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**" Study on the metabolism of chloro-phenylalanines in crop and weed plants in
relation to the formation of herbicidal products."**

by

DOMINIQUE LAINET, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

05/09/1991.

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in relation to the formation of herbicidal products. "**

**Submitted by Dominique Lainet, B.Sc.
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Thesis Supervisor

Chair, Department of Biology

Carleton University

October 1991

Abstract

Metabolism of nine ring-substituted chloro-phenylalanines was examined in excised shoots of one crop species, *Vicia faba* and two weeds, *Cirsium arvense* and *Sinapis arvensis*. Gas chromatography-mass spectroscopy analysis of purified methanolic extracts of the fed shoots showed that most but not all of the chloro-phenylalanines were metabolized to the corresponding chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids in the three species. The results are discussed in relation to the potential use of certain chloro-phenylalanines as selective herbicides through a process of lethal synthesis in which these chloro-phenylalanines would be metabolized to the corresponding chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids which are known to exhibit growth-regulating and herbicidal activity.

The relationship between structure and metabolism of the chloro-phenylalanines was also investigated. Computer-modelling analysis showed that those compounds which were metabolized to the greatest extent shared a common steric geometry, which was different from that found for the less metabolized chloro-phenylalanines.

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1. Introduction

The ability of certain ring-substituted chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids to exhibit growth-regulating and, in some cases, herbicidal activity has been known since the 1940's and several studies to relate physiological activity with the substitution pattern of these compounds have been carried out (e.g. Pybus *et al.* 1959a,b, Ugochukwu and Wain 1968).

In the 1970's, studies on the metabolic pathways responsible for the biosynthesis of phenylacetic acid (PAA) in plants showed that it was formed from the amino acid, L-phenylalanine, and that cinnamic acid was also a product of phenylalanine metabolism (Wightman 1973, Wightman and Rauthan 1975).

Earlier investigations by Wain and Wightman (1954) had shown that plants can form a product that has herbicidal activity from an inactive precursor and that not all plants necessarily possess the same ability, which means that this phenomenon, which is termed lethal synthesis, can be used as a means for the selective control of weeds in certain crops. The formation of highly active 2,4-D from inactive 2,4-dichlorobutyric acid (2,4-DB) by some weeds while certain leguminous crops do not form 2,4-D and are thus unharmed by the 2,4-DB is a good example of the possible use of lethal synthesis as a biochemical method for the selective control of weeds.

In view of the known growth-regulating activity of some of the chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids and of the well

demonstrated formation of phenylacetic and cinnamic acids from phenylalanine and of benzoic acid from cinnamic acid, Taylor and coworkers synthesized a series of ring- substituted chloro-phenylalanines and examined their ability to serve as substrates for the aminotransferase enzyme of the PAA bio-synthesis pathway and also investigated the effect of the substitution pattern on this ability (Taylor *et al.* 1987, Taylor and Wightman 1987a,b,c). They also examined the *in vivo* metabolism of the chloro-phenylalanines in six crop and weed species to see if any differential lethal synthesis of physiologically active chloro-phenyl metabolites could be found among those plants (Taylor *et al.* 1988). These authors reported that highly purified bushbean aspartate-aromatic aminotransferase, the enzyme responsible for the first step in the synthesis of PAA from phenylalanine, could use some of the chloro-phenylalanines as a substrate and that the activity of the enzyme with the chloro-phenylalanines varied with their ring-substitution pattern. They also found that excised shoots of the six species studied produced various quantities of Cl-phenylacetic, Cl-benzoic and Cl-cinnamic acids when fed with solutions of the chloro-phenylalanines. The amounts of chloro-phenyl metabolites produced depended both on the species and the chloro-phenylalanine fed to the plant. The suggested pathway for the formation of chloro-phenyl metabolites from chloro-phenylalanines is shown in Figure. 1.

The present study was undertaken with the purpose of extending the range of crop and weed species in which metabolism of chloro-phenylalanines has been studied and, as a secondary aspect, to investigate the relationship between

chemical structure and metabolism of the chloro-phenylalanines. From what was previously found by Taylor *et al.* (1988) in their metabolism experiments, it is expected that the shoots of the three species will have the ability to metabolize most of the nine chloro-phenylalanine to the corresponding chloro-phenylacetic, chloro-benzoic and/or chloro-cinnamic acids. Within each species, the extent of metabolism can be expected to vary with the substitution pattern of the chloro-phenylalanines. Also, for certain of the chloro-phenylalanines it is thought that some differences between the three species in their ability to metabolize these compounds will be found. From the results of Taylor and Wightman's (1987c) study on the ability of the different phenylalanines to serve as substrate for the aspartate-aromatic aminotransferase it would be expected that some general relationship between structure and metabolism of the chloro-phenylalanines will be found.

Figure 1. Proposed biochemical pathways for the conversion of D,L-phenylalanine and D,L-chloro-phenylalanines to their corresponding phenylacetic, benzoic and cinnamic acids in shoot tissues. From Taylor *et al.* (1988).

2. Literature Review

2.1 History, Uses and Physiology of Auxinic Herbicides.

2.1.1 History and General Uses

The introduction of auxin-like compounds as herbicides dates back to the early 1940's and results from work by both British and American scientists (Fletcher and Kirkwood 1982). In England, during World War II, in the course of investigations on the role of auxins in plant growth, Slade, Templeman and Sexton (1945), sprayed mixed stands of oats and the weed *Brassica sinapis* with α -naphthylacetic acid (NAA) and noticed that this substance killed the charlock but left the oats untouched. Further experiments with other weed and crop species showed that NAA selectively killed many dicots while leaving the monocots unharmed. A search of related compounds with similar selective action showed 2-methyl-4-chlorophenoxyacetic acid (MCPA) to be highly active.

Another team of British scientists, Nutman, Thornton and Quastel (1945), while investigating the role of IAA in the formation of root nodules, noticed that, at low concentration, IAA killed red clover but did not affect cereals. Realising that IAA could be used as a selective weed killer they tested other related compounds and found 2,4-dichlorophenoxyacetic acid (2,4-D) to be very active. 2,4-D and MCPA were further tested by Blackman (1945) in field trials and confirmed to be potent selective herbicides.

During the same period 2,4-D was also being investigated in the United

States. In 1944, Marth and Mitchell reported that it killed the weeds *Taraxacum officinale* and *Plantago* spp. in lawns, while Hamner and Tukey (1944) reported field trials of 2,4-D as an herbicide in cereal crops. By 1946, 2,4-D and MCPA were introduced as commercial herbicides and are still widely used today for the selective control of dicot weeds in cereals crops and turf (Fletcher and Kirkwood 1982).

Following the discovery of 2,4-D and MCPA as selective herbicides, many related compounds were tested for selective herbicidal activity and some of them were subsequently introduced as commercial herbicides. 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) which resembles 2,4-D in its herbicidal properties but is more active against many woody species was introduced in 1944 (Fletcher and Kirkwood 1982). Chlorfenac (2,3,6-trichlorophenylacetic acid) was introduced in 1958 as a total weed killer for non-crop areas (Fletcher and Kirkwood 1982). 2,3,6-TBA (2,3,6-trichlorobenzoic acid) was introduced as an herbicide in 1954 and, along with Dicamba (3,6-dichloro-*o*-anisic acid) which was introduced in 1958, is mostly used, in mixture with various phenoxyalkanoics, to extend the spectrum of weeds controlled by these compounds. Another benzoic acid derivative, Chloramben (3-amino-2,5-dichlorobenzoic acid), was introduced in 1958 and is used in pre-emergence control of weeds in soybean, groundnuts, maize, carrots and other non-cereal crops (Fletcher and Kirkwood 1982).

Another important step in the history of the use of auxin-like compounds as selective herbicides was brought about by the work of Wain and his colleagues at

Wye college. Fawcett *et al.* (1954) first reported evidence that the aliphatic side-chain of *w*-phenoxyalkylcarboxylic acids can be degraded through β -oxidation in plant tissues. Wain and Wightman (1954), in comprehensive experiments with homologous series of 4-chloro-, 2,4-dichloro- and 2,4,5-trichloro-substituted phenoxyalkylcarboxylic acids, confirmed the existence of a β -oxidation system in several plants; the chloro-acids with an even number of side-chain methylene groups were converted to the inactive corresponding chloro-phenols, while the ones with an odd numbers of methylene groups were converted to the active corresponding chloro-phenoxyacetic acid (Fig.2). These authors found that the activity of the enzymes of the β -oxidation system was dependent on the type of plant tissue and on the number and position of the ring substituents of the acid. They proposed that this could form the basis for using some of these compounds in selective weed control. Wain (1955) reported the testing of 2,4,5-trichlorophenoxybutyric and -caproic acids on a range of crop and weed plants. He showed that some of the crops were not affected by the Cl-phenoxybutyric or -caproic acids, since these compounds, which are inactive *per se*, were not degraded to the highly active 2,4,5-trichlorophenoxy acetic (2,4,5-T), while many of the weeds associated with the crops were destroyed by the compounds due to their ability to metabolize the trichloro-acids to the toxic end product, 2,4,5-T.

The work of Wain and his colleagues led to the introduction of the phenoxybutyric herbicides 2-methyl-4-chlorophenoxybutyric acid (MCPB) and

Figure 2. β -oxidation of *w*-phenoxycarboxilic acids.

a) β -oxidation of 2,4-dichlorophenoxybutyric acid, with an odd number of methylene groups, resulting in the production of highly active 2,4-dichlorophenoxyacetic acid.

b) β -oxidation of 2,4-dichlorophenoxypropionic acid, with an even number of methylene groups, resulting in the production of an inactive phenol. From: Wain and Wightman (1954).

a)

b)

2,4-dichlorophenoxybutyric acid (2,4-DB) which are used for the selective control of broad-leaved weeds in certain leguminous crops. The transformation, through metabolism by a plant or other organism, of an inactive compound to an active one, is termed lethal synthesis or lethal metabolism. This phenomenon has been widely used in the design of other pesticides.

Even today, 2,4-D and related compounds, such as 2,4-DB, are amongst the most widely used of herbicides. This is not surprising since the crops in which they are used, cereals and certain leguminous crops, are the main source of food for both man and animals (Hassall 1990).

2.1.2 Physiological Aspects

Auxin-like herbicides are systemic herbicides and most of them are applied through foliage-spray. Translocation from the leaves to the various parts of the plants is normally in the symplast (Hassall 1990). The major routes of metabolism or detoxification are through ring hydroxylation and/or conjugation with glucose or amino acids (Hassall 1990).

The difference, from one plant species to another, in susceptibility to this class of herbicides can depend on physiological and/or morphological factors. Among the possible factors are species differences in the rate of uptake and translocation, in the rate and type of detoxification of the herbicide, and in the ability to transform an inactive precursor into a corresponding active substance (Hassall 1990, White *et al.* 1990).

