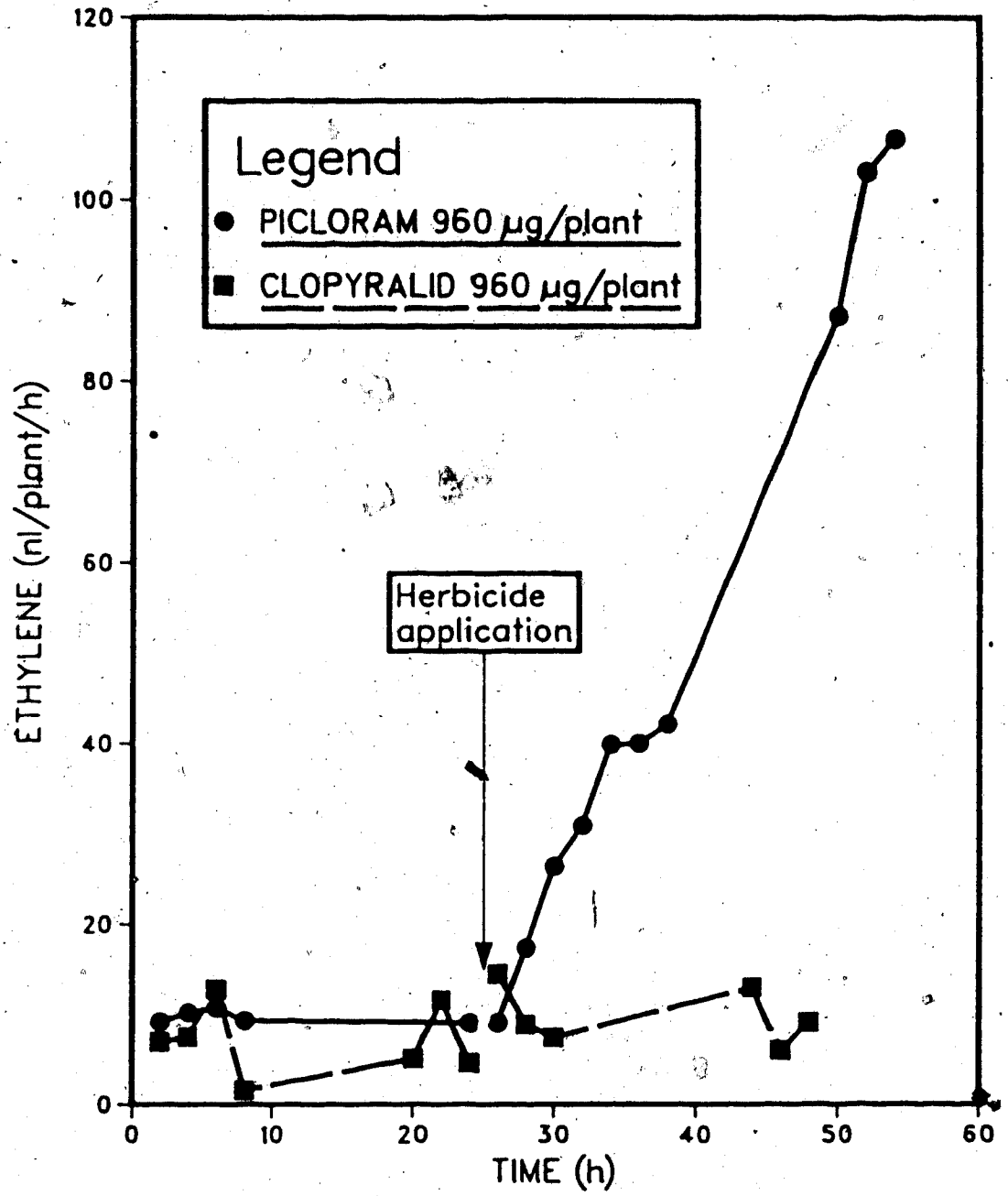


Figure 4. Effect of picloram or clopyralid treatment on ethylene production by rapeseed plants. The X-axis indicates the time lapse from insertion of the plant into the cuvette. Each plant (four-leaf stage) was allowed to equilibrate for several hours in the cuvette before measurements of ethylene production were initiated. After 24 hours, each plant received 960 µg of picloram or clopyralid, applied in 200 µl (twenty 10-µl drops) to the third leaf.

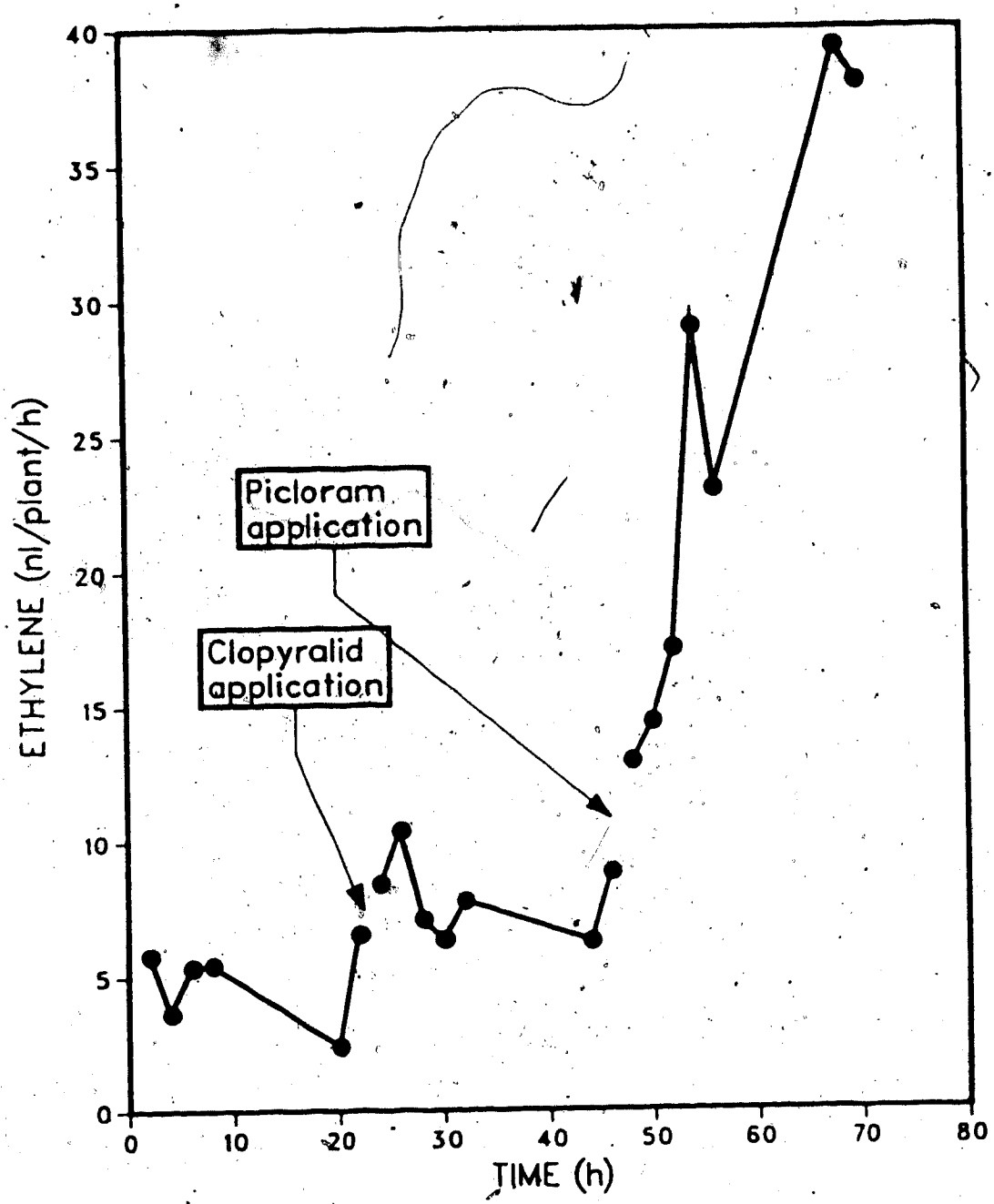


Figure 5. Effect of a sequential treatment of clopyralid followed by picloram on ethylene production by a rapeseed plant. Conditions as in Figure 4.

hours after picloram treatment. The apparent increase in the rate of ethylene production before picloram treatment (Figure 5) represents fluctuations in basal rates of ethylene production (for comparison see Figure 4).

Having determined that ethylene production increases in response to the herbicide to which the plant is susceptible, an experiment was done to assess whether this increase in ethylene production could be prevented with AVG, a known inhibitor of ethylene biosynthesis (128). The treatment of leaves and cotyledons of rapeseed with AVG prevented the generation of ethylene above basal levels by the plant after receiving a picloram dose of 250 μg (Figure 6). In contrast, a plant that did not receive the AVG pretreatment generated approximately six times more ethylene 20 hours after the picloram treatment (Figure 6).

In sunflower, a species susceptible to both herbicides, ethylene production increased in response to both chemicals. The data are presented only for clopyralid. Clopyralid (10 μg) increased ethylene production in sunflower several-fold (Figure 7). When AVG (31 μM) was applied before treatment with clopyralid, AVG prevented generation of ethylene above levels recorded before the herbicide treatment was applied (Figure 7). Even when a massive dose (960 μg/plant) of clopyralid was applied to sunflower plants that were pretreated with a 31 μM dose of AVG, there was no increase in ethylene generation above basal levels (Figure 8). In sunflower plants that did not receive an AVG pretreatment