The main symptoms of phytotoxicity caused by auxin-like herbicides are:

petiole and stem epinasty, lateral swelling of parenchyma cells leading to callus tissue, tumor and adventitious root formation, arrest of root elongation and swelling of the roots tips and, eventually, plugging and collapse of the vascular system. All these changes contribute in bringing about the death of the plant (Hassall 1990, Ashton and Crafts 1973).

The exact mechanism leading to abnormal growth is still unknown. It is thought that auxin-like herbicides act as persistent auxins; their concentrations not fluctuating in response to stimuli in the same way as that of endogenous auxins do. High concentrations of auxins cause symptoms similar to those elicited by 2,4-D (Hassall 1990). Also, transgenic tobacco plants synthesizing abnormally high levels of natural auxins, displayed growth abnormalities similar to those resulting from an application of 2,4-D (Klee *et al.* 1987). Both IAA and 2,4-D, when given at sub-lethal doses, can differentially induce the expression of certain genes (Hagen *et al.* 1984, Takahashi *et al.* 1989). Several-fold increases in RNA levels have been observed following application of herbicidal doses of 2,4-D (Hassall 1990). The herbicide, through its persistent auxin effect, could cause uncontrolled and prolonged expression of various genes, thus leading, directly or indirectly, to the growth abnormalities and eventual death observed in susceptible plants (Hassall 1990). For example, one of the many effects caused by the application of auxinic herbicides to susceptible plants is an auxin-stimulated increase in ethylene biosynthesis through the induction of ACC synthase. Ethylene has been shown to be responsible for some of the abnormal growth seen in susceptible plants. Also,

cyanide is a by-product of ethylene biosynthesis. It has been suggested that increases in cyanide levels could contribute to the death of a plant by stimulating inefficient alternate respiratory pathways, as well as inhibiting various enzymes such as nitrate reductase (Tittle *et al.* 1990, Chkanikov *et al.* 1990). In addition to their effect on gene expression, auxin-like herbicides probably affect other aspects of plant growth. For example, Goldsworthy and Mina (1991) suggested that part of the phytotoxic effect of 2,4-D might be attributed to its capacity to destroy pre-determined polarity in cells.

2.2 Structure-Activity Relationships

Numerous studies have been carried out on the structure-activity relationships of auxin-like growth-promoting substances such as phenoxyacetic, phenylacetic, benzoic and cinnamic acids and their derivatives. Some general structural requirements for activity have emerged and within each sub-group of compounds, e.g. phenylacetic acids, activity has been correlated to type and position of substituents. A comprehensive theory, rationalizing the link between structure and activity of all the molecules that possess auxin-like growth-promoting properties, has still however to be found. Over the years many theories have been proposed, notably by Koepfli, Thimann and Went 1938, Muir and Hansch 1951 and 1952, Veldstra 1944 and 1953, Porter and Thimann 1965, Kaethner 1977 and Katekar 1979, but none have satisfactorily explained all of the observed correlations between the chemical structure of different compounds and their physiological activity, or inactivity.

Two of the basic structural requirements for auxin-like activity are the presence of an unsaturated aromatic ring and a carboxyl group or a group that can convert to a carboxyl group. This group may be attached directly to the unsaturated ring or through a chain of one or more carbon atoms (Koepfli *et al* 1938, Muir and Hansch 1951). These two requirements are basic in the sense that they are shared by all the main groups of auxin-like growth-regulators. When indolylacetic, naphthylacetic, phenoxyacetic, phenylacetic, benzoic and cinnamic acid derivatives are compared, however, it is found that ring-substituent positional requirements for activity vary from one group to the other (Pybus *et al.* 1959 a and b, Ugochukwu and Wain 1967, Katekar and Geissler 1982).

Only the physiological activity and structure-activity relationships of phenylacetic, benzoic and cinnamic acids and their derivatives will be considered in detail since these compounds are the ones with which this study is concerned. To give the reader a basis of reference as to the activity level of the different compounds, activity of the derivatives will be compared to that of 2,4-dichlorophenoxyacetic acid (2,4-D), the powerful synthetic auxin and well-known herbicide. The proposed auxin receptor structure (Katekar 1979) and how it explains the activity/inactivity of some of the auxin-like compound will also be discussed.

2.2.1 Phenylacetic Acid and its Derivatives.

Haagen-Smit and Went (1935) were the first to examine the growth-regulating activity of phenylacetic acid (PAA) and found it to be active in the pea

curvature test. In the same year, Zimmerman and Wilcoxon (1935) reported that phenylacetic acid could induce responses such as root initiation, swelling and bending of stems and leaf epinasty in a variety of plant species, when applied locally in a lanolin paste or given in a water solution. Since then, more evidence has been found that PAA might be involved in a number of physiological processes in plants. Phenylacetic acid at a concentration of 10^{-4} M is more active than IAA in inducing lateral root primordia in primary roots of pea seedlings (Wightman *et al.* 1980). It can support the growth of tobacco callus tissue cultures in a similar way to IAA or 2,4-D, although it must be used at a higher concentration due to its lower auxin activity (Milborrow *et al.* 1975, Leuba and LeTourneau 1990). Exogenously applied PAA, like IAA, can cause thickening of the upper part of decapitated pea epicotyls and inhibit the growth of lateral buds (Prochazka *et al.* 1983). It can also delay abscission in cotton petioles (Suttle and Mansager 1986) although its effect in the last two phenomena is more short term than that of IAA. PAA has also been shown to induce ethylene formation in wheat seedlings (Machackova *et al.* 1981), and the presence of PAA was found to increase IAA stimulation of membrane bound ATPase isolated from pumpkin hypocotyls (Scherer 1981). Recent work has demonstrated that PAA may be involved in regulation of auxin transport (Morris and Johnson 1987).

Following the discovery of the growth promoting activity of PAA (Haagen-Smit and Went 1935) some of its derivatives were also examined for physiological activity. Pybus *et al.* (1958, 1959b) investigated the influence of chlorine-ring-

substitution on the plant growth-regulating activity of PAA. They used the wheat cylinder, pea segment and pea curvature tests as their assay systems. In general, results from the three tests were in agreement and the results from the wheat cylinder test are shown in Table 1.

Mono-ring-substitution, whatever the position, increased activity over that of the parent molecule. The 2- and 3-Cl-derivatives showed activity comparable to that of 2,4-D while the 4-Cl-derivative exhibited lower activity. Introduction of a second or third chlorine substituent into the ring led to active compounds in all cases, except for the 3,5-Cl-derivative. The 2,3- and 2,3,6-Cl-derivatives showed higher activity than the powerful 2,4-D. Activity of the 2,6-Cl-derivative was comparable to that of 2,4-D, while activity of the 2,5- and 3,4-Cl-derivatives was lower than 2,4-D but still higher than that of the 2,4-Cl-derivative which showed activity similar to that of the mono-substituted 4-Cl-derivative. In summary, the order of activity of the chloro-substituted PAA's was found to be: 4-, 2,4- < 2,5-, 3,4- < 2-, 3-, 2,6- $\approx$ 2,4-D < 2,3- and 2,3,6- (Pybus *et al.* 1959b). The comprehensive results of Pybus *et al.* (1959b) agree with those of other workers. In an earlier study, Weintraub and Norman (1949) had shown that in general halogen- and nitro-groups increase the activity of phenylacetic acid, while methyl-, hydroxyl- and amino-groups have the opposite effect. In a later study, Chamberlain and Wain (1967) also obtained results similar to those of Pybus *et al.* (1959b) for the monochloro-substituted derivatives. The 2,3-, 2,6- and 2,3,6-Cl-PAA were tested on crop and weed plants and showed selective herbicidal activity,

Table 1. Growth-regulating activity of phenylacetic and chloro-substituted phenylacetic acids in the wheat cylinder test. Results from Pybus *et al.* (1959b).

Activity is expressed as percent increase in length over that of the water control.

* Activity significantly different from water control at 0.1% level.

Compound tested	Wheat coleoptile cylinder test concentration tested (M)			
	10^{-6}	10^{-5}	10^{-4}	10^{-3}
2,4-D	37*	51*	45*	32*
Phenylacetic acid (PAA)	0	0	20*	42*
2-chloro-PAA	25*	48*	56*	50*
3-chloro-PAA	20*	51*	47*	46*
4-chloro-PAA	0	18*	50*	36*
2,3-chloro-PAA	57*	60*	58*	40*
2,4-chloro-PAA	3	20*	45*	44*
2,5-chloro-PAA	2	37*	46*	36*
2,6-chloro-PAA	13*	47*	54*	41*
3,4-chloro-PAA	4	40*	36*	8
3,5-chloro-PAA	0	0	6	0

with 2,3- and 2,3,6-Cl-PAA being as effective as 2,4-D (Pybus *et al.* 1958).

2.2.2 Benzoic Acid and its Derivatives

Growth-promoting benzoic acids were first reported by Zimmerman and Hitchcock (1942) who found some of the disubstituted halogen-nitro-benzoic acids able to produce morphogenetic effects in various plants. Pybus *et al.* (1959a) studied the entire series of methyl- and chloro-substituted benzoic acids using the same three physiological tests they had used with the phenylacetic acids. Some of their results are shown in Table 2. Benzoic acid itself was found to be inactive. Introduction of either chlorine or methyl in a single ring-position did not generally lead to activity. Di-substitution, however, lead to the 2,3-, 2,5- and 2,6-dichloro- or dimethyl-substituted compounds being physiologically active, with the 2,5-Cl derivative being as active as 2,4-D. Di-chloro compounds were more active than dimethyl-substituted ones. Tri-chloro-substitution, particularly in the 2,3,6-positions, gives a compound with activity greater than 2,4-D. So with chloro-substituted benzoic acids, the order of activity is as follows: 2,6- < 2,3- < 2,5- $\approx$ 2,4-D < 2,3,6-. It is of interest to note that in this series, substitution in the para-position does not confer any activity to the parent compound. The results obtained by Pybus *et al.* (1959a), as to the general substitution pattern that leads to activity in benzoic acids, agree with observations made in earlier studies by other workers (Muir and Hansch 1951, 1952, Bentley 1950) and the pattern seems to hold for other halogen- or nitro-substituents, not just chlorine. For example, Zimmerman and Hitchcock (1942) found that the 2-bromo-3-nitro- and

Table 2. Growth-regulating activity of benzoic and chloro-substituted benzoic acids in the wheat cylinder test. Results from Pybus *et al.* (1959a).

Activity is expressed as percent increase in length over that of the water control.

* Activity significantly different from water control at 0.1% level.

Compound tested	Wheat coleoptile cylinder test concentration tested (M)			
	10^{-6}	10^{-5}	10^{-4}	10^{-3}
2,4-D	37*	51*	45*	32*
Benzoic acid (BZA)	0	0	0	0
2-Chloro-BZA	0	0	0	10*
3-chloro-BZA	0	0	0	0
4-Chloro-BZA	0	0	0	0
2,3-chloro-BZAA	0	0	29*	27*
2,4-chloro-BZA	0	0	0	0
2,5-chloro-BZA	0	24*	43*	36*
2,6-chloro-BZA	0	0	0	54*
3,4-chloro-BZA	0	0	0	0
3,5-chloro-BZA	0	0	0	0

2-chloro-5-nitro- derivatives were physiologically active compounds.

2.2.3 Cinnamic Acid and its Derivatives

Each cinnamic acid and its derivatives can exist in two geometric isometric forms known as the *cis*- and *trans*- forms. The two series of isomers vary in their plant growth-promoting activity; *cis*-cinnamic acid and most of its derivatives are active while the *trans*- form of cinnamic acid and its derivatives generally are not (Koepfli *et al.* 1938, Haagen-Smit and Went 1935, Ugochukwu and Wain 1968). Ugochukwu and Wain (1968) did a systematic study of the effect of halogen ring-substitution on the growth-regulating activity of *cis*-cinnamic acid. Part of their results are shown in Table 3. They found that unsubstituted cinnamic acid was quite active. Mono-substitution with a chlorine atom in the 2- or 4- position yielded derivatives with higher activity than the parent molecule, while substitution in the 3- position gave a compound that was active but not as much as the unsubstituted acid. Of the di-substituted cinnamics, 2,6-Cl- was a highly active derivative showing activity greater than any of the the active mono-chloro-substituted compounds. The 2,4-Cl-derivative was the second most active of the di-substituted compounds with activity levels lower than those of the 2-Cl- and 4-Cl-derivatives but similar to the unsubstituted acid. All of the other dichloro-substituted cinnamic acids showed activities lower than that of the parent compound. The order of activity of the chloro-substituted cinnamic acids can be summarized as follows: 3,5-, 2,5- < 2,3- < 3,4-, 3- < 2,4- < 2,4-D $\approx$ 4-, 2- < 2,6-. The first two compounds in this list show almost no physiological activity.

Table 3. Growth-regulating activity of cinnamic and chloro-substituted cinnamic acids in the wheat cylinder test. Results from Ugochukwu and Wain (1968).

Activity is expressed as percent increase in length over that of the water control.

* Activity significantly different from water control at 0.1% level.

Compound tested	Wheat coleoptile cylinder test concentration tested (ppm)		
	1	10	100
Cinnamic acid (CNA)	1	38*	45*
2-Chloro-CNA	24*	49*	52*
3-chloro-CNA	1	25*	34*
4-Chloro-CNA	19*	50*	49*
2,3-chloro-CNA	6	14*	2
2,4-chloro-CNA	2	38*	22*
2,5-chloro-CNA	1	5	8*
2,6-chloro-CNA	37*	56*	5T
3,4-chloro-CNA	2	27*	T
3,5-chloro-CNA	0	8*	0

2.2.4. The Auxin Receptor Site

Katekar (1979), taking into account the activity/inactivity of several series of auxin-like compounds, designed a composite auxin receptor site and discussed the structure-activity relationship of the various compounds in relation to his proposed receptor site. According to Katekar, since the auxin receptor is, by hypothesis, complementary to the 3-indolyl acetic acid molecule, it can be represented as in Fig. 3.

The receptor is divided into three main areas. The first is designated the 'carboxyl acceptor' and is the region that binds the carboxyl group of the interacting molecule. The requirement for a carboxyl group, or a group that can mimic a carboxyl group, is basic to activity (Koeplfli *et al.* 1938) and Katekar has suggested that the conformational requirements of the receptor in the carboxyl acceptor area might be highly specific and determine how the remainder of the molecule can interact with the rest of the receptor and thus be determinant for activity.

The second main area is called the ' α -area' and is the portion of the receptor that accepts the methylene carbon of IAA. It is suggested that the α -area might exert a steric effect but that it might not necessarily bind to the α -atom. The absence of steric hindrance in this position in the interacting molecule might be more important for activity than the actual presence of a specific group, e.g. the methylene hydrogen of IAA. The third main area of the receptor site is referred to as the 'electron acceptor' (Ar1, Ar2 and a-f in Fig. 3)

and corresponds to the area that accepts the indole ring.

As demonstrated by the activity of some of the benzoic acids in which all carbon atoms and single atom substituents are planar, the receptor site must be able to interact with molecules having the carboxyl atom, the atom overlying the α -area and the aromatic ring, coplanar. The shape of the receptor is therefore proposed as being planar except for the part that accepts the oxygen or OH atoms of the carboxyl group, as shown in Fig. 3. The absolute configuration is thought to be complementary to the S form of arylpropionic acids.

It is hypothesized (Katekar 1979) that the area of the receptor site that accepts the indole ring of IAA is electrophilic in nature and interacts with the π electrons of the aromatic ring, or of the electron-rich substituents. This view is at variance with most of the preceding structure-activity theories in which the plant substrate was postulated as being nucleophilic, but it agrees with the observations that the effect of substituents on the activity of a parent molecule is to increase that activity if they have available electrons, but to decrease it if they don't. The electron acceptor area is thought to extend beyond the boundaries defined by the indole ring since monochloro-substituted indoleacetic acids show increased activity over the parent molecule (Porter and Thimann 1965). The whole electron receptor area is sub-divided in areas designed as Ar1 and Ar2, which correspond to the pyrrole and benzene ring of the IAA molecule, and surrounding areas labelled a-f which are accessory regions (Fig. 3).

Katekar (1979) suggested that if the carboxyl acceptor and α -area

Figure 3. Structure of 3-indolylacetic acid and of the auxin receptor site in plant cells, as proposed by Katekar (1979).

- a) structure of 3-indolylacetic acid
- b) Top view of the receptor site.
- c) Side view of the receptor site.

Figure 4. Overlap of different growth-regulating molecules with the proposed auxin receptor site. From: Katekar (1979).

- a) indolylacetic acid
- b) phenylacetic acid
- c) phenoxyacetic acid
- d) 2,3-dichlorophenylacetic acid
- e) 2,3,6-trichlorobenzoic acid
- f) 2,4-dichlorophenoxyacetic acid.

requirements are met, then, subject to any shape and conformational requirements imposed by the receptor site, (e.g. steric restraint in some of the accessory regions), activity will result when areas Ar1 and/or Ar2 are covered by the interacting molecule. Fig. 4 shows examples of how some molecules would fit in the postulated site. If only Ar1 or Ar2 is covered, the compound will exhibit only weak activity. Where only Ar1 or Ar2 is covered by an aromatic ring, e.g. phenylacetic acid, substitution with electron-rich substituents, if it has the effect of covering part of the remaining area, will increase activity. This would explain the higher activity of 2,3-Cl-PAA, compared to the lower activity of unsubstituted PAA or the monosubstituted derivatives when substituted in the 2- or 3- position (Fig. 4).

Even if the electron acceptor site is large and non-selective enough to accept a wide range of auxin-like molecules, there still appear to be some size and steric limitations. Steric obstruction is postulated to occur between the 'c' and 'd' areas and also in the 'a' and 'f' areas, (Fig. 3). The effect of steric obstruction is manifested by reduced activity when a molecule impinges on the obstructed areas. Such steric obstruction in the 'c-d' areas would explain the inactivity of para-substituted benzoic acids.

Katekar's (1979) theory explains many of the previously unexplained structure-activity observations but its weakness lies in the fact that it does not explain the activity of cis- isomers of cinnamic acid and its derivatives, unless partial coverage of the 'Ar1' area along with coverage of the 'b' area is sufficient

to confer activity to an interacting molecule.

2.3 Phenylacetic Acid Biosynthesis

Although the growth-regulating activity of synthetic phenylacetic acid has been known since the mid-1930's (Haagen-Smit 1935, Zimmerman and Wicoxon 1935), evidence of its biosynthesis and occurrence as a natural auxin in plants did not come until the mid-1970's.

Early studies on the metabolic pathway of IAA biosynthesis showed that the aromatic amino acid, L-tryptophan, is the precursor of this auxin. The main pathway of IAA synthesis, referred to as the arylpyruvate pathway, was found to involve the formation of 3-indolepyruvic acid and 3-indoleacetaldehyde as intermediates (Wightman and Cohen 1968, Schneider *et al.* 1972, Wightman 1973). The initial conversion of L-tryptophan to 3-indolepyruvic acid is a transamination reaction and it was shown in several plants that this reaction is catalysed by a multispecific aminotransferase capable of transaminating all three aromatic amino acids in addition to aspartic and glutamic acids (Forest and Wightman 1972, Wightman 1973). It was also known by the early 1970's that L-phenylalanine could be converted in plant cell-free extracts to phenylethylamine and that further metabolism of this amine yielded phenylacetaldehyde and phenylacetic acid (Clarke and Mann 1957). Also, in fungi, phenylalanine was shown to be converted to phenylacetic acid (Moore and Towers 1967, Moore *et al.* 1967).

In the light of this information, Wightman and Rauthan (1975) studied the metabolism of ^{14}C -phenylalanine in tomato and other plant shoots to investigate

the possibility that shoot tissue may be able to convert L-phenylalanine to phenylacetic acid via a sequence of reactions similar to those involved in the formation of IAA. After feeding ^{14}C -L-phenylalanine solutions to excised tomato shoots, the radioactive metabolites recovered in an acid ether extract of the homogenized shoots were malonylphenylalanine, phenyllactic acid, phenylpyruvic acid, phenylacetic acid and cinnamic acid. Examination of a basic ether extract showed the presence of ^{14}C -labelled phenylethylamine (Wightman and Rauthan 1975). This metabolite is the decarboxylation product of phenylalanine and would arise from phenylalanine decarboxylase activity. In some species, decarboxylation of tryptophan to form tryptamine is the first step of an auxilliary pathway to IAA synthesis (Schneider and Wightman 1974). The neutral ether fraction from tomato shoots was also examined by Rauthan and Wightman (1975) and some radioactive phenylacetaldehyde was found. After similar tracer experiments with several crop plants, Wightman and Rauthan (1975), concluded that phenylacetic acid is formed in shoots during the metabolism of exogenously supplied ^{14}C -L-phenylalanine and from the nature of the radioactive intermediates detected, it appears that the biosynthetic pathways from phenylalanine to phenylacetic acid are in fact similar to those reported earlier for the conversion of L-tryptophan to 3-indolylacetic acid. The suggested pathways are shown in Fig. 5.