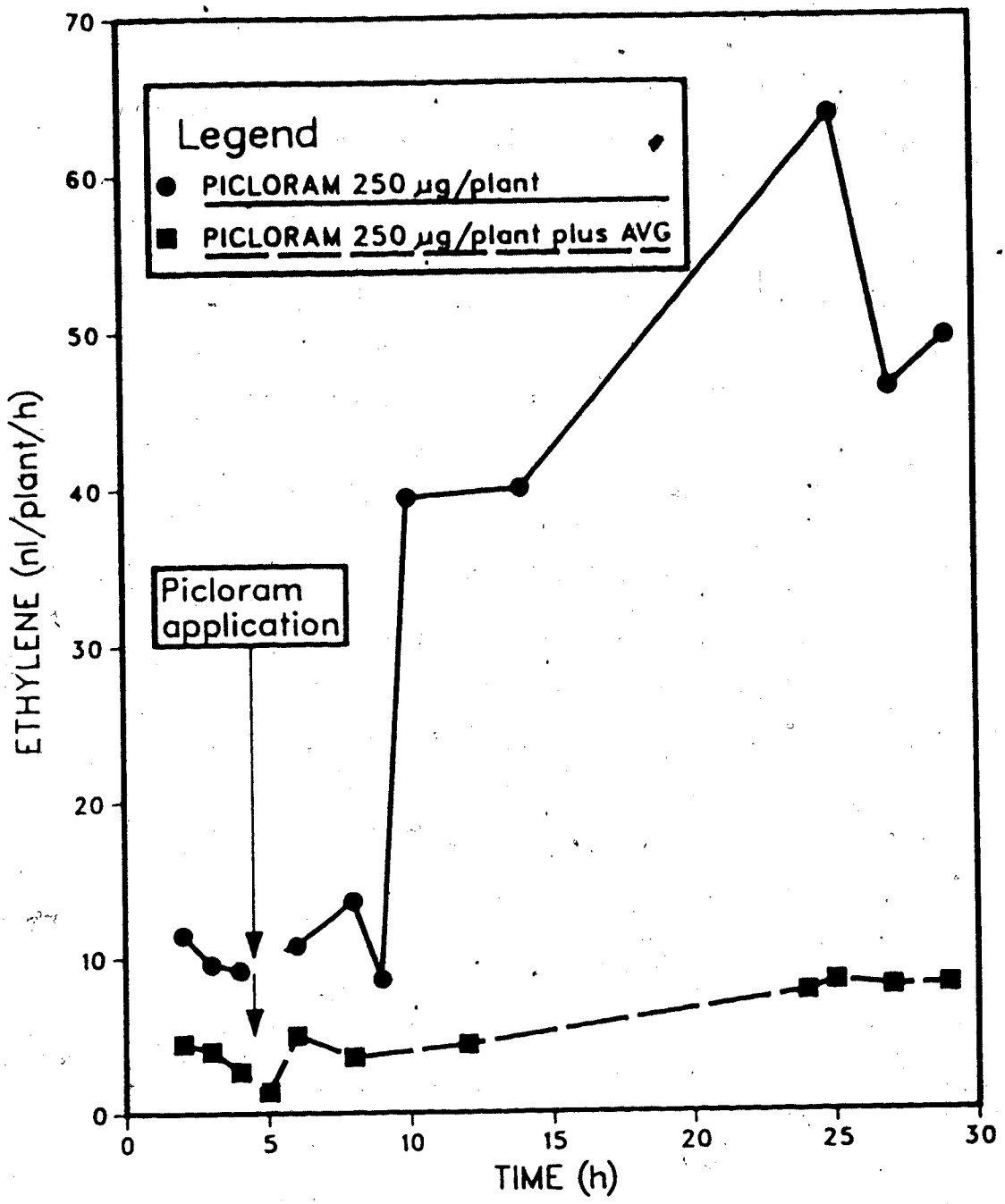


Figure 6. Effects of picloram or a pretreatment with AVG followed by picloram on ethylene production by rapeseed plants. AVG (125 µM) solution was sprayed on the entire plant 48 and 24 hours prior to insertion into the cuvette. Herbicide dose was delivered in 100 µl of solution (ten 10-µl drops).

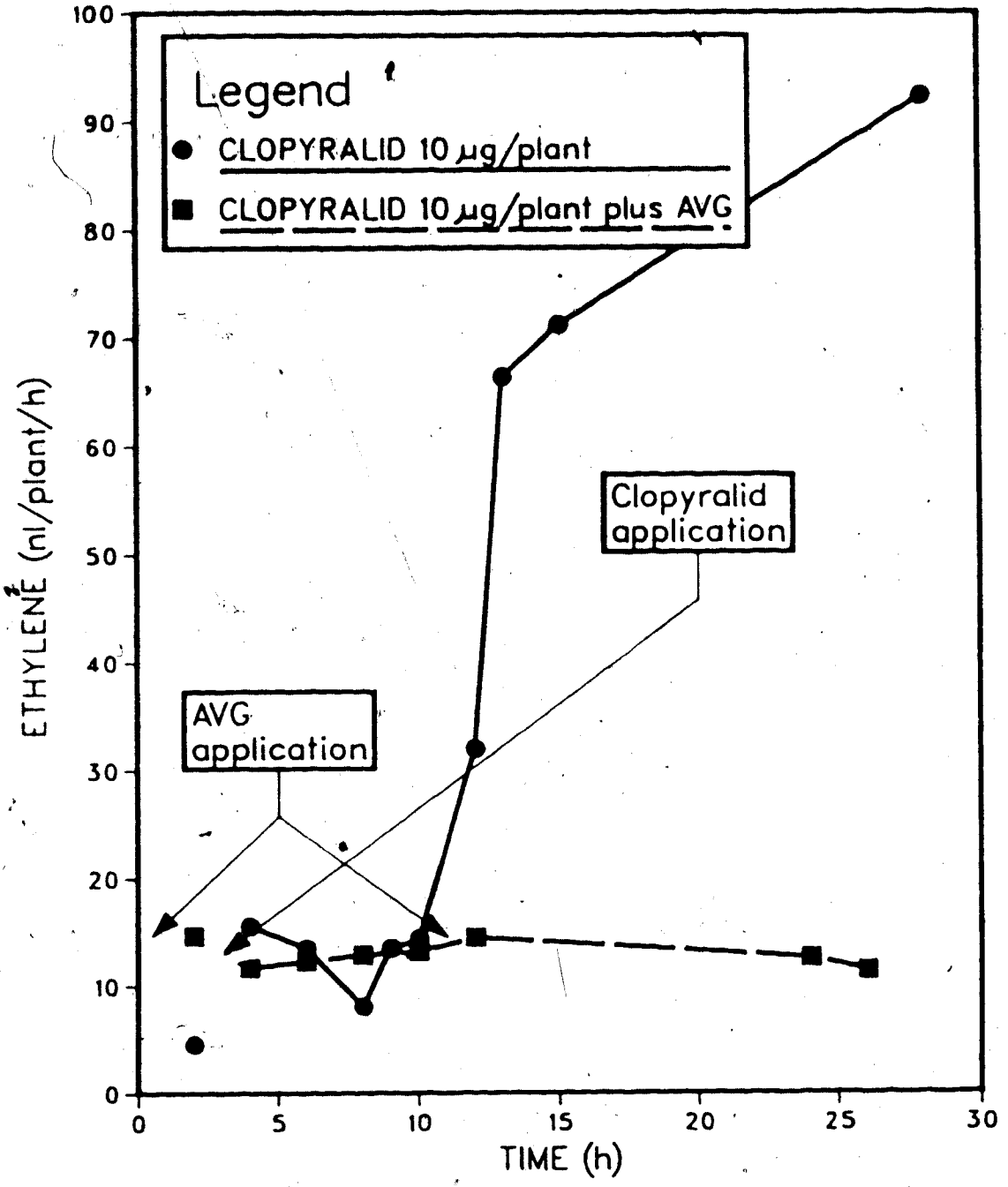


Figure 7. Effect of clopyralid treatment on ethylene production by sunflower plants. One leaf of the second leaf pair received 10 µg herbicide delivered in 100 µl of solution. AVG (31 µM) was applied after insertion of the plant into the cuvette.

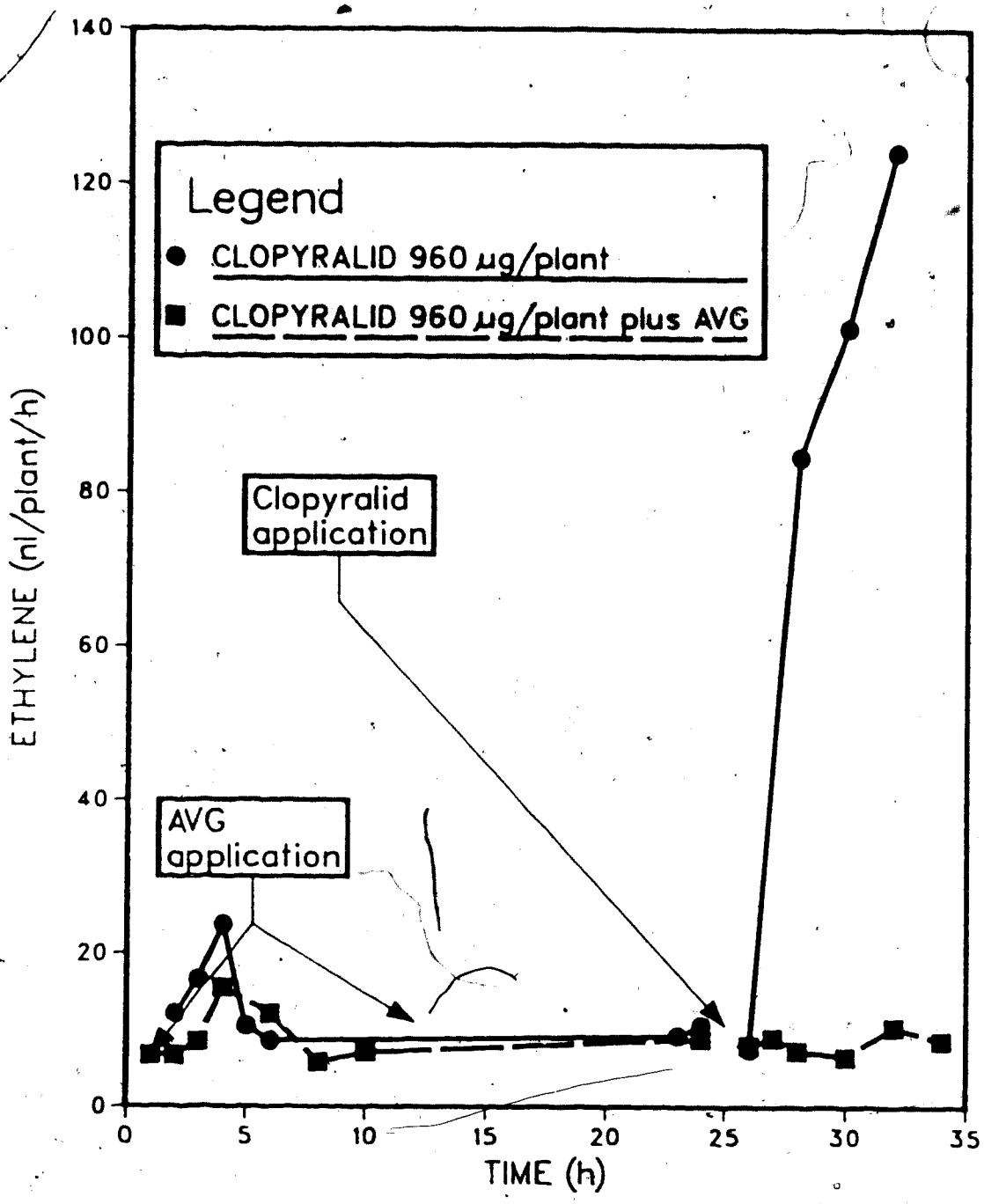


Figure 8. Effect of clopyralid treatment on ethylene production by sunflower plants. One leaf of the second leaf pair received 960 µg herbicide delivered in 200 µl of solution. AVG (31 µM) was applied to the plant after its insertion into the cuvette.

the ethylene levels generated rose more than 12 times above basal level 5 hours after herbicide treatments were applied to the plants.