Camirand *et al.* (1983) using tobacco plants and gas chromatography (GLC) for the identification of metabolites, confirmed the production of all the phenyl-metabolites detected by Wightman and Rauthan (1975) following a feeding of

Figure 5. Pathway of phenylacetic acid biosynthesis. From: Wightman and Rauthan (1975)

shoots with L-phenylalanine. These authors also found that L-tyrosine was metabolized to *p*-hydroxy-phenylacetic acid (*p*-OH-PAA) through a metabolic pathway similar to the ones operating in the conversion of L-tryptophan and L-phenylalanine to IAA and PAA, respectively. *p*-OH-PAA was also produced following a feeding with L-phenylalanine through *in vivo* hydroxylation of one of the intermediates in the pathway leading to PAA. However, the reverse phenomenon, namely, dehydroxylation of L-tyrosine metabolites to yield PAA from tyrosine, was not found.

In another phase of this study, Wightman and Rauthan (1975) investigated the natural occurrence of phenylacetic acid in tomato and in four other crop plants. Using paper chromatography for analysing acidic ether extracts of the shoots and a bioassay technique for identifying auxin compounds, they obtained the first evidence that phenylacetic acid occurs with 3-indoleacetic acid as the natural auxins of these plants. Confirmation of the presence of PAA as a natural auxin in many plant species was later reported by Wightman and Lighty (1982) from experiments using bioassay in combination with GLC and GC-MS analytical techniques.

As shown in Fig. 5, the first step in the arylpyruvate pathway to PAA is a transamination reaction. Taylor and Wightman (1987a) purified the enzyme responsible for L-phenylalanine aminotransferase activity in shoots of bushbean seedlings. The enzyme was resolved into two forms, called isozyme I and II, which behaved differently in ion exchange and hydrophobic chromatography. Isozymes I

and II were also able to transaminate many compounds in a series of chloro-substituted phenylalanines; the structure-metabolism relationships of these chloro-phenylalanines will be discussed later.

Isozymes I and II were further characterized by Taylor and Wightman (1987b). They were found to have molecular weights of 100,000 and 110,000 daltons, respectively, and these values are similar to those reported for other plant aminotransferases which generally fall into two size classes of around 50,000 or 110,000 daltons (Wightman and Forest 1978, Ireland and Joy 1985). Isozymes I and II were not resolved into their subunits but aminotransferases of this size are usually composed of two subunits of equal size (Ireland and Joy 1985). pH optima for isozymes I and II were of 8.5-9 and 8.5, respectively. This is also similar to other plant transaminases, the majority of which show pH optima around 8 (Wightman and Forest 1978, Ireland and Joy 1985). Of the common keto acids used as substrates by plant aminotransferases, isozymes I and II were unable to use glyoxylate or pyruvate but could use both 2-oxoglutarate and oxaloacetate. Isozyme I exhibited no preference between these two keto-acids while isozyme II showed more activity with 2-oxoglutarate, at least when D,L-phenylalanine was used as the amino acid substrate. Specificity for the amino acid donor was also tested, using 2-oxoglutarate as the keto acid acceptor. From this examination, the two isozymes were found to be primarily aspartate aminotransferases, which also showed relatively low activities (0.1-5% of that observed with aspartate) with the three aromatic amino acids (Taylor and

Wightman 1987b). The same situation was observed earlier by Forest and Wightman (1972) using a purified aspartate-aromatic aminotransferase from bushbean roots, and has been reported for animal tissue, where much of the aminotransferase activity observed with the three aromatic amino acids, can be attributed to the secondary activity of aspartate aminotransferase (Mason and Deshmukh 1985). Such activity of animal aspartate aminotransferase varies from as low as 0.1% to as high as 30% of the activity observed with L-aspartate, depending on the source from which the enzyme is isolated and the aromatic acid used as a substrate (Mason and Deshmukh 1985).

Beside L-aspartate and the three aromatic acids, L-glutamate was the only other amino acid that could serve as a substrate for the bushbean Isoenzymes I and II. The two isozymes exhibited different specificities toward the aromatic amino acids with isozyme I preferring L-phenylalanine over L-tryptophan or L-tyrosine while, the reverse was true for isozyme II (Taylor and Wightman 1987b). Other plant, microbial and animal aspartate-aromatic aminotransferases which are present in multiple forms also exhibit differential specificity for their amino acid and ketoacid substrates (Mason and Deshmukh 1985, De-Eknamkul and Ellis 1987, Yagi *et al.* 1990). All three forms of the enzyme responsible for L-tyrosine aminotransferase activity in cell cultures of *Achusa officinalis* are multispecific but the preferred amino acid and ketoacid substrate is different for each form (De-Eknamkul and Ellis 1987). Kinetics studies with isozyme II, using L-phenylalanine and 2-oxoglutarate as the two substrates, suggest that there is no

cooperativity and only one binding site per subunit. Competition studies indicate that aspartate, glutamate and the three aromatic amino acids all compete for the same site on the enzyme (Taylor and Wightman 1987b).

Taylor and Wightman (1987b) also examined the effect of different inhibitors and cations on the bushbean aspartate-aromatic aminotransferases isozymes I and II. Hydroxylamine, which binds with free carbonyl groups and thus acts as an antagonist to the cofactor pyridoxal-5-phosphate (PLP), inhibited the phenylalanine aminotransferase activity of the two enzymes. Inhibition of enzyme activity by hydroxylamine has been reported for most plant aminotransferases (Wightman and Forest 1978, Ireland and Joy 1985) suggesting the presence of a PLP cofactor in such plant enzymes as has been demonstrated for animal aminotransferases (Arnone *et al.* 1985). No evidence of significant inhibition or stimulation by cations was found with isozyme I but isozyme II, however, was strongly inhibited by Ca^{2+} . This finding is unusual since most plant transaminases are unaffected by cations (Ireland and Joy 1985). Isozymes I and II were found to be localized in the cytoplasm and are responsible for over 95% of the L-phenylalanine aminotransferase activity in the cells of bushbean shoots. Mitochondria and chloroplasts were shown to contain their own form of phenylalanine-aspartate aminotransferase but these two forms did not account for more than 5% of the L-phenylalanine aminotransferase activity of the cells. In this connection, purified chloroplasts and mitochondria from sunflower plants, which contain both IAA and PAA, have been shown to synthesize these two

compounds when supplied with the precursor amino acids, L-tryptophan and L-phenylalanine, thus indicating the presence of an aromatic aminotransferase in both organelles (Wightman and Frégeau 1982, Frégeau and Wightman 1983).

Studies on the active site and catalytic mechanism of aspartate aminotransferase have been made almost exclusively with purified enzyme from an animal source. It appears that no detailed study of the catalytic site and mechanism of action have been made on this enzyme purified from a plant source. There is evidence, though, that the catalytic mechanism in the plant enzyme is a basic ping-pong bi-bi mechanism, similar to that found in the corresponding animal enzyme and involves an aldimine to ketimine conversion (Ireland and Joy 1985, Taylor and Wightman 1987b) .

The preference of the enzyme for dicarboxylic amino acid substrates is mainly determined by the interaction of these substrates with two arginine residues forming part of the active site. The electronic interactions between the carboxylate groups and the positively charged Arg groups is thought to help the transition from an open to a close conformation of the enzyme, and to stabilize the closed conformation which is necessary for an efficient aldimine to ketimine conversion (Arnone *et al.* 1985, Kirsh *et al.* 1984). Taylor and Wightman (1987c) have suggested that the ability of aspartate aminotransferase to act also on aromatic amino acids, albeit with much lower efficiency, comes from the affinity of the electron-rich ring of the aromatic amino acids for one of the arginine residues of the active site, which would normally be occupied by the distal carboxylate of

aspartic acid.

Taylor and Wightman (1987c) also carried out a study to relate the structure of a series of ring- and side chain-substituted phenylalanines to their activity as substrates for the bushbean shoot aspartate-aromatic aminotransferase isozymes I and II. Side-chain substitution of phenylalanine, whether at the amino-N or α -carboxyl group, was found to inactivate the amino acid as a substrate for both isozymes. Substitution on the nitrogen of the amino group would prohibit the formation of the transaldimination intermediate due to the lack of a protonated amino group, while substitution at the α -carboxyl group is thought to prevent an important ionic pairing with a strongly positive amino acid residue at the active site of the enzyme thus preventing formation of the 'enzyme-substrate' complex.

When the effects of ring-substitution were studied, isozyme II was found to transaminate all of the halogen substituted derivatives containing fluoro-, chloro-, iodo- and bromo- substituents. For the chloro- and fluoro-substituted analogues moving the substituent from the 2- to the 3- and then the 4-position of the phenyl ring lead to increasing ability of the analogue to serve as substrate for the enzyme. Substitution in the 4- position, whether with an halogen atom or with an amino group, which was the most active of the substituents, resulted in transamination rates 1.5 to 5 times greater than those observed with unsubstituted D,L-phenylalanine. The 4-nitro- analogue was totally inactive as an aminotransferase substrate. Isozyme I was less reactive than isozyme II with the different ring-substituted analogues. The 2-fluoro and the 2-Cl derivatives were not

transaminated. Halogen or amino substitution in the 4-position led to good rates of transamination with isozyme I, but not as high as those obtained with isozyme II, the amino-substituted analogue being the only one showing greater activity as a substrate than the parent amino acid. Again, 4-nitro-D,L-phenylalanine was inactive (Taylor and Wightman 1987c).

2.4 Benzoic Acid Biosynthesis.

The formation of benzoic acid in plants results from side-chain degradation of cinnamic acid (Gross 1985). The exact enzymatic sequence leading from cinnamic to benzoic acid, however, is still a matter of debate. Zenk (1979) proposed that it occurs through a reaction sequence analogous to the β -oxidation of fatty acids. According to Zenk's hypothesis, cinnamic acid is first activated through the formation of a cinnamoyl-CoA thioester by the action of a cinnamate-CoA ligase. The activated cinnamoyl-CoA then enters a β -oxidation pathway leading to benzoyl Co-A, which would then be split by a thiol esterase to give benzoic acid. This sequence of reactions requires the presence of ATP and CoA. Results of experiments by Alibert and coworkers (1971, 1972), where the degradation of cinnamic acid to benzoic acid in cell-free extracts from *Quercus pedunculata* roots was stimulated upon addition of ATP and CoA, support Zenk's hypothesis. Alibert *et al.* (1972) suggested that the location of benzoic acid synthesis was in the microbodies.

Loffelhardt and Kindl (1975, 1976) working with some higher plants and a blue-green alga, found that the main enzymatic system responsible for the

formation of benzoic acid was thylakoid-bound and that some of the enzymes involved were tightly coupled. Contrary to what had been observed in *Quercus* by Alibert and co-workers (1971, 1972), no stimulation of benzoic acid synthesis was found upon addition of ATP or CoA. Similarly, in cell-free extracts of potato tubers and *Cucumis sativus*, the conversion from *p*-coumaric acid to *p*-hydroxybenzoic acid was not stimulated by addition of CoA or ATP (French *et al* 1976, Hagel and Kindl 1975) and these authors considered that the conversion might occur through a non-oxidative pathway. No thorough investigation of these two possible pathways appears to have been done in recent years and consequently, whether one or the other, or perhaps both pathways operate in benzoic acid biosynthesis in plants remains an open question. The two possible pathways are shown in Fig. 6.

Another possible route for benzoic acid formation would be from further metabolism of PAA, through a set of reactions similar to that leading to the formation of indole-3-carboxylic acid from IAA. The peroxidase-catalysed decarboxylative pathway of IAA degradation leads, via indole-3-methanol and indole-3-aldehyde, to indole-3-carboxylic acid (Grambow and Langenbeck-Schwich 1983, Wiese and Grambow 1986, Sandberg *et al.* 1984). Should PAA be further metabolized in plants in a similar fashion as IAA, the equivalent product to indolecarboxylic acid would be benzoic acid (Taylor *et al.* 1988).

Figure 6. Proposed pathways for the biosynthesis of benzoic acid from cinnamic acid.

2.5 Cinnamic Acid Biosynthesis

The elimination from phenylalanine of -NH_3^+ and the (pro-3s)-hydrogen to yield trans-cinnamate is catalyzed by phenylammonia-lyase (PAL). The formation of cinnamic acid from phenylalanine is the first step in phenylpropanoid metabolism that leads to the formation of a wide range of secondary metabolites which are involved in defense responses and stress adaptation in plants (Hanson and Havir 1981). Cinnamic acid is converted, through a sequence of hydroxylation and methylation reactions, to several substituted acids that can be activated by conversion to their corresponding esters of coenzymeA. These activated esters then enter different biosynthetic pathways leading to lignins, flavonoids, stilbenes, benzoic acids and other compounds (Grisebach 1981). Considering its primary position in these important metabolic pathways, it is not surprising to find that PAL activity is regulated by various environmental factors such as light, irradiation, pathogen infection and wounding (Hanson and Havir 1981).

PAL has been purified from a variety of sources, both plant and fungal. The native enzyme is tetrameric with a sub-unit molecular weight of 83,000 or 77,000 daltons depending on the plant species from which it is extracted (Hanson and Havir 1981, Bolwell *et al.* 1985). It is mostly a cytosolic enzyme, but association with membranous structures has been reported (Shaw *et al.* 1990). Within a given plant species, PAL can be present in multiple forms which have different physical and kinetic properties (Bolwell *et al.* 1985, Lopez-Valbuena *et al.* 1991). PAL purified from *Vicia faba* leaves was found to be present in three isoforms (Lopez-Valbuena *et al.* 1990). *In vivo* labelling and *in vitro* translation

studies have demonstrated that environmental control of PAL induction is at the level of *de novo* synthesis of the enzyme (Bolwell et al. 1985). PAL is encoded by a small family of genes and it has been shown, through RNA blot hybridization and nuclear run-off experiments, that environmental stimuli such as wounding, light or fungal elicitors rapidly stimulate transcription of PAL genes (Lois et al. 1989). The stimulation of gene transcription can be differential. In *Phaseolus vulgaris*, transcription of all three PAL genes was induced by wounding, but only PAL1 and PAL2 genes were induced by fungal elicitor treatment (Cramer et al. 1989).

Even though PAL is a tetrameric enzyme it contains only two active sites (Hanson and Havir 1981). The enzyme has been reported as being allosteric and exhibiting negative cooperativity between the two active sites (Hanson and Havir 1981). Bolwell *et al.* (1985), however, suggested that this apparent negative cooperativity might have been the result of kinetic analysis being performed on enzyme preparations that were not sufficiently purified and thus contained many forms of the enzyme. In their study of PAL from *P. vulgaris*, these authors found that initial rate analysis of PAL activity in the eluate from ion-exchange chromatography gave non-linear Edie-Hofstee plots, typical of plots previously interpreted as showing negative cooperativity of the enzyme from a variety of sources. Further purification of the enzyme by chromatofocussing, however, yielded four forms of the enzyme. Each of these forms when assayed individually exhibited normal Michaelis-Menten kinetics and no apparent cooperativity.

The catalytic activity of PAL can be modulated, either positively or negatively, by some of the end-products of the diverging metabolic pathways that have PAL as common initial step (Dubery 1990). PAL from potato and also probably other species is glycosylated. In the potato enzyme, mannose residue(s) occur on an N-linked oligosaccharide side chain. The enzyme appears to be glycosylated in more than one site with fucose, possibly part of an O-linked side chain (Shaw *et al.* 1990). These authors suggested that glycosylation was important for folding of the protein into the conformation resulting in proper orientation of the active site residue for maximal catalytic activity of PAL.

The enzyme's active site contains an electrophilic center which combines with the amino group of phenylalanine to facilitate its removal. Labelling of the active site with nitromethane followed by HCl hydrolysis of the inactivated enzyme and study of the hydrolysis products has given strong evidence that the prosthetic group is a dehydroalanine residue (Havir and Hanson 1975).

Based on results from studies using deuterium and nitrogen-15 isotope effect, an E1cb catalytic mechanism with formation of a carbanion intermediate has been postulated for PAL. The suggested catalytical mechanism is shown in Fig. 7 (Hermes *et al.* 1985). The actual substrate of PAL is the unprotonated phenylalanine with the free amino group available for a nucleophilic addition to the dehydroalanine residue of the enzyme thus leading to the formation of the activated enzyme-substrate complex. The pro-s hydrogen is removed; forming a carbanion intermediate. The amino group then leaves the substrate, the double

Figure 7. Postulated catalytical mechanism of phenylammonia-lyase. From: Hermes *et al.* (1985).

bond forms and trans-cinnamate, the product, is released. Following release of trans-cinnamate, deamination of the prosthetic group on the enzyme occurs through hydrolysis and the base resulting from catalytic removal of the pro-S hydrogen of phenylalanine exchanges with the solvent thus restoring the enzyme to its original form. The exact way by which the carbanion intermediate is stabilized is still unknown. When carbanions are formed α to a carboxyl group, the electrons appear to be delocalized into the carboxyl group to give an aci-carboxylate structure. In the proposed catalytic mechanism of phenylammonia lyase, however, the negative charge resulting from removal of the pro-S hydrogen would have to be temporarily delocalized into the phenylalanine ring. Possibly, a positively charged group in the enzyme active site is in a suitable position to stabilize any negative charge that is delocalized into the ring (Hermes *et al.* 1985).

2.6 Computer Modelling

Computerized molecular modelling is now a widely used technique in the field of biochemistry and organic chemistry. In the field of medical biochemistry it is used in various ways; to explore the conformational, steric and electrostatic properties of agents that interact with biological receptors, to map receptor sites based on complementarity with ligands, to guide the design of new drugs based on models of the receptor site (Yadav *et al.* 1989, Boudon *et al.* 1989). The contribution of computerized molecular modelling to the field of drug design is especially interesting since it reduces the need to work, by trial and error, through the synthesis and experimental evaluation of numerous candidate molecules, which

is a time-consuming and costly process (Wolfe *et al.* 1987). Computer modelling is also very helpful in organic chemistry where it can be used to deduce the conformation of molecules and predict pathways and feasibility of reactions (Straathof *et al.* 1989, Entrena *et al.* 1989). Now that both computers and software programs are becoming more and more powerful, molecular modelling is beginning to be used in DNA research (Foley *et al.* 1989).

Computer modelling of molecules is based on the ability to calculate theoretical energies associated with a given molecule. The methods used for these calculations can be divided in three general categories; empirical or molecular mechanics, semi-empirical (molecular orbitals) or *ab-initio*. The software program used in this study is a molecular mechanics-based one and, for this reason, only this type of method will be considered in more detail.

The term molecular mechanics thus refers to a calculational method designed to give accurate *a priori* structures and energies for molecules. Molecular mechanics, or force field calculations as they are sometimes referred to, are based on a simple classical-mechanical model of molecular structure. In simple terms, the molecular mechanics model considers the molecule as a group of balls connected by springs. The molecule will be in its "normal" or low-energy configuration when the sum of the forces on the springs is at its minimum. The set of functions and parameters that are considered to describe the properties of the model are referred to as a force field. The exact content of a force field will vary from author to author (Burkert and Allinger 1982). One of the most widely

used force field is Allinger's MM2 (Allinger 1977). In this force field the following basic relationships apply:

$$E_s = K_s/2 (l - l_0)^2$$

$$E_b = K_b/2 (\theta - \theta_0)^2$$

$$E_{vdw} = K_v (c_1 \exp(-c_2 r/r_v) - c_3 (r/r_v)^6)$$

$$V_t = \{ V_1/2 (1 + \cos w) + V_2/2 (1 - \cos 2w) + V_3/3 (1 + \cos 3w) \},$$

where the first function represents the bond stretching energy, E_s , which is a parabolic function centered about the equilibrium distance " l_0 ", with the steepness of the curve modified by the stretching constant, K_s , and with " l " representing the length of the bond considered. The second function, which considers the bending energy, is similar to the stretching energy function. The third function considers the steric energy of non-bonded atoms, not including atoms linked through a central atom, through the calculation of Van der Waals energies. " r " is the interatomic distance, r_v is the sum of the Van der Waals radii of the two atoms concerned while K_v , a constant which determines the steepness of the curve, is the geometric mean of the hardness parameters of the two atoms. The fourth function represents the torsional energy, V_t . This quantity adds steric energy as groups interact in a torsional sense. " w " represents the dihedral angle, while V_1 , V_2 and V_3 are one, two and three-fold torsional barriers, respectively. Other interactions which are considered in the model are the dipole moment and the stretch-bend energies, the last one compensating for the interaction of two atoms linked to a central atom (Allinger 1977).

The MMP2 force field and program which was used in this study, was also designed by Allinger in 1985 and is a modified and improved version of MM2. MMP2 is specially adapted for molecules with a conjugated π system and some of the functions have been improved through inclusion of additional terms. A detailed description of the MMP2 functions and of how the computer program works can be found in Clark (1986).

While the force field defines the mechanical model used to represent the molecule, the purpose of the computer program is to use the functions and parameters of the force field to determine the optimum structure and energy. Input to the program defines a starting structure. The first step is the determination of the interatomic distances, bond angles and torsional angles in the starting structure. The values obtained are then used, in combination with the functions, to calculate an initial steric energy, which is an integration of the potential energies calculated for all the bonds and atoms; stretching, bending, Van der Waals, torsional and stretch-bend energies. The program then optimizes the geometry of the molecule through a minimization of the steric energy and comes up with the steric geometry which corresponds to the low-energy conformation (Clark 1986).