It might be argued that an increase in ethylene production is a result of localized tissue wounding caused by application of the herbicide solution to a plant. Therefore, an experiment was designed to minimize any ethylene production that might be a result of tissue wounding. A 50 µg dose of clopyralid was applied to the stem and cotyledons of a sunflower plant (Figure 9). The treated zone of the plant was situated below the cuvette, which enclosed the true leaves of the plant. Five hours after treatment with clopyralid, ethylene concentration in the cuvette began to rise and was still increasing 20 hours after treatment.

As is evident from the data presented in Figures 4 to 9, there was considerable variation in the absolute rates of ethylene production between different plant species and within a species, in spite of selection for uniformity of plants in terms of age and size. Such variations in basal rates of ethylene production become apparent when measurements are made using a continuous flow system (9). In closed systems where ethylene can be quantified only by sealing the plant for several hours in a chamber before the samples are withdrawn, the net measurement of ethylene accumulation tends to obscure the variability in basal rates of ethylene produced. Even with this variation, the times at

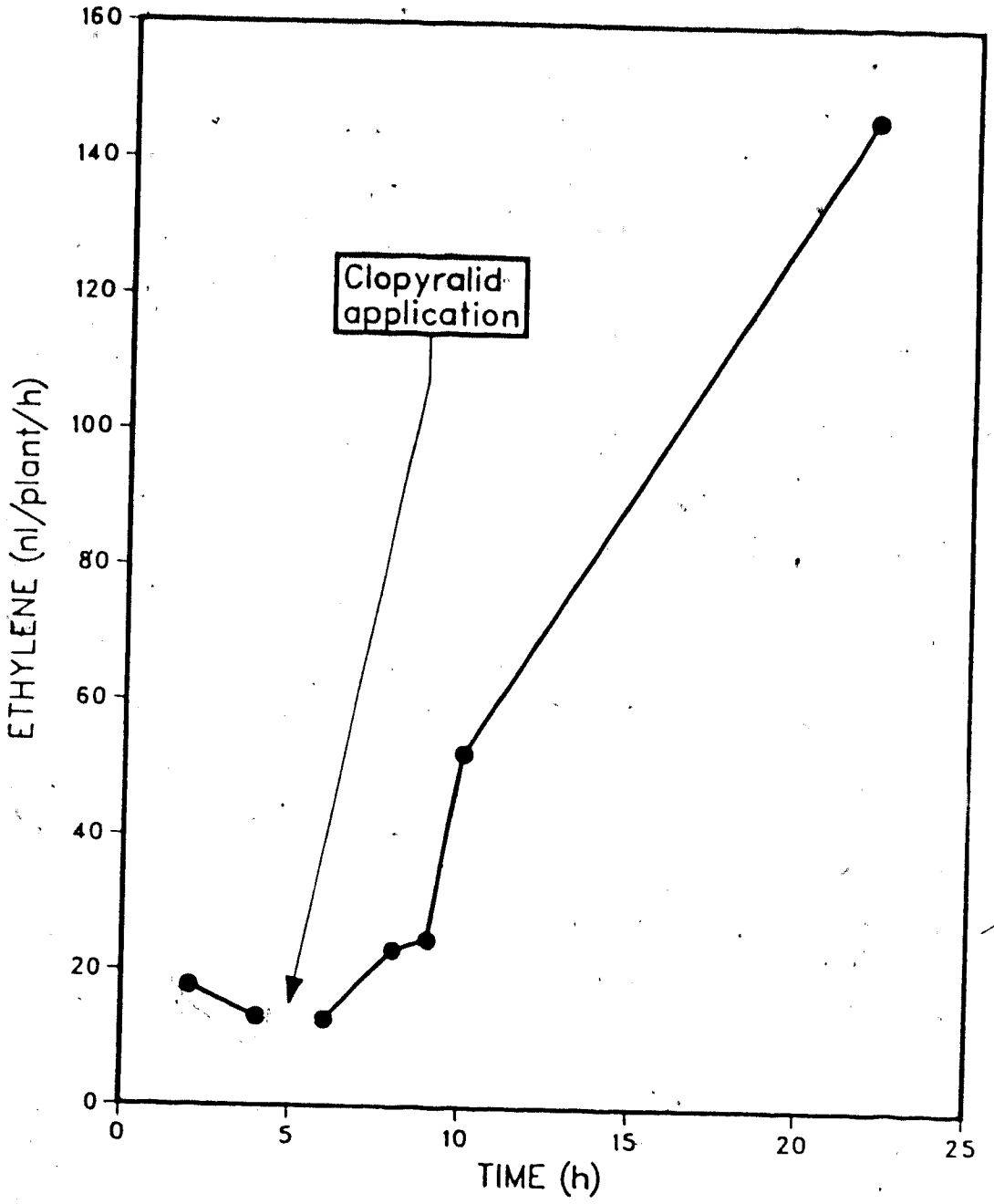
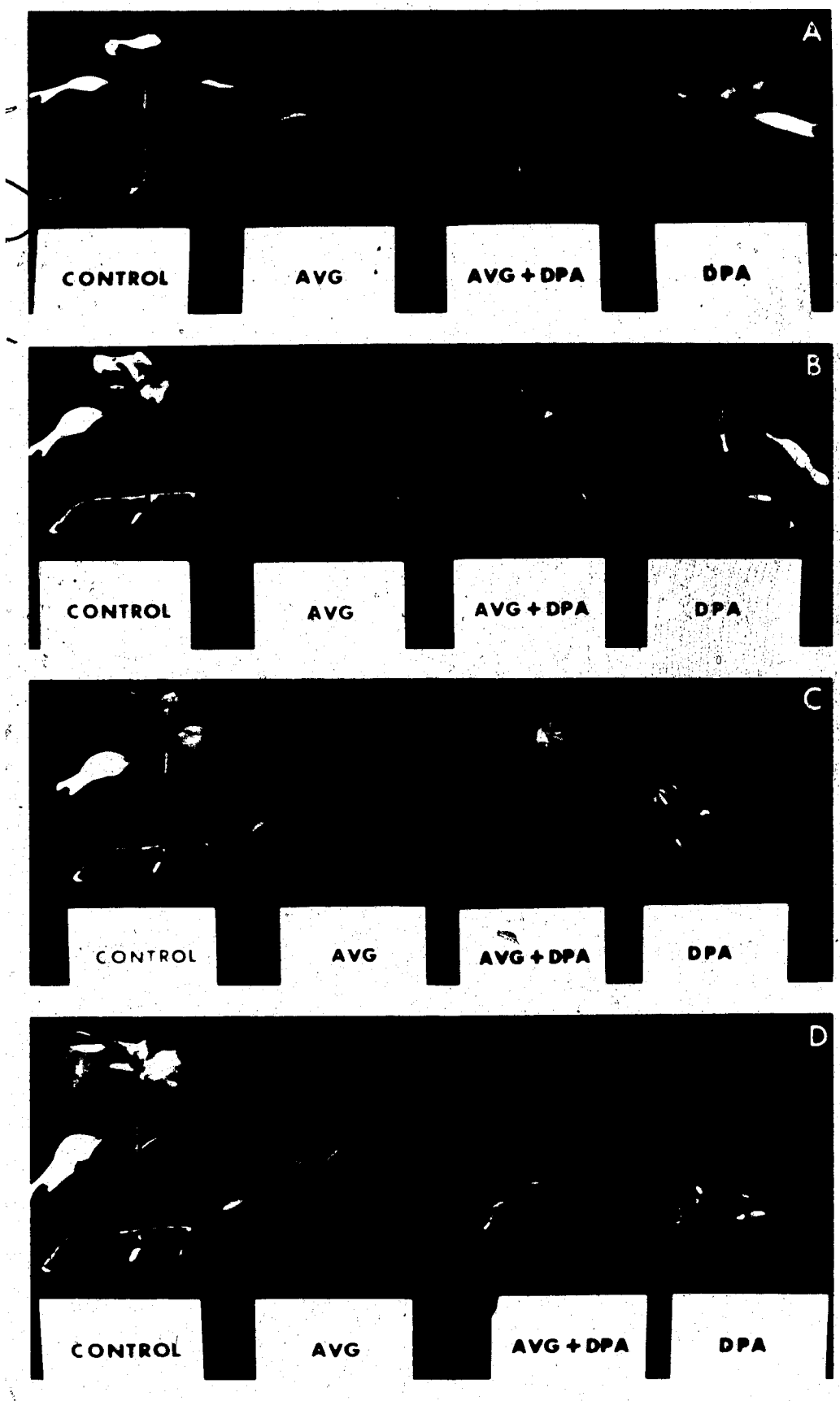


Figure 9. Effect of clopyralid on ethylene production when applied to sunflower cotyledons. Cotyledons were isolated outside the sealed cuvette. A herbicide dose of 50 µg was delivered in 100 µl of solution.

which changes in ethylene production occurred in response to a treatment were consistent for all replications of an experiment.

For detailed experiments on the changes in morphology induced by various treatments, sunflower was chosen as the representative plant because of its susceptibility to both herbicides. Leaves of sunflower plants became epinastic 4 hours after a 50 μg clopyralid dose was applied to the cotyledons (Figure 10). The symptoms were virtually identical to the ones observed when plants were fumigated with ethylene. Symptoms progressed rapidly and stem curvature was apparent 8 hours after clopyralid treatment. Treatment of plants with AVG, before the application of herbicide, delayed the development of symptoms. The plant treated with AVG plus clopyralid displayed hyponasty of cotyledons and leaves, as well as leaf rolling, 8 hours after treatment, although some hyponasty was evident after 4 hours. Stem bending did not occur until approximately 16 hours after the treatment with AVG plus clopyralid treatment. Application of AVG alone did not appear to affect the morphological status of a sunflower plant, although 24 hours after treatment with AVG, the leaves emerging from the apex were somewhat chlorotic with rolling evident at leaf margins. Symptoms were observed for 5 days after herbicide treatment; there was little change in morphological characteristics of the plant after 24 h. The morphology of sunflower plants treated with clopyralid was affected

Fig. 10. Morphological changes in sunflower treated with AVG, clopyralid (DPA), or AVG + clopyralid (AVG + DPA). Pictures represent symptoms 0, 4, 12, and 24 hours after clopyralid was applied (A,B,C,D). AVG (125 μM) was applied to leaves until run-off, 24 and 12 hours before and 0, 4, 12, and 24 hours after cotyledons were treated with clopyralid (50 μg/100 μl, ten 10-μl drops).


A
CONTROL
AVG
AVG + DPA
DPA
B
CONTROL
AVG
AVG + DPA
DPA
C
CONTROL
AVG
AVG + DPA
DPA
D
CONTROL
AVG
AVG + DPA
DPA

regardless of whether an AVG pretreatment was applied. Leaves of plants treated with AVG plus clopyralid remained hyponastic, while crown tissue showed little hypertrophy. Leaves of clopyralid-treated plants were epinastic, while crown tissue showed marked hypertrophy.

4.3.2 Discussion

Data presented in Sections 4.1 and 4.2 show that the difference in rate of ethylene production in response to either herbicide treatment cannot be attributed to differences in absorption, translocation, or metabolism of the herbicides in rapeseed plants. These results, along with the fact that the two herbicides have similar but slightly different chemical structures, suggest that the compounds may interact differently with some target site within resistant and susceptible species. In the case of susceptible species, the target site is activated and sets in motion the biosynthesis of ethylene. In resistant species, the herbicide may reach the target site but has no subsequent effect.

It could be argued that the commercial formulations used in the ethylene experiments were designed to optimize selectivity differences within rapeseed plants while the formulation used in the absorption and translocation studies was designed to favour penetration. Therefore, it was important to determine if rapeseed plants would respond similarly, in terms of the amount of ethylene generated,

when the commercial formulations were compared with the formulation used in the absorption and translocation of the radiolabeled herbicides used in earlier experiments (see Section 3.1). Consequently, in a control experiment, a dose of 300 μg/plant of clopyralid or picloram, spiked with the corresponding radiolabeled herbicide, was dissolved in a solution containing 10% ethanol plus 0.5% Tween 20 and applied to rapeseed plants. Ethylene production levels rose at least 22-fold 24 hours after picloram application, whereas there was no increase in ethylene evolution above basal levels in rapeseed plants treated with clopyralid. The total recovered radioactivity that was translocated acropetally out of the treated leaf 24 hours after application of ^{14}C-clopyralid and ^{14}C-picloram was 32.9 ± 3.1 and 19.7 ± 4.3, respectively, and are similar to the results shown in Tables 2 and 3 where the commercial formulation was not used. These findings indicate that the selectivity differences within rapeseed plants to the commercially formulated mixtures containing clopyralid and picloram, were not a result of differences in the amount of absorption and subsequent translocation of the two herbicides.

In another experiment an attempt was made to inhibit the generation of ethylene by rapeseed plants in response to picloram treatments by pretreating the plants with doses of clopyralid. The results indicate that there was no clear indication of competitive inhibition of the herbicidal

action of picloram on rapeseed plants by clopyralid. Furthermore, ethylene production was not maintained at basal levels when picloram was applied after clopyralid as shown in Figure 5.

This is the first experimental work in which herbicide-induced ethylene production has been shown to occur in intact plants using a continuous-flow system. This approach eliminates the potential pitfalls of previously published reports of experiments, using closed systems, where it is often very difficult to interpret the data because of other interfering factors such as changes in the gaseous environment around the plant. Moreover, closed systems are not suitable for following the time course of changes in the rate of ethylene production. This makes it difficult to distinguish whether changes in the rate of ethylene production occur before or after the development of symptoms in response to herbicide treatment. The data presented in this paper indicate that the application of picloram and clopyralid to susceptible species induces the increased biosynthesis of ethylene. This increase in ethylene production preceded or coincided with the onset of morphological changes induced by application of a herbicide to the susceptible species. However, when clopyralid treatments were applied to a resistant species, rapeseed, ethylene production did not increase above basal levels.

Wounding has been shown to increase ethylene production (1). Visible wounding of the treated leaf was apparent only

in some cases when a 960 μg dose of clopyralid per plant was applied to rapeseed or sunflower. This localized wounding, when observed, occurred several hours after the increase in rate of ethylene production started. Moreover, increased ethylene production was observed with the herbicide treatment regardless of whether wounding was visible or not. In order to further reduce the effects of possible wounding on ethylene production, sunflower cotyledons were treated with clopyralid and isolated below the sealed cuvette. (Hallmen (55) has shown that ^{14}C-picloram, applied to the cotyledons of sunflower, will move acropetally 24 hours after treatment.) The treatment of isolated cotyledons with clopyralid led to an increase in ethylene production from the portion of the plant enclosed in the cuvette approximately 4 hours after treatment. Thus, ethylene production cannot be attributed to localized wounding of the treated tissue.

Abeles (2) found that 2,4-D stimulated ethylene production in corn and soybean. Ethylene had an inhibitory effect on growth of the two plant species. However, CO_2, a competitive inhibitor of ethylene action, could not be demonstrated to reverse the supposed ethylene effect. Several other researchers indicated that the manifestation of auxinic-type herbicide action is independent from, or in addition to the action of these compounds in inducing ethylene production (5, 6, 88). The results indicate that picloram- and clopyralid-induced ethylene production could

well be responsible for some of the morphological symptoms displayed by susceptible plants.

This conclusion is supported by the work with AVG (Figures 6, 7, 8, and 10). Furthermore, Yu and Yang (128) suggested that AVG inhibits ethylene biosynthesis by inhibiting the conversion of methionine to 1-aminocyclopropane-1-carboxylic acid (ACC). AVG has been used with non-auxinic type herbicides to determine whether herbicide symptoms can be attributed to increased ethylene production brought about by the herbicide treatment (97). In the present experiments, treatment of plants with AVG prevented the generation of ethylene induced by massive doses of picloram or clopyralid applied to rapeseed and sunflower plants. AVG delayed the development of symptoms after herbicide treatment. When the symptoms did develop, they were quite distinct from those induced by the herbicide alone. AVG acted to delay stem curvature and prevented epinasty. Hypertrophy was less severe when AVG was applied as a pretreatment before sunflower was treated with clopyralid. However, leaves were hyponastic 4 hours after herbicide treatment and remained in this position for at least 5 more days. Conversely, when only the herbicide was applied, epinasty and stem curvature were apparent 4 hours after treatment. Furthermore, the morphological manifestations of epinasty and stem curvature, brought about by herbicide treatment, appeared soon after or simultaneously with the rise of ethylene levels above basal

levels in plants sealed in the cuvette. Morphological responses to ethylene fumigation or clopyralid treatment were similar. These results, taken together, suggest that enhanced ethylene biosynthesis in response to herbicide application is a factor involved in the resulting morphological changes.

4.4 Identification of the Water-Soluble Metabolite(s) of Picloram and Clopyralid

The following experiments were conducted to determine how many water-soluble herbicide metabolites were produced within a plant species, whether the metabolites were the same in the different species, and to determine the structure of the metabolite(s).

4.4.1 Preliminary Identification by TLC of the Metabolite(s) Produced in the Three Plant Species

Aqueous extracts from rapeseed, sunflower, and Canada thistle plants treated with ^{14}C-picloram or ^{14}C-clopyralid and harvested 144 hours later were subjected to TLC on silica gel plates using the solvent system n-butanol:NH_4OH:H_2O (8:1:1 v/v/v). One metabolite was detected for each of the herbicides; it was the same in all three plant species. The picloram and clopyralid metabolites had Rf values of 0.62 and 0.65, respectively. There was some trace of a second metabolite of each herbicide, with an Rf of 0-0.05.

Nicotinic acid which is found in plants can be converted to nicotinamide, a known precursor of NAD and NADPH. Therefore, it was hypothesized that the herbicide metabolite may be produced by the same mechanism used by the plants to to convert nicotinic acid to nicotinamide; the metabolite produced being the carboxylic acid amide of picloram or clopyralid.

Nicotinic acid, nicotinamide, and the aqueous extracts from rapeseed, sunflower, and Canada thistle plants previously treated with ^{14}C-picloram or ^{14}C-clopyralid were chromatographed (n-butanol:NH_4OH:H_2O (8:1:1)) on a silica gel TLC plate. The Rf value of nicotinic acid (0.20) was found to be similar to that of picloram and clopyralid. The Rf of nicotinamide (0.52) was similar to that of the herbicide metabolite produced by all three plant species.

On the basis of these results the carboxylic acid amides of picloram and clopyralid were synthesized (section 3.8.1). The synthetic amides of the herbicides had Rf values identical to the unknown metabolites of clopyralid and picloram. The Rf values are shown in Table 10.

A superficial examination of the data (Table 10) may lead one to conclude that the herbicide metabolite was an amide derivative. However, in light of the following information this conclusion was questioned. First of all, the Rf values for the metabolites of picloram and clopyralid indicate that these two metabolites are less polar than either of the corresponding herbicides. Aqueous solutions

Table 10. Rf values of various compounds chromatographed on silica gel TLC plates and developed in the solvent system n-butanol:NH_4OH:H_2O (8:1:1).

Compound	Rf value[1]
Nicotinic acid	0.20
Picloram	0.19
Clopyralid	0.25
Nicotinamide (Sigma Chemical Co.)	0.52
Nicotinamide (synthetic)[2]	0.52
Amide of picloram (synthetic)[2]	0.62
Amide of clopyralid (synthetic)[2]	0.65
Picloram plant metabolite[3]	0.62
Clopyralid plant metabolite[3]	0.65

[1]Slighty different Rf values were obtained each time the plant extracts and known compounds were chromatographed.
[2]Synthetic: this compound was synthesized in the lab as described in Section 3.8.1.
[3]Refers to plant extracts from rapeseed, sunflower, and Canada thistle plants.

were fortified with nicotinamide, one of the synthetic amides of either herbicide, or the plant metabolite eluted from TLC plates that were developed in n-butanol:NH_4OH:H_2O (8:1:1 v/v/v). The aqueous solutions were then partitioned against dichloromethane. All the compounds present in the aqueous phase moved into the organic phase. These results were contrary to the findings in Section 4.2 in which no metabolite in the aqueous phase would partition into dichloromethane. Second, there was streaking of the radioactive zones on silica gel TLC plates. The streaking began at the region where the carboxylic acid herbicide was

found and stretched into the region where the suspected amide metabolite was located. When this streaked radioactive zone was eluted from the TLC plate and re-chromatographed, two distinct spots were found that co-migrated with the amide of the herbicide and the carboxylic acid herbicide. Third, when less concentrated NH_4OH was used in the solvent system a second unknown metabolite with an Rf of 0-0.05 became predominant.