3. Material and Methods

3.1 Plant Material.

Metabolism of the chloro-phenylalanines was examined in one crop plant, broad bean (*Vicia faba* var. Toto) and two weeds : Canadian thistle (*Cirsium arvense* (L.) Scop.) and wild mustard (*Sinapis arvensis* L.). Broadbean seeds were purchased from Stoke's seeds. Canadian thistle and wild mustard seeds were collected during the summer 1988 in the region of Aylmer (P.Q.). The thistle plants were located in a stretch of non-cultivated land while the wild mustard plants were found in an oat field. The weed seeds were stored for several weeks in the dark at 4°C before their capacity to germinate was examined.

All seedlings were grown in an air-conditioned greenhouse at 25/20°C day/night temperatures and 16h photoperiods, with daylight supplemented with 300W Philips photoflood incandescent lights. The soil mixture consisted of 1/3 perlite, 1/3 peat moss and 1/3 washed sand. Plants were fertilized once a week with "plant prod" (20-20-20 nitrogen-phosphorus-potassium, plus minor elements and chelated irons) and were watered once or twice daily as required to keep the soil to full water-holding capacity. Except for *V. faba*, all seeds were germinated in plastic trays or 10-inch pots which were kept in a growth cabinet (under 16h photoperiod provided by white-fluorescent and incandescent lights and at 25/20°C day/night temperatures) until the seedlings had reached the two-leaf stage. At that point, the plantlets were repotted at a density of one and four plants per five-inch pot for *C. arvense* and *S. arvensis*, respectively, and then transferred to the

greenhouse. *V. faba* seeds were sown directly in 6-inch pots, 5 seeds per pot, and allowed to germinate in the greenhouse. When the seeds had germinated, the smallest seedling of the five was removed leaving four plants per pot. *C. arvense* seeds were either pre-chilled for two months prior to sowing or forced to germinate without pre chilling by exposing the sown, but not soil-covered, seeds to a 12 h photoperiod of high intensity light ($250 \text{ microeinsteins m}^{-2} \text{ sec}^{-1}$) and day/night temperatures of 30/18°C in a growth cabinet equipped with white fluorescent lights until germination occurred. The trays were then transferred to normal growth cabinet conditions as stated above. *S. arvensis* seeds were subjected to a 24 h imbibition period in a 10^{-6} M gibberellic acid solution prior to sowing.

Metabolism experiments were normally performed on plants before they had reached the flower bud stage and were 3, 5 and 8 weeks old for *V. faba*, *S. arvensis* and *C. arvense* respectively.

3.2 Metabolism Experiments

Each metabolism experiment was carried out by placing excised shoots of the crop or weed species in two beakers containing solutions of the different D,L-chloro-phenylalanines and allowing the shoots to absorb and metabolize the solutions under controlled conditions over a period of 24 h. The ring-substituted chloro-phenylalanines used in this study were synthesized by Dr. D.C. Taylor (see Taylor *et al.* 1987) and the different isomers are listed in Table 4 along with their melting points.

Table 4. Chloro-ring-substitution patterns and melting points of the chloro-phenylalanines used in this study.

Melting points from Taylor *et al.* (1987).

Substitution pattern	Melting point (°C)*
2-chloro-	216-219
3-chloro-	220-221
4-chloro-	228-229
2,3-dichloro-	238-240
2,4-dichloro-	239-241
2,5-dichloro-	237-238
2,6-dichloro-	257-259
3,4-dichloro-	213-216
3,5-dichloro-	229-230

Three control treatments were used in each experiment. In the first, the shoots were excised and immediately frozen. In the second and third controls, the excised shoots were fed either distilled water or unsubstituted DL-phenylalanine, respectively, using the same experimental procedure as that used in the chloro-phenylalanine feedings. Comparison of the levels of acidic metabolites found in the distilled water-fed plants to those found in the freshly excised and frozen shoots (first control) indicates the effects, if any, of excision and experimental manipulation on shoot metabolism. The purpose of the third control is to verify that the normal biochemical pathways that lead to conversion of phenylalanine to phenylacetic, benzoic and cinnamic acids are, in fact, active in the plant species being studied. So, in each experiment there was a total of 12 treatments; 3 controls and 9 different chloro-phenylalanine solutions. A total of nine metabolism experiments were performed: 3 repeats of each of *V. faba*, *C. arvense* and *S. arvensis*.

At the start of each experiment, ten shoots of the plant species under study were excised (approximately 23 cm below the youngest leaf) and weighed individually in order to calculate an average shoot fresh weight. The number of shoots required to obtain a total fresh weight of approximately 50g per treatment was then determined. This total shoot weight resulted in 4, 8 and 16 shoots for *C. arvense*, *V. faba* and *S. arvensis*, respectively, being used with each treatment solution.

50 ml treatment solutions, each at a concentration of 1×10^{-4} M were

prepared for D-L-phenylalanine and each of the D-L-chloro-phenylalanines treatments. 50 ml of distilled water was also prepared for the third control treatment. For each treatment, the 50 ml solution was divided equally into two 150 ml beakers. The appropriate number of shoots was then excised (at approximately 25 cm down from the tip of the youngest leaf) and placed in a beaker of water at room temperature. The basal 2 cm of each shoot was then recut underwater to remove any air locks that might have formed in the vascular system and the shoots were left to stand in water for about five minutes. The shoots were then transferred to beakers containing the experimental solutions, an equal number of shoots being placed in each of the two beakers and the total weight of the shoots was recorded. The beakers were randomly placed in a growth cabinet at 25°C and under 250 microeinsteins m⁻² sec⁻¹ light intensity, and the time was recorded as time 0. This procedure was repeated for each of the 11 treatments.

When the shoots had absorbed most of the solutions (or as much as possible without the level of the solution falling below the cut ends of the shoots), they were supplied with a 25 ml aliquot of distilled water and the time recorded. This was repeated a second time and after the second aliquot of water had been absorbed, the beakers were filled with 150 ml of distilled water and the plants left to metabolize for a total of 24h from the start of the experiment. At this time, the amount of solution absorbed since the last water addition was recorded, the shoots were re-weighed and quickly frozen with liquid nitrogen. A 50g sample of shoots

was freshly excised and immediately frozen at the start of each experiment to serve as the zero time or unfed (first) control. All frozen samples were stored at -22°C until extraction of the acidic metabolites could be undertaken. Taylor *et al.* (1988) using a similar experimental procedure for feeding D,L-phenylalanine solutions to plant shoots found that in all cases, over 95% of the D,L-phenylalanine was taken up by the shoot tissue.

3.3 Extraction of Acidic Metabolites

For each sample, the frozen plant material along with 250ml of "grinding medium" (containing 1000 ml methanol, 125 ml permanganate double distilled water, 125 ml 1M ammonium acetate buffer at Ph 6.5 and 10 ml 2-mercaptoethanol) and the appropriate internal standards were homogenized for 1 min at maximum speed in the glass container of a Waring blender. The internal standards used were 2,6-dichlorophenylacetic acid, 2,6-dichlorobenzoic acid and 2,6-dichlorocinnamic acid (20 µg of each) for each of the three control treatments and the three monochloro-phenylalanine treatments. 4-chlorophenylacetic acid, 4-chlorobenzoic acid and 4-chlorocinnamic acid (20 µg of each) were used as internal standards for each of the dichloro-phenylalanine treatments. Each homogenate was transferred to a 600ml beaker and left to stir overnight on a magnastirrer at 4°C to allow for maximum extraction of the metabolites.

During the next day, each homogenate was filtered through a Buchner funnel, using Whatman No.1 paper disc, and the plant residue was washed twice with 50 ml volumes of re-distilled methanol. The filtrate and methanol washings

were transferred to a 1000 ml round flask and concentrated to the aqueous fraction on a rota-evaporator . This concentrated fraction (approximately 60 ml) along with a 20 ml water washing of the evaporator flask were transferred, in equal amounts, to two centrifuge tubes and frozen to precipitate the remaining chloroplast fragment proteins and pigments.

In the next step, the aqueous fraction was thawed and the precipitated pigments and any other solid material were sedimented by centrifugation at 12,000 rpm (12,300 x g) for 15 min in a Sorvall RC2-B refrigerated centrifuge. For each sample the two supernatants were collected, pooled and the pH adjusted to 2.5 with 3N phosphoric acid. The sample was then extracted three times with equal volumes of re-distilled ether and the combined extracts were rota-evaporated to 3 ml. The sample was then transferred, using a pasteur pipette, to a 4 ml silicon-glass vial and evaporated further by putting it in a Reactitherm (Lab-Line) Multi Block Heater (# 2090) at 35°C under a flow of nitrogen gas. The two 2 ml acetone washings of the rota-evaporator flask were added to the sample as it evaporated. Each sample was evaporated to a final volume of 0.5 ml.

High pressure liquid chromatography (HPLC) of each sample was preceded by a purification step using a Prepsep C-18 mini-column (Fisher brand). A 5cc syringe linked to the mini-column by a piece of tubing was used to draw the solvents and sample through the column at a rate of about one drop per second. Pre-conditioning of the mini-column was accomplished by flushing twice with 2 ml of 0.1N formic acid in HPLC-grade methanol followed by two flushings with 2

ml 0.1N formic acid in distilled water. The concentrated sample was made up to 4ml with 0.1N formic acid in 20% methanol, filtered using a Millipore filter with .45 μ m pore size (HAWP 01300) and applied to the Prepsep mini-column. The eluant was discarded. The adsorbed sample was first washed with 2 ml of 0.1N formic acid in water followed by a wash with 0.5 ml of 0.1N formic acid in 35% methanol. The 35% methanol wash removes some of the unwanted UV absorbing compounds (Taylor et al. 1988). The sample was then eluted from the column with 0.5 ml of 0.1N formic acid in methanol, transferred to a 4ml vial and made up to 1 ml with 0.1N formic acid in distilled water. The resulting sample was Milipore-filtered just prior to injection in the HPLC.

HPLC was carried out using a Beckman system comprising two model 110 pumps connected to a Partisil 10 ODS-3 M9\25 Whatman column coupled to a dual wavelength UV detector (Autex model 152) set at 254 nm and a linear chart recorder (Recordall series 5000). Solvent flow was maintained at 2.5 ml/min at 1000-2000 PSI and the solvent gradient was controlled by a system controller-programmer (Beckman model 420). Reverse phase chromatography was performed using the following elution gradient: from 20% to 50% solvent B (0.1N formic acid in methanol) in solvent A (0.1N formic acid in water) from time 0 to 10 min, isocratic elution for 8 min, from 50% to 65% B in A from 18 to 28 min, isocratic elution for 22 min and then from 65% to 100% B in A from 50 to 55 min. Isocratic flow of 100% B was continued for 10 min and then from 100% down to 20% B in A from 65 to 72 min at which time the column was ready for

injection of the next sample to be analyzed (see Fig. 8). For each sample the HPLC eluate was collected from 2 min before the UV peak of the first expected metabolite to 4 minutes after the UV peak of the last expected metabolite according to the elution times of the authentic standards of metabolites shown in Table 5. The collected fraction was rota-evaporated to a few drops and transferred to a 4 ml vial. The rota-evaporator flask was washed twice with 2 ml of acetone and the washes added to the sample. The content of the vial was evaporated down to dryness at 35°C under a stream of nitrogen gas and stored in the freezer until derivatized.

Pentafluorobenzylbromide (PFBr) was used as the derivatizing agent. The procedure used was adapted from that previously described by Taylor *et al.* (1988). Approximately 20-25 mg of potassium carbonate and 1.5 ml of an acetone solution of PFBr (5 μ l PFBr per ml of solution) were added to each of the concentrated samples. The vials were sealed and left to react in a Reactitherm block at 60°C for 1.5 h. The samples were then left to cool down and the residual solvent was evaporated under a flow of nitrogen. To each sample, 1 ml of distilled water was added to redissolve the reaction residue and the sample was then extracted three times with 1ml volumes of HPLC-grade ethyl acetate. The combined extract was evaporated under nitrogen to a volume of 0.8 ml, transferred to a 1 ml Pierce reacti-vial and evaporated down to dryness. The samples were stored in the freezer until they were taken to GC-MS Center at Ottawa University for qualitative and quantitative analysis of the metabolites

Figure 8. Reverse phase HPLC elution gradient.

Table 5. HPLC retention times of chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids.

Chloro-substitution pattern	HPLC retention times (min)		
	Phenylacetic acids	Benzoic acids	Cinnamic acids
Unsubstituted	17.0	18.3	24.2
2-chloro-	22.3	19.3	30.7
3-chloro-	23.3	27.3	31.6
4-chloro-	23.8	27.2	31.4
2,3-chloro-	28.0	17.2	-
2,4-chloro-	30.5	30.1	-
2,5-chloro-	28.2	28.1	-
2,6-chloro-	30.1	17.7	34.4
3,4-chloro-	31.8	38.0	-
3,5-chloro-	33.1	38.1	-

present.

3.4 Qualitative and Quantitative Analysis of Metabolites

The GC-MS analysis of PFB-derivatized metabolites was performed on a VG 7070E Analytical Mass Spectrometer using a 30m Megabore DB5 gas chromatography column. 10 μ l of ethyl acetate was used to redissolve each derivatized sample and aliquots of 0.2 to 0.6 μ l were injected in the GC-MS. After a 2 min isothermal period at the starting temperature of 220°C, a 10°C / min gradient of increasing temperature was applied until the last compound had come out of the GC column and entered the Mass Spectrometer. Identification of each suspected metabolite was achieved by comparing its MS fragmentation pattern with that given by the PFB derivative of the authentic compound.

Standard curves for the quantification of the metabolites were constructed by using mass ratios (mass of metabolite / mass internal standard) as the independent variables and area ratios (area for metabolite / area for internal standard), where area represents the area under the molecular ion peaks of the metabolite and internal standard PFB derivatives observed on the total ion current (TIC) from the GC-MS analysis, as the dependent variables. One standard curve was prepared for each of the possible acidic metabolites. Each standard curve was prepared by using three different mass ratios (20/20 μ g, 10/20 μ g and 2/20 μ g) with four replicates per ratio. For each sample, the appropriate amounts of the authentic compound and internal standard dissolved in methanol were put in a 4 ml vial, evaporated to dryness and derivatized according to the procedure

described earlier. The derivatized samples were analyzed by GC-MS to obtain the corresponding areas. The area ratios were calculated and plotted against the mass ratios to obtain the linear regression equation. An example of standard curve is shown in Fig. 9. From these equations and the area ratios obtained from the GC-MS analysis of the plant samples, the amount of each acidic metabolite produced in the shoots during a metabolism experiment were determined using the following equation:

$$\text{Amount of metabolite } (\mu\text{g}) = \frac{\frac{\text{Area metabolite}}{\text{Area internal standard}} - A}{B} \times 20 \mu\text{g}$$

where

- A and B are the constants from the linear regression equation of the standard curve of the given metabolite ,
- area metabolite and area internal standard are obtained from the GC-MS analysis of the water, phenylalanine or chlorophenylalanine-fed shoot sample,
- 20 μg is the amount of internal standard added at the beginning of the extraction procedure .

The detectability levels were estimated for the monochloro-substituted metabolites and were found to be 500, 23 and 2230 ng for mono-Cl-PAA's, -Cl-BZA's and -Cl-CNA's respectively.

Figure 9. Standard curves for the quantification of 4-Cl-phenylmetabolites.

- a) Standard curve for the quantification of 4-Cl-PAA.
- b) Standard curve for the quantification of 4-Cl-BZA.
- c) Standard curve for the quantification of 4-Cl-CNA.

a)

b)

c)

3.5 Chemicals

All of the ring-substituted mono- and dichloro-phenylalanines used in this study were synthesized and characterized by Dr D.C. Taylor as described in Taylor *et al.* (1987b). The synthesis was done by acetamidomalonate condensation with the appropriate chlorobenzyl halides, followed by acid hydrolysis. The resulting chloro-phenylalanines were characterized as to their melting point, IR spectra, ^1H NMR spectra and the mass spectra of their PFB derivatives.

The mono- and dichloro-phenylacetic acids, benzoic acids and cinnamic acids with ring-substitution patterns corresponding to those of the series of chloro-phenylalanines were required for use as internal standard and/or authentic standards for the identification and quantitation of the expected products of chloro-phenylalanine metabolism. They were either synthesized by Dr. D.C. Taylor, as described in Taylor *et al.* (1987), or purchased when commercially available.

Phenylacetic acid and all of the monochloro-phenylacetic acids were purchased from Aldrich Chemical Company. All of the dichloro-phenylacetic acids were synthesized from the corresponding benzyl halides by a nucleophilic displacement reaction with NaCN in DMSO. The resulting dichloro-phenylacetonitriles were hydrolyzed in base to give the corresponding dichloro-phenylacetic acids.

Benzoic acid and all of the mono- and dichloro-benzoic acids, except for the 2-chloro- and the 2,6-dichloro-benzoic acids were purchased from Aldrich

Chemical Company. The 2-chloro- and 2,6-dichloro-benzoic acids were synthesized from the corresponding benzyl halides by oxidation with Jones reagent.

Cinnamic acid, the monochloro-cinnamic acids and 2,6-dichloro-cinnamic acid were purchased from Aldrich Chemical Company. Pentafluorobenzylbromide, the derivatizing agent, was purchased from Supelco Canada Ltd. All other chemicals used in this study were purchased either from Mallinckrodt, B.D.H. Laboratory Chemicals or Sigma Chemical Company and were of analytical grade.

3.6 Computer Modelling of Chloro-phenylalanines

Computer modelling analysis of the chloro-phenylalanines was performed by Dr. Lee Collier from the Chemistry Department (Carleton University) using the molecular modelling software system "Tribble", (Quantum Chemistry Program Exchange, bulletin 529). MMP2, a molecular mechanics-based routine which is part of "Tribble" software system, was used for conformational analysis of the chloro-phenylalanines. The program "Tribble" was run on a VAX 750 computer.

4. Results

4.1 General results from metabolism experiments; time required for absorption of solutions and observations made on plants during the feeding and metabolism periods.

Vicia faba and *Sinapis arvensis* shoots absorbed the experimental solutions at approximately the same rate, within 2.5 hours, and successive 25 ml aliquots of distilled water were then added at about 2.5 hs intervals. *Cirsium arvense* shoots were slower in absorbing the solutions, taking 4hs, and aliquots of distilled water were added at intervals of about 4 hs. All treatments, except distilled water, led to a slight amount of wilting of the shoots during the first few hours of the feeding period. *Sinapis arvensis* and *Vicia faba* shoots appeared to be more sensitive than *Cirsium arvense* shoots to the experimental manipulations, showing a greater amount of wilting.

4.2 Metabolism of Phenylalanine and Chloro-Phenylalanines in Crop and Weed Plants.

4.2.1 Metabolism of D,L-Phenylalanine in the Three Plant Species

The initial endogenous levels of phenylacetic acid (PAA), benzoic acid (BZA) and cinnamic acid (CNA) found in excised, unfed shoots of the three plant species studied, as well as the amounts of these acids found in excised shoots after 24h feeding with water, or D,L-phenylalanine, are shown in Table 6. On average the initial endogenous amounts of PAA and BZA ranged from about 15 to 125 x

Table 6. Amounts of phenyl metabolites found in three replicate experiments with unfed shoots of crop and weed plants and with excised shoots fed for 24 hr with either distilled water or 10^{-4} M solution of D,L-phenylalanine (PHE).

(1) Amounts estimated from ratio of areas of molecular ion peaks (metabolite / internal standard) in the TIC scan of PFB-derivatized HPLC fractions, relative to ratios of known amounts of metabolite and internal standard.

(2) 50 g samples of shoots were supplied with either 10^{-4} M solutions of D,L-phenylalanine (50ml) or with distilled water. Unfed shoots were frozen at -22°C immediately after harvesting.

Species treated	Feeding solutions (2)	Metabolites produced (10^{-2} nmoles * g^{-1} fresh weight) (1)					
		Phenylacetic acid		Benzoic acid		Cinnamic acid	
		replicate data	mean	replicate data	mean	replicate data	mean
<i>Vicia faba</i>	UNFED	---		273		0	
		---	125	145	349	0	0
		125		630		0	
	WATER	262		589		---	
		207	242	158	379	0	0
		257		389		0	
	PHE	555		926		0	
		234	442	209	494	0	0
		537		347		0	
<i>Cirsium arvense</i>	UNFED	0		15		0	
		42	14	0	7	0	0
		0		---		0	
	WATER	0		22		0	
		126	52	166	75	0	0
		30		58		0	
	PHE	0		406		0	
		0	0	---	342	0	0
		0		278		0	
<i>Sinapis arvensis</i>	UNFED	---		288		0	
		41	46	170	184	0	0
		50		94		0	
	WATER	37		301		14	
		17	55	0	155	0	5
		110		165		0	
	PHE	55		406		35	
		---	55	---	418	---	12
		---		430		0	

10^{-2} nmoles per g fresh weight of shoot tissue for PAA, and from about 10 to 350 $\times 10^{-2}$ nmoles per g fresh weight shoot tissue for BZA.

In *Vicia faba*, PAA and BZA were detected in all three control treatments; unfed shoots (UNFED), water- (WATER) and D,L-phenylalanine- (PHE) fed shoots. In each treatment, the levels of BZA detected were higher than those of PAA. Cinnamic acid was not found in any of the control treatments. A comparison of the three treatments for the amounts of PAA and BZA detected in the shoots (Table 6) shows that the phenylalanine feeding led to an over three-fold increase in the amount of PAA detected over the initial endogenous level. The level of BZA also appeared to increase as a result of PHE feeding, but to a lesser extent. In shoots that were excised and fed with water for 24 h, the amounts of PAA and BZA detected were intermediate between those found in the unfed and PHE-fed shoots.