Fourth, the aqueous extracts from rapeseed, sunflower, and Canada thistle plants harvested 144 hours after treatment with ^{14}C-picloram or ^{14}C-clopyralid were run in one of two solvent systems that did not contain NH_4OH (Table 11). The results show that for each herbicide applied one common metabolite was produced by all three plant species (Table 11). The Rf value of the unknown metabolite of each herbicide did not correspond to that of the acid amide of picloram or clopyralid. In another experiment, each TLC plate was spotted with the plant extracts from the three species, picloram, clopyralid, and the acid amide of the herbicides. A plate was incubated for 1.5 hours in a chamber filled with ammonia vapours and developed in one of the two solvent systems listed in Table 11. Depending on which herbicide was applied to the three plant species, the unknown herbicide metabolite that was found in the aqueous extract had an Rf value corresponding to the acid amide of that herbicide.

Table 11. Rf values of various compounds chromatographed on silica gel plates and developed in solvent systems without NH_4OH.

Compound	Solvent system #1[1]	Solvent system #2[1]
	---------Rf values[2]---------	
Picloram standard	0.51	0.45
Clopyralid standard	0.52	0.46
Amide of picloram	0.80	0.82
Amide of clopyralid	0.87	0.78
Picloram plant extract after ammonialysis[3]	0.80	0.82
Clopyralid plant extract after ammonialysis[3]	0.87	0.78
Picloram plant extract[3]	0.35-0.50	0-0.05
Clopyralid plant extract[3]	0.35-0.50	0-0.05

[1]Solvent system #1: isopropanol:acetic acid:H_2O (18:1:1 v/v/v).
Solvent system #2: CH_2Cl_2:methanol:acetone:acetic acid (8:1:1:1 v/v/v/v).
[2]Rf values were slightly different each time the chromatographic procedure was repeated.
[3]Refers to plant extracts from rapeseed, sunflower, and Canada thistle plants.

4.4.2 Purification and Identification by GC-MS of the Ammonialysed Water-Soluble Metabolite from Rapeseed Plants

The aqueous extracts from plants treated with picloram and clopyralid were exposed to ammonia to convert the water-soluble herbicide metabolites therein to the corresponding carboxylic acid amides. These amides are stable compounds that can be easily separated from the aqueous extracts and purified by TLC and gas chromatography as described in Section 3.5. The amide derivatives could be used to determine whether there was any alteration in functional groups other than the carboxyl group after the picloram and clopyralid had undergone metabolism and were subjected to ammonolysis.

The water-soluble metabolites of picloram or clopyralid were isolated from the detached leaves of rapeseed plants (Section 3.5) and subjected to ammonolysis to produce the corresponding amide of the herbicide that was previously applied to the plant. After purification of the amide (Section 3.5.1), the mixture was injected into a combined GC-MS. The resulting mass fragmentation patterns of the authentic standards of the carboxylic acid amide of picloram and clopyralid are shown in Figures 11(A) and 12(A), respectively. The mass fragmentation patterns of the water-soluble plant metabolites of picloram and clopyralid following ammonolysis are shown in Figures 11(B) and 12(B), respectively, and appear to be identical to the patterns

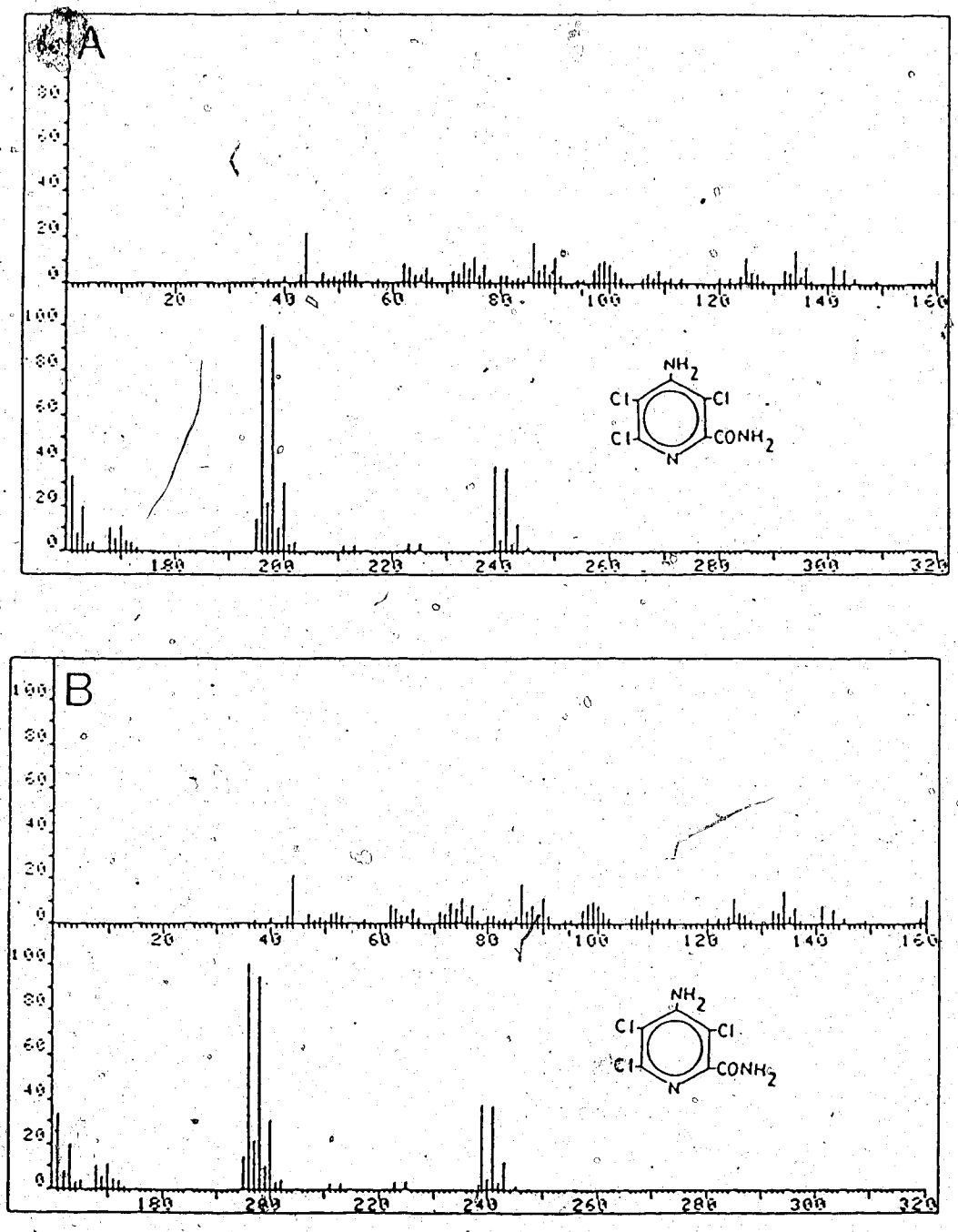


Figure 11. Mass spectral fragmentation patterns of the synthetically produced carboxylic acid amide of picloram (A) and the water-soluble metabolite of picloram after exposure to ammonia vapours (B).

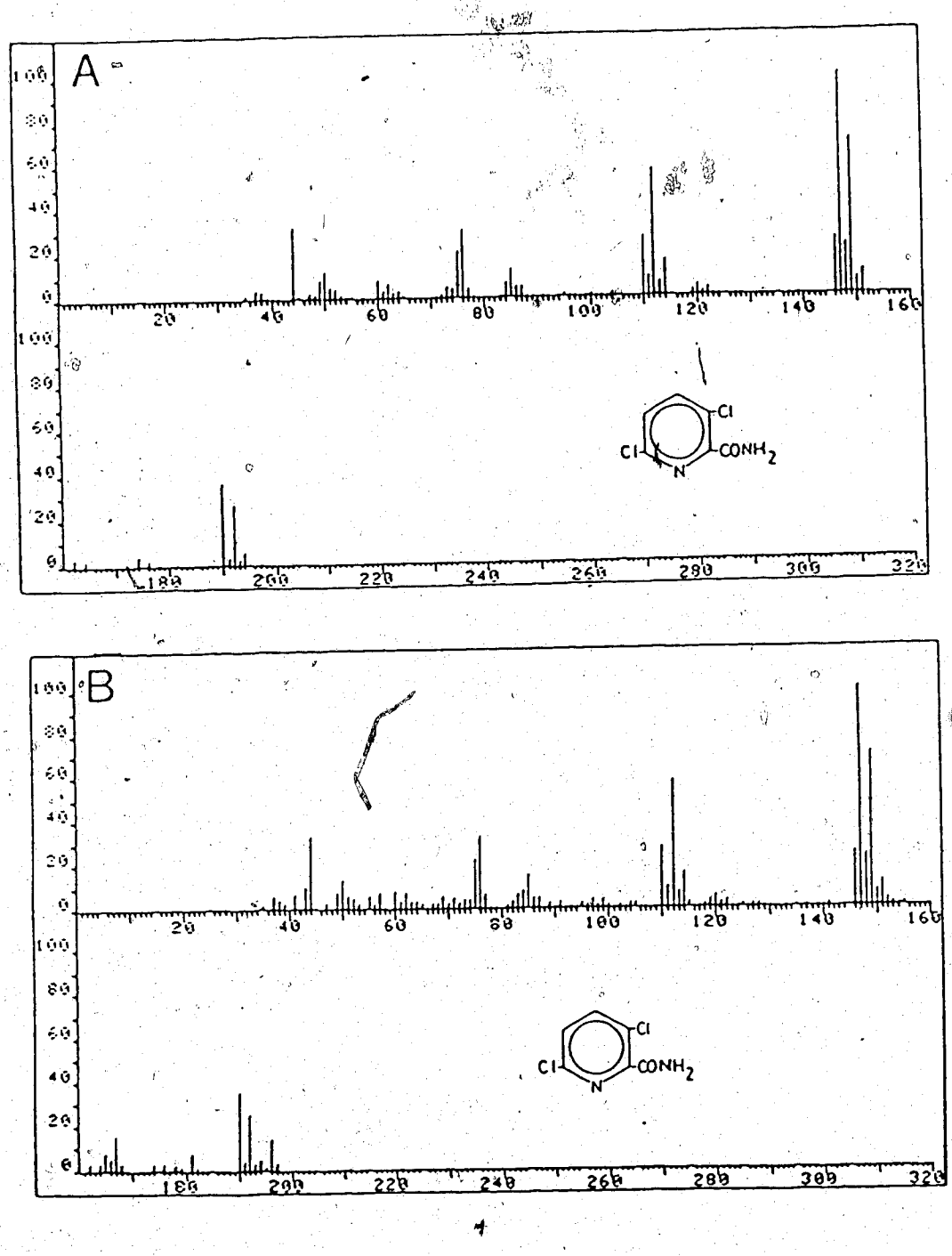


Figure 12. Mass spectral fragmentation patterns of the synthetically produced carboxylic acid amide of clopyralid (A) and the water-soluble metabolite of clopyralid after exposure to ammonia vapours (B).

produced by the synthetic amide standards of the respective herbicides.

4.4.3 Purification and Identification by GC-MS of the Water-Soluble Metabolite from Rapeseed Plants

A large scale production and purification of the plant metabolite of picloram and clopyralid was undertaken as described in Section 3.5. Once the metabolite of either herbicide was purified it was acetylated or silylated. The acetylated derivative was then subjected to one more purification procedure on silica gel TLC plates that were developed in dichloromethane:methanol (9:1 v/v). The acetylated or silylated derivatives of the water-soluble metabolites of picloram and clopyralid were then injected into a combined GC-MS using the conditions described for the analysis of the amide derivatives of the herbicides (Section 3.5.1). This GC-MS procedure with the silylated or acetylated derivatives of the water-soluble metabolite of either herbicide was unsuccessful. A mass fragmentation pattern revealed a compound that was similar to picloram or clopyralid. After several unsuccessful attempts to obtain a mass fragmentation pattern using the silylation or acetylation methods the procedure was abandoned.

In another experiment, the purified water-soluble metabolite of clopyralid was dissolved in a small quantity of glycerol and subjected to fast atom bombardment (FAB) mass spectrometry. The mass fragmentation pattern that

resulted indicated the presence of sodium glycerol. There was no indication of any other compounds. These results indicated that there was not enough of the metabolite present obtain a good fragmentation pattern by this method.

4.4.4 Effect of pH on the Stability of the Herbicide Metabolite

During preliminary experiments with the metabolites of the herbicides it became evident that the stability of the water-soluble metabolite was affected by the pH of the solution in which it was dissolved. Therefore, the relationship between pH and the stability of the herbicide metabolite was determined by incubating samples of the water-soluble metabolite of clopyralid for 3 hours in solutions of various pH. The results presented in Figure 13 show that above pH 6 there was rapid conversion of the metabolite back to clopyralid. When samples of the water-soluble metabolite of picloram and clopyralid were incubated at pH 4 and 5 for as long as 48 hours, there was no conversion to the corresponding herbicide.

4.4.5 In Vitro Synthesis of the Herbicide Metabolite

The *in vitro* synthesis of the water-soluble metabolite was attempted using the methods of Frear et al. (47) and Kopcewicz et al. (68). Both procedures were conducted at pH 5 and and then repeated at pH 7. Regardless of the pH at

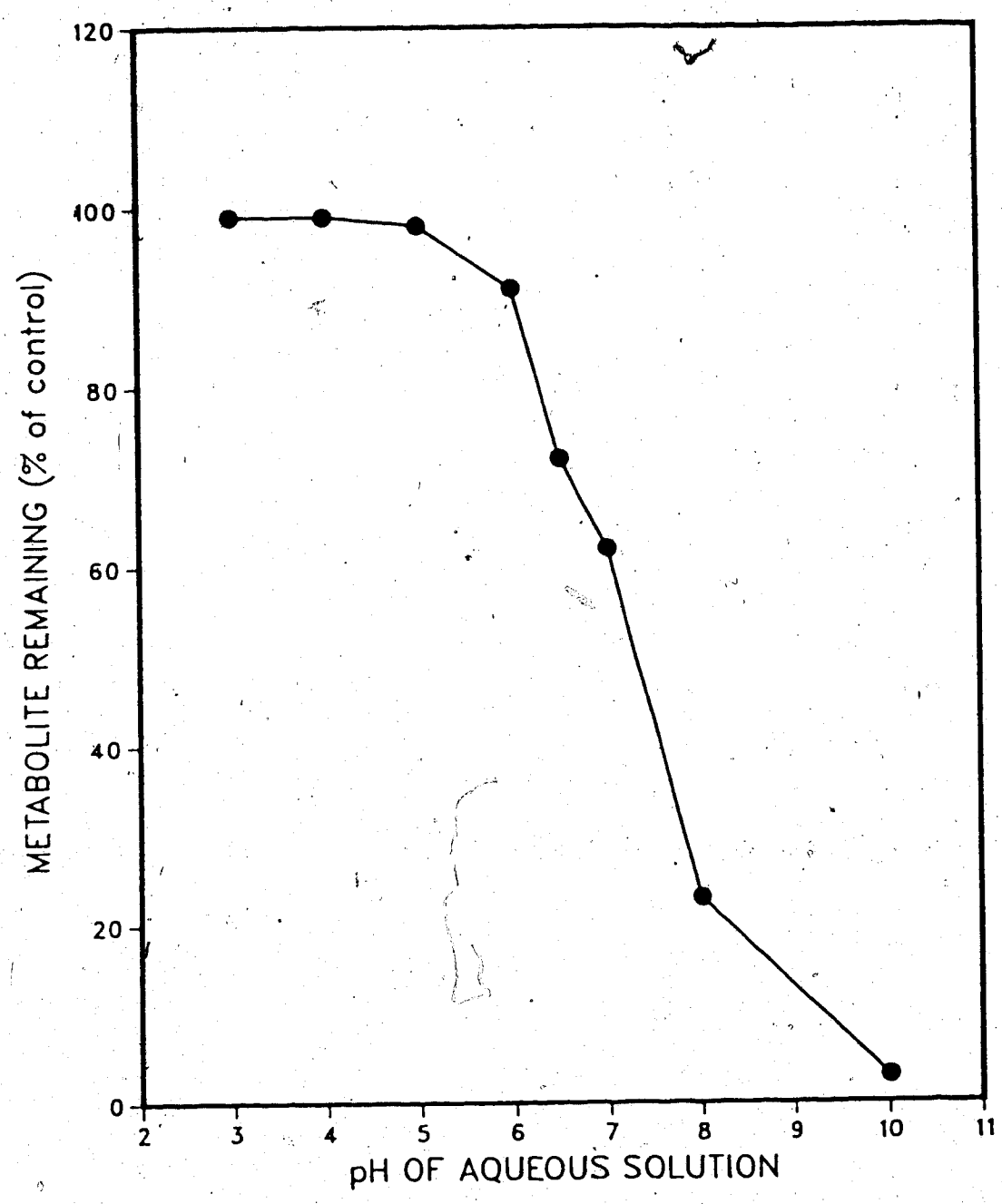


Figure 13. Effect of pH on the stability of the water-soluble metabolite of clopyralid after a 3-hour incubation in an aqueous solution. Standard errors of the means were less than 2%.

which both procedures were conducted, the results proved to be negative, that is, no water-soluble metabolite of clopyralid could be synthesized *in vitro*.

4.4.6 Chemical Synthesis and Chromatography of the Suspected Water-Soluble Metabolite

Characterization by GC-MS of the structure of the water-soluble metabolite of picloram and clopyralid was not successful (Section 4.4.3). Therefore, an attempt was made to determine the metabolite's structure by chemical synthesis of the suspected plant metabolite. Once synthesized, the compound was used as a standard to determine if it had the same Rf value as the unknown herbicide metabolite produced by the plant, as determined by TLC.

The glucose ester of clopyralid was synthesized and purified as described in Section 3.8.2. It was subjected to several tests to confirm that it was the glucose ester of clopyralid. One of the tests involved the addition of the glucose metabolite to an aqueous solution to determine whether it could be partitioned into dichloromethane. As suspected, the results indicated that none of the glucose ester of clopyralid could be partitioned into the organic phase. Another test that was performed was chromatography of the synthetic glucose ester of clopyralid, along with several other standard compounds, in different TLC solvent systems (Table 12). In some cases, the compounds were

Table 12. Rf values of various compounds after TLC on silica gel plates[1].

Compound	Solvent #1[2] (no ammonia)	Solvent #1[2] (ammonia)	Solvent #2[3] (no ammonia)	Solvent #2[3] (ammonia)
	(Rf values)			
Glucose	0.00-0.05 T	0.00-0.05 T	0.00-0.28 T	0.00-0.24 T
Sucrose	0.00-0.02 T	0.00-0.02 T	0.00-0.23 T	0.00-0.19 T
Gentiobiose	0.00-0.02 T	0.00-0.02 T	0.00-0.16 T	0.00-0.16 T
Clopyralid	0.46 UC	0.49 UC	0.51 UC	0.52 UC
Amide of Clopyralid	0.91 UC	0.87 UC	0.83 UC	0.82 UC
Clopyralid conjugate with glucose	0.31 TUC	0.00-0.05 T 0.87 UC	0.51 UC 0.62 UC	0.00-0.24 T 0.82 UC
Clopyralid conjugate with sucrose	0.00-0.07 TUC 0.17 TUC	0.00-0.02 T 0.87 UC	0.40-0.52 TUC	0.00-0.20 T 0.82 UC
Clopyralid conjugate with gentiobiose	0.00-0.05 TUC	0.00-0.02 T 0.87 UC	0.00-0.16 T streaked TUC	0.00-0.16 T 0.82 UC
Water-soluble metabolite from rapeseed	0.00-0.05 TUC	0.00-0.05 T 0.87 UC	0.07-0.33 T 0.35-0.50 UC	0.00-0.20 T 0.82 UC

[1]Rf values followed by the appropriate letter indicate whether the compounds were located on the plates by visualization with ultraviolet light at 254 nm (U), by scanning for ^{14}C radioactivity with a Geiger-Mueller TLC plate scanner (C), and/or reaction of the sugars with thymol/H_2SO_4 indicator (T). In some cases the compounds were applied to the plate and exposed to NH_3 vapours prior to chromatography.
[2]Solvent 1: dichloromethane:methanol:acetone:acetic acid (8:1:1:1 v/v/v/v).
[3]Solvent 2: isopropanol:acetic acid:H_2O (18:1:1 v/v/v).

exposed to ammonia vapours for 1.5 hours to ammonialyse any ester bonds. The compounds were located on the silica gel TLC plates by visualization with ultraviolet light (254 nm) which confirms the presence of clopyralid or a derivative of the herbicide, by scanning the plate for ^{14}C radioactivity which confirms the presence of ^{14}C-clopyralid, and/or by chemical reaction with thymol/H_2SO_4 to confirm the presence of sugars. The results of these three tests are shown in Table 12.

The chemically synthesized glucose ester of clopyralid had an Rf value different from any of the other synthetic standards or the plant metabolite, after chromatogaphy in either of the two solvent systems (Table 12). This synthetic glucose metabolite gave a positive test for the presence of both a sugar and clopyralid. When the synthetic compound was exposed to ammonia vapours prior to chromatography, two distinct products resulted (Table 12). These products were glucose and the amide of clopyralid. Taken together, the results confirm that the synthesized compound was the glucose ester of clopyralid. Furthermore, this glucose ester of clopyralid did not have the same Rf value as the herbicide metabolite produced by the plant, suggesting they are two different compounds. Therefore, it was hypothesized that the water-soluble plant metabolite may have involved conjugation of clopyralid with a sugar moiety that was more polar than glucose, such as a disaccharide. (Polarity was assessed in terms of migration of the compounds on TLC

plates that were developed in the solvent systems used in Table 12. The most polar compound has the lowest Rf value.)

The synthesis of the sucrose metabolite is described in Section 3.8.2. The results in Table 12 indicate that two compounds are present after the chromatography of this synthetic sucrose-metabolite in the first solvent system. The two compounds are probably isomers of the synthetic sucrose-herbicide metabolite. In the second solvent system there was streaking of the sucrose metabolite. However, in both solvent systems the two compounds representing the synthetic sucrose esters of clopyralid gave positive tests for both sucrose and clopyralid. Ammonialysis of these two compounds produced the amide derivative of clopyralid and sucrose. The sucrose ester of clopyralid has an Rf value which is more like the Rf value of the water-soluble plant metabolite than the glucose ester of the herbicide.

The synthetic gentiobiose metabolite has an Rf value identical to that of the plant metabolite when developed in the first solvent system (Table 12). When the gentiobiose metabolite was run in the second solvent system there were two isomers of this metabolite, as indicated by positive tests for sugar and clopyralid. However, the plant metabolite did not co-migrate with either of the two isomers of the synthetic gentiobiose metabolite although their Rf values were similar to that of the plant metabolite. In order to determine conclusively that the synthetic metabolites of gentiobiose were different from the plant

metabolite, both compounds were chromatographed on silica gel plates and developed in n-butanol:acetic acid:H_2O (12:3:5). The results were similar to those obtained using the second solvent system noted in Table 12; both isomers of the synthetic metabolite having a slightly different Rf value than that of the plant metabolite. When the compounds were exposed to ammonia vapours, gentiobiose and the amide derivative of clopyralid were the products resulting from the ammonolysis of the two isomers of the gentiobiose esters of clopyralid.

4.4.7 Enzymatic Hydrolysis of the Water-Soluble Metabolite

The naturally produced herbicide metabolite was incubated with α- and β-glucosidase and hesperidinase to determine if the herbicide was conjugated with a sugar. The incubation mixture was subjected to TLC to determine if the plant metabolite had been hydrolyzed. All enzyme incubation mixtures were chromatographed against controls that contained no enzyme.

The water-soluble herbicide metabolite produced by the plant was not hydrolyzed by any of the enzyme preparations after a 4-hour incubation. However, incubation for 16, 24, and 48 hours with β-glucosidase and hesperidinase resulted in hydrolysis of the plant metabolite. The product formed had the same Rf value as the synthetic glucose ester of clopyralid and will henceforth be designated as the intermediate metabolite .

The intermediate metabolite created by enzymatic hydrolysis was isolated and subjected to ammonolysis. The resulting compound co-migrated identically with the amide of clopyralid, indicating that the intermediate metabolite may be a monosaccharide ester of clopyralid.

None of the plant metabolite remained after a 48-hour incubation with β-glucosidase. The only compounds remaining were the intermediate metabolite and clopyralid. Hesperidinase did not hydrolyze all of the plant metabolite to the intermediate metabolite . None of the plant metabolite was hydrolyzed in the control solutions or the solutions containing α-glucosidase, as long as 48 hours after initiation of incubation.

The previous results indicate that β-glucosidase and, to a lesser extent, hesperidinase hydrolyze the plant metabolite to the intermediate metabolite . The quantity of plant metabolite hydrolyzed by β-glucosidase was 100%, since none of this metabolite was detected by scanning the plate with the Geiger Mueller scanner. Furthermore, it appears from the TLC results that little of the plant metabolite was hydrolyzed to clopyralid by β-glucosidase or hesperidinase after 24 or 48 hours of incubation. However, it is difficult to determine the quantity of the plant metabolite that was hydrolyzed to clopyralid because there was always some clopyralid present on the TLC plates after chromatography. The presence of this clopyralid is a result of an inability to completely remove all the herbicide that was not

metabolized by the plant. In addition some free herbicide may result from the chemical hydrolysis of a small portion of the plant metabolite thus forming clopyralid during chromatography in the acid solvent systems. Consequently, the quantity of the water-soluble metabolite enzymatically hydrolyzed to clopyralid could not be determined by scanning the TLC plates for ^{14}C. Therefore, an alternate method was used in which the metabolite was incubated with α-glucosidase, β-glucosidase, or hesperidinase. The quantity of the plant metabolite converted to clopyralid was determined by partitioning the incubation mixture against dichloromethane to extract all the free herbicide (Table 13).

The results of the dichloromethane extraction experiment revealed that α-glucosidase did not hydrolyze the primary metabolite to clopyralid 4 or 24 hours after treatment (Table 13). However, β-glucosidase and hesperidinase hydrolyzed significant quantities of the plant metabolite to clopyralid 24 hours after initiation of the incubation. Hesperidinase did not convert as much of the water-soluble metabolite to clopyralid as did β-glucosidase (Table 13). The fraction of metabolite hydrolyzed by hesperidinase and β-glucosidase to clopyralid was 13.2 and 20.3%, respectively, after a 24-hour incubation.

The hesperidinase enzyme preparation also contained 0.003 units/mg of β-glucosidase. Therefore, it was difficult to determine whether the intermediate metabolite and

Table 13. Enzymatic hydrolysis of the water-soluble metabolite of clopyralid. The water-soluble herbicide conjugate was incubated for 4, 24, or 48 hours in sodium citrate buffer (pH 5.0) with α-glucosidase, β-glucosidase, or hesperidinase. The conversion of the clopyralid metabolite back to the parent herbicide was determined by partitioning the aqueous phase, after it was adjusted to pH 1.6, three times against dichloromethane'.

Time (h)	Enzyme	Clopyralid (%)	Metabolite (%)	Difference from control (%)
4	Control	14.5 (0.5)	84.5 (0.5)	
	α-glucosidase	13.9 (0.6)	86.1 (0.6)	-1.6
	β-glucosidase	17.7 (1.2)	82.3 (1.2)	+2.2
	Hesperidinase	19.0 (2.2)	81.0 (2.1)	+3.5
24	Control	15.0 (0.7)	85.0 (0.7)	
	α-glucosidase	16.8 (1.3)	82.2 (1.3)	+2.8
	β-glucosidase	35.3 (1.0)	64.7 (1.0)	+20.3
	Hesperidinase	28.2 (2.8)	71.8 (2.8)	+13.2
48	Control	16.1 (2.6)	83.9 (2.6)	
	β-glucosidase	35.4 (0.2)	64.6 (0.2)	+19.3

'Standard errors of the means are in parentheses.

clopyralid that resulted from the hydrolysis of the plant metabolite were a result of the hesperidinase or β-glucosidase activity. Consequently, the hesperidinase enzyme was not included in the 48-hour incubation study.

4.4.8 Discussion

The results (Tables 10 and 11) taken together indicate that one metabolite was formed from each of the two

herbicides. Each herbicide metabolite was the same in all three plant species. Because the picloram and clopyralid metabolite were the same in all three plant species, only the metabolites isolated from rapeseed plants were used for further investigations on the structure and properties of the metabolite. The metabolite is very hydrophilic and probably represents picloram or clopyralid conjugated by means of an ester bond to a very polar compound. Some researchers have suggested that picloram is conjugated with sugar(s) (27, 72, 77, 114).

Upon exposure to ammonia vapours the metabolite of picloram and clopyralid isolated from the three plant species undergoes ammonolysis producing the carboxylic acid amide of the respective herbicide. Therefore, the use of NH_4OH in the TLC solvent system must be avoided to prevent the artifical formation of the carboxylic acid amide of picloram and clopyralid.

IAA has been shown to conjugate with myo-inositol or glucose by means of an ester link through the carboxylic acid group of IAA (73,122). When this metabolite was exposed to ammonia vapours the molecule was cleaved yielding the carboxylic acid amide of IAA. Furthermore, Hallmen (57) found that chromatography in solvents that contain ammonium hydroxide leads to decomposition of the water-soluble metabolite of picloram. He found that this lability made it impossible to purify these compounds by TLC in solvent systems containing ammonium hydroxide.

The mass spectral fragmentation patterns of the acid amides of the herbicides indicate that the carboxylic acid group is the only substituent group that undergoes alteration during the conversion of picloram and clopyralid to the water-soluble metabolite (Figures 11 and 12).

There is no clear reason why the fragmentation patterns for the water-soluble metabolite of picloram and clopyralid could not be obtained (Section 4.4.3). It is possible that the water-soluble metabolite was not stable under the temperature conditions at which the GC-MS was operated. However, decreasing the temperature did not solve the problem. It is also possible that the metabolite of the two herbicides breaks down in the silylation and acetylation mixture, since it usually took several days before the mixture was subjected to GC-MS. The silylating or acetylating solution contains pyridine which is known to be slightly basic. Prolonged incubation in this solution may cause deterioration of the ester bond of the water-soluble metabolite due to pH effects, resulting in conversion of the plant metabolite to the parent acid (see Section 4.4.4).

The results of the experiment on the effect of pH on the stability of the herbicide metabolite (Section 4.4.4) indicate that the metabolite may be biosynthesized and isolated by the plant in an organelle such as the vacuole, which is able to maintain a pH of approximately 5. In addition, it is unlikely that the metabolite of either herbicide is mobile in the phloem since the pH of the phloem

is regarded as being above 6.5. The plant metabolite would be hydrolyzed in the phloem at this pH.

Because of the instability of the water-soluble metabolite above pH 6 it is not surprising that *in vitro* synthesis of the metabolite was not successful at pH 7 (Section 4.4.5). However, the lack of success in biosynthesizing the metabolite of clopyralid at pH 5 cannot be explained. It is possible that enzymes required for the *in vitro* synthesis of the metabolite were not isolated during the procedure or became inactive when the plant tissues were macerated in the process of isolating the enzymes. Second, the proper substrates may not have been provided to successfully create the metabolite *in vitro*. Finally, there is no way of knowing with certainty what the compound is to which the herbicides are conjugated. Therefore, it is possible that the methods used in the attempt to produce the water-soluble herbicide metabolites were not specific enough to result in production of the metabolite.

Corner and Swain (30) successfully synthesized the glucose esters of *p*-coumaric, caffeic, ferulic, and sinapic acid. These esters were formed in good yield when the acids were incubated at pH 8.4 at 37°C with a 2-mole excess of uridine-5'-diphosphoglucose (UDPG) and an acetone powder that was prepared from the leaves of geranium. However, using the same conditions, no glucose esters were synthesized from the same four acids and UDPG using the

acetone powders from three other plant species, which included cabbage, chicory, and barley.

A review of the literature revealed that amongst water-soluble metabolites of many herbicides and plant growth regulators, such as 2,4-D, MCPA, IAA, dicamba, chlorsulfuron, and metribuzin, it is quite common to find glucose conjugates. In fact, esterification of 2,4-D, picloram, diphenamid, and IAA to glucose through the carboxylic acid moieties of these compounds is one the most common types of the water-soluble metabolites formed. Therefore, based on the evidence presented previously in this thesis that clopyralid may be conjugated to a water-soluble compound through an ester bond, it was hypothesized that the most likely metabolite was the glucose ester of clopyralid.

The suspected glucose conjugate of clopyralid was synthesized and purified as described in Section 3.8.2. The synthesis of the benzoic acid ester of glucose (6-benzoyl-D-glucose) has been described by Bock et al. (14) using a similar method.

The Rf value of the glucose ester of clopyralid was markedly different than that of the water-soluble plant metabolite (Table 12). However, both the gentiobiose and sucrose esters of clopyralid had Rf values which, although not indentical to, were at least similar to that of the water-soluble plant metabolite. These results support the hypothesis that the plant metabolite is a disaccharide ester

of clopyralid. Furthermore, several researchers have found metabolites of diphenamid (63) and 3-phenoxybenzoic acid (84) that were conjugated by means of an ester bond to gentiobiose.

Sucrose is not a reducing sugar. Furthermore, sucrose is not available for conjugation with other compounds in the plant, such as clopyralid, since the fructose and glucose subunits are joined through the anomeric carbons of both monosaccharides. Therefore, a sucrose ester of clopyralid could not be synthesized by the plant. However, in the present experiments sucrose was used as one of the sugars in the chemical sythesis of the metabolite because this allowed the production of a synthetic metabolite that was more polar than the glucose-herbicide conjugate.

The enzyme hydrolysis experiments (section 4.4.7) further support the hypothesis that the herbicide metabolite produced by the plant may be a disaccharide ester of clopyralid. Since the plant metabolite was hydrolyzed by β-glucosidase to form both the intermediate metabolite and clopyralid, it appears that the plant metabolite has at least one glucose sub-unit. However, it is not clear why β-glucosidase will not completely hydrolyze the plant metabolite to clopyralid.

If the water-soluble herbicide metabolite is a disaccharide ester of clopyralid, it is possible that the sugar farthest from the herbicide molecule is glucose. This sugar may be easily hydrolyzed by β-glucosidase to yield the

intermediate metabolite . However, the sugar subunit adjacent to the herbicide may not be glucose. Therefore, when the water-soluble metabolite was hydrolyzed yielding the intermediate metabolite the enzyme could not remove the sugar remaining on the intermediate metabolite to produce the free herbicide. If this was the case, it is not clear why 20% of the original plant metabolite is hydrolyzed to clopyralid 24 and 48 hours after initiation of incubation. One possible explanation is that the sugar moiety of the intermediate metabolite may be susceptible to slow hydrolysis. It is also conceivable that the sugar moiety of the intermediate metabolite may be glucose. However, this glucose unit may not be readily removed from the metabolite if the herbicide molecule inhibits the enzyme's activity. A second possibility is that the intermediate metabolite may not be enzymatically hydrolyzed to the herbicide. Rather, lowering the pH to 1.6 to partition the free herbicide into the organic phase may result in chemical hydrolysis of the intermediate metabolite. Finally, there may be two different plant metabolites that involve disaccharides, both of which have a glucose attached at the farthest position from clopyralid. When this glucose is hydrolyzed, two intermediate metabolites result. The hydrolysis of 20% of the plant metabolite to clopyralid may actually repesent complete hydrolysis of the water-soluble metabolite that has two glucose units. The remaining intermediate metabolite may actually result from hydrolysis of glucose from the

water-soluble plant metabolite that has a monosaccharide other than glucose attached adjacent to the herbicide. This second intermediate metabolite may resist hydrolysis by β-glucosidase.

Several researchers have identified other types of water-soluble metabolites of picloram. For example, Maroder and Prego (77) speculated that the N-glucoside of picloram was formed in *Prosopis ruscifolia*, on the basis of studies involving enzyme hydrolysis, electrophoresis, and acid/base hydrolysis. Chkanikov et al. (27) supported these findings by use of mass spectrometry and other physicochemical methods. They found that picloram was converted to N-(2-carboxy-3,5,6-trichloropyridine-4-yl)-glucosamine (N-glucoside of picloram) in sunflower plants. The extent of conversion to the N-glucoside in sunflower exceeded 70% of the recovered dose. In addition 20% of the recovered dose was converted to the glucose ester of picloram. Only in sunflower plants was the N-glucoside of picloram more prevalent than the glucose ester of picloram. In other work, Kudaikina et al. (72) isolated the glucose ester of picloram (1-O-(4-amino-3,5,6-trichloropicolyl)-D-glucopyranoside) in sunflower and bean plants and identified it by mass spectrometry and chromatography. Chkanikov et al. (28) also found that picloram formed ether-soluble conjugates with mustard oils in radish and mustard plants.

Results presented in this thesis indicate that the water-soluble metabolite of clopyralid, isolated from

rapeseed plants, is a disaccharide ester of clopyralid. There is no evidence for the formation of either the non-polar metabolites of picloram and clopyralid or the N-glucoside of picloram in rapeseed, sunflower, and Canada thistle plants. The formation of the N-glucoside of clopyralid is not possible since this herbicide does not have an amino substituent group. Furthermore, there was no evidence that the glucose ester of either herbicide was formed in the three plant species.

Summary and Conclusion

There was no difference between the amounts of ^{14}C-picloram and ^{14}C-clopyralid that were absorbed by rapeseed, sunflower, and Canada thistle plants. Approximately 60% or more of the recovered radioactivity from picloram and clopyralid treatments moved acropetally out of the treated leaf 144 hours after herbicide was applied to sunflower and rapeseed plants. Less than 5% of the ^{14}C-label from either herbicide treatment moved basipetally in sunflower and rapeseed plants.

More than 55% of ^{14}C-label from picloram and clopyralid moved acropetally out of the treated leaf in Canada thistle plants 144 hours after treatment. The acropetal translocation pattern of the two herbicides resembled that in the two annual species. Significantly more radioactivity moved basipetally 144 hours after application of clopyralid than picloram. This observation may explain why clopyralid is more effective than picloram in controlling Canada thistle shoot regrowth from root buds.

Significantly more of both ^{14}C-picloram and ^{14}C-clopyralid was converted to water-soluble metabolites in rapeseed than in sunflower and Canada thistle plants. There was no difference between the amounts of ^{14}C-picloram and ^{14}C-clopyralid that were metabolized in rapeseed plants 144 hours after treatment. However, even with the same amount of metabolism of both herbicides in rapeseed plants, a 5 kg/ha dose of clopyralid had no herbicidal affect, whereas 100

g/ha of picloram caused herbicide symptoms. Therefore, differences in the extent of metabolism between the two herbicides do not explain sensitivity differences within rapeseed plants. In sunflower plants, significantly more picloram than clopyralid was metabolized 144 hours after treatment and yet, sunflower plants are very sensitive to both herbicides. Taken together, results of the metabolism experiments indicate that differences in sensitivity between species to the two herbicides cannot be attributed to differences in the extent of herbicide metabolism.

Picloram applied to the third leaf of a rapeseed plant increased ethylene evolution several-fold. Clopyralid had no effect on ethylene production in rapeseed plants. In sunflower, both picloram and clopyralid elevated ethylene levels. Ethylene biosynthesis induced by the herbicide treatment was not restricted to treatment areas. When clopyralid was applied to the cotyledons of sunflower, the herbicide treatment resulted in an increased rate of ethylene production from the true leaves. Increased ethylene production preceded or coincided with the onset of morphological responses induced by a herbicide application to a susceptible species. Treatment with aminoethoxyvinylglycine (AVG) before picloram or clopyralid application prevented the increase in ethylene production. Pretreatment with AVG also delayed the development of morphological changes induced by picloram or clopyralid. It appears that enhanced ethylene biosynthesis after application of picloram

or clopyralid to susceptible species was a factor involved in the subsequent morphological changes.

Taken together, the results of the absorption, translocation, metabolism, and ethylene experiments indicate that differences in sensitivity within and between susceptible species to picloram and clopyralid are a result of differences in the way the two herbicides interact with some target site in the plants.

Thin layer chromatography indicated that the herbicide metabolite that is formed after application of picloram or clopyralid was the same in all three plant species. Mass spectrometry and TLC showed that the metabolite is formed by conjugation of a very polar compound through the carboxylic acid moiety of picloram and clopyralid. Enzymatic hydrolysis with β-glucosidase showed that the metabolite may be a disaccharide ester of the herbicide. This disaccharide appears to have a glucose subunit in it.

In view of these findings, further experiments should be conducted *in vitro* with cell suspensions from rapeseed and sunflower plants to determine how various concentrations of radiolabeled picloram and clopyralid are taken up by cells, where the herbicides accumulate in cells, and whether ethylene production increases only in cells from species that are susceptible to picloram and clopyralid. These experiments may elucidate whether rapeseed plants have a different susceptibility to picloram than clopyralid at a cellular level, thereby testing the hypothesis proposed in

this thesis that picloram and clopyralid react differently with some target site in susceptible and resistant plant species.

Experiments should also be conducted to conclusively determine whether the water-soluble metabolite of picloram and clopyralid is the same in all three plant species and whether any other water-soluble metabolites can be found in these species. This would require that the metabolites from all three plant species be isolated and characterized by GC-MS. Furthermore, it should be established whether the metabolite is phytotoxic and whether it is translocated within the plant or sequestered within some organelle in cells.

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