In *Cirsium arvense*, initial endogenous amounts of PAA and BZA were similar and relatively low, less than 15×10^{-2} nmoles per g fresh weight. Both PAA and BZA were also detected in water-fed shoots with BZA being present in slightly higher amounts than PAA. In PHE-fed shoots, however, no PAA was found but BZA was detected in appreciable amounts. CNA was again not detected in any of the control treatments. Comparison of the three treatments for the amounts of PAA and BZA produced shows that, contrary to what was found in *Vicia faba*, feeding of *Cirsium arvense* with phenylalanine did not lead to an increase in the average amount of PAA found in the shoots (Table 6). The level

of BZA, however, showed approximately a 45-fold increase, compared to initial levels, following the PHE feeding. As in *V. faba*, excision and feeding of *C. arvense* shoots with water led to a definite increase in the endogenous levels of PAA and BZA, but the increase in BZA was not as high as the one observed with the PHE treatment.

In *Sinapis arvensis*, as in the other two species, unfed shoots were found to contain both PAA and BZA, but no detectable amounts of CNA (Table 6). BZA was found in greater amounts than PAA. Water- and PHE-fed shoots contained both PAA and BZA and, contrary to the other two species, some CNA was detected in both of these treatments in *Sinapis arvensis*. BZA once again appeared to be the most prominent metabolite. When the three treatments are compared for the amount of PAA detected, no appreciable difference is found between the amount produced in PHE-fed shoots and the initial level in unfed shoots. In contrast, BZA levels increased significantly, compared to initial levels, following feeding of the shoots with phenylalanine. CNA levels also appeared to be slightly higher in PHE-fed shoots. In water-fed shoots, levels of the three metabolites were found to be slightly higher for PAA and CNA and lower for BZA than the initial levels in unfed shoots. When amounts of PAA, BZA and CNA found in water-fed shoots are compared to those detected in PHE-fed shoots, the amounts of PAA were almost identical in the two treatments, while BZA and CNA were detected in higher amounts in the PHE-fed shoots than in water-fed shoots (Table 6).

4.2.2 Metabolism of Chloro-Phenylalanines in the Crop Plant, *Vicia faba*.

Results from experiments on the metabolism of chloro-phenylalanines in excised shoots of *Vicia faba* are summarized in Table 7. Considering that three replicates with variances as high as the ones observed here do not form a basis for conventional statistics it was decided to use a notional value (as described in note 3 of Table 7 legend) to represent the range of values obtained from the repeat experiments. Metabolism of 2-Cl-PHE led to the production of all three corresponding acids, namely, 2-Cl-PAA, 2Cl-BZA and 2Cl-CNA. The three metabolites were produced in approximately the same amounts. Feeding with 3-Cl-PHE led to the production of both 3-Cl-BZA and 3-Cl-CNA; however, no 3-Cl-PAA was detected. 3-Cl-BZA was produced in much greater amounts than 3-Cl-CNA. Metabolism of 4-Cl-PHE gave rise to 4-CL-PAA, 4Cl-BZA and 4Cl-CNA, with 4-Cl-BZA being present in much greater amounts than either of the other two metabolites.

All of the dichloro-phenylalanines were metabolized to their corresponding dichloro-PAA and/or dichloro-BZA, except for 2,6-Cl₂-PHE (Table 7). Appreciable amounts of 2,6-Cl₂-CNA, however, were detected. The presence of corresponding dichloro-cinnamic acids was not determined for the other dichloro-phenylalanine treatments since the authentic standards were not available. 2,3-, 2,4- and 2,5-Cl₂-PHE all gave rise to their corresponding dichloro-PAA and dichloro-BZA and in each case, the dichloro-PAA metabolite was detected in greater or equal amounts than the corresponding dichloro-BZA, although the

Table 7. Amounts of chloro-phenyl metabolites found in 3 replicate experiments with excised shoots of *Vicia faba* fed with 10^{-4} M solutions of different D,L-chloro-phenylalanines for 24 h.

(1) Amounts estimated from ratio of areas of molecular ion peaks (metabolite / internal standard) in the TIC scan of PFB-derivatized HPLC fractions, relative to ratios of known amounts of metabolite and internal standard.

(2) 50 g samples of *Vicia faba* shoots were supplied with 10^{-4} M solutions of chloro-phenylalanines (50 ml) followed by distilled water for a total period of 24 h.

(3) Notional value represents the approximate average of the replicate data falling within the following range:

range	notional value
undetectable	0
1-19	10
20-99	50
100-299	200
300-1000	500

--- indicates a missing data point.

N.D. indicates that the metabolite was not determined.

0 indicates that when the metabolite was undetectable, although it may have been produced and subsequently lost during the extraction, a value of 0 was used in the computation of the notional value.

Chloro-phenylalanine supplied ⁽²⁾	Metabolites produced (10 ⁻² nmoles /g ⁻¹ fresh weight) ⁽¹⁾					
	Chloro-phenylacetic acid		Chloro-benzoic acid		Chloro-cinnamic acid	
	replicate data	notional value ⁽³⁾	replicate data	notional value ⁽³⁾	replicate data	notional value ⁽³⁾
2-chloro-	101		78		164	
	0	50	43	50	0	50
	0		0		0	
3-chloro-	0		341		33	
	0	0	88	200	0	10
	---		---		---	
4-chloro-	0		138		44	
	23	10	121	200	0	50
	---		---		---	
2,3-dichloro-	56		22		N.D.	
	0	50	27	10	N.D.	N.D.
	56		0		N.D.	
2,4-dichloro-	55		26		N.D.	
	46	200	26	50	N.D.	N.D.
	201		175		N.D.	
2,5-dichloro-	41		32		N.D.	
	23	50	38	50	N.D.	N.D.
	39		0		N.D.	
2,6-dichloro-	0		0		0	
	0	0	0	0	318	200
	0		0		0	
3,4-dichloro-	0		53		N.D.	
	0	0	62	200	N.D.	N.D.
	0		204		N.D.	
3,5-dichloro-	29		674		N.D.	
	0	10	53	500	N.D.	N.D.
	0		234		N.D.	

differences in amounts between the two metabolites were not large (Table 7). 3,4-Cl₂-PHE was metabolized to 3,4-Cl₂-BZA but not to 3,4-Cl₂-PAA. 3,5-Cl₂-PHE was metabolized to both 3,5-Cl₂-PAA and 3,5-Cl₂-BZA but contrary to what had been found for the 2,3-, 2,4- and 2,5-Cl₂-PHE treatments, much more 3,5-Cl₂-BZA was synthesized than 3,5-Cl₂-PAA.

Comparison of the nine chloro-phenylalanines with respect to their metabolism to the corresponding Cl-PAA's, shows that all of them, except 2,4-Cl-PHE, yielded amounts of the corresponding Cl-PAA that were either below detection or relatively low (Table 7). Some differences, however, can be seen. Metabolism of 2,4-Cl₂-PHE to 2,4-Cl₂-PAA, for example was much higher (with a notional value of 200×10^{-2} nmoles detected per g fresh weight of shoot tissue) than the metabolism found with 3-, 4-, 2,6-, 3,4- and 3,5-Cl₂-PHE, which all yielded undetectable or very low amounts (less than 10×10^{-2} nmole per g fresh weight) of the corresponding chloro-PAA's. 2-, 2,3- and 2,5-Cl-PHE, which gave a notional value of 50×10^{-2} nmoles of Cl-PAA per g fresh weight, showed a rate of conversion to their chloro-PAA that was intermediate between that of 2,4-Cl₂ and the other chloro-phenylalanines.

A similar comparison of the nine chloro-phenylalanines with respect to their metabolism to Cl-BZA's showed that the amounts produced ranged from undetectable levels for 2,6-Cl₂-BZA, to relatively high amounts for 3,5-Cl₂-BZA (Table 7). The amount of 3,5-Cl₂-BZA produced (with a notional value of 500×10^{-2} nmoles per g fresh weight of shoot tissue) was much higher than the amounts

of 2,3-, 2,5- and 2,6-Cl₂-BZA's detected, which were always under 40×10^{-2} nmoles per g fresh weight of shoot tissue. The 3-, 4-, and 3,4-Cl₂-PHE treatments gave moderately high amounts of the corresponding BZA's, with a notational value of 200×10^{-2} nmoles per g fresh weight. 2-Cl-PHE was metabolized to 2-Cl-BZA yielding a notational value of 50×10^{-2} nmoles per g fresh weight, which was similar to the amounts of chloro-BZA's produced by the 2,4-Cl-PHE-fed shoots.

Comparison of the chloro-phenylalanines for which metabolism to the corresponding Cl-CNA's was determined shows that the notational values for the different Cl-CNA's ranged from 10 to 200×10^{-2} nmoles per g fresh weight of shoot tissue for 3-Cl- and 2,6-Cl₂-PHE, respectively (Table 7). 4-Cl-CNA production, even with a notational value of 50×10^{-2} nmoles per g fresh weight, was similar to the production of 3-Cl-CNA from 3-Cl-PHE while 2-Cl-production appeared slightly higher if the data from the three replicates are considered.

4.2.3 Metabolism of Chloro-Phenylalanines in the Weed Plant, *Cirsium arvense*.

Results from metabolism of the nine chloro-phenylalanines in excised shoots of *Cirsium arvense* are summarized in Table 8. This weed plant was found to have the capacity to metabolize all three monochloro-phenylalanines to some extent. Feeding with 2-Cl-PHE yielded 2-Cl-BZA, but no 2-Cl-PAA or 2-Cl-CNA were produced. 3-Cl-PHE and 4-Cl-PHE, on the other hand, were both metabolized to the corresponding Cl-PAA, Cl-BZA and Cl-CNA. In these two treatments, the corresponding Cl-BZA was found in greater amounts than the corresponding Cl-PAA, while Cl-CNA production was intermediate but closer to

2

of/de

2

PM-1 3 1/2"x4" PHOTOGRAPHIC MICROCOPY TARGET
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Table 3. Amounts of chloro-phenyl metabolites in 3 replicate experiments with excised shoots of *Cirsium arvense* fed with 10^{-4} M solutions of different D,L-chloro-phenylalanines for 24 h.

(1) Amounts estimated from ratio of areas of molecular ion peaks (metabolite / internal standard) in the TIC scan of PFB-derivatized HPLC fractions, relative to ratios of known amounts of metabolite and internal standard.

(2) 50 g samples of *Cirsium arvense* shoots were supplied with 10^{-4} M solutions of chloro-phenylalanines (50 ml) followed by distilled water for a total period of 24 h.

(3) Notional value represents the approximate average of the replicate data falling within the following range:

range	notional value
undetectable	0
1-19	10
20-99	50
100-299	200
300-1000	500

--- indicates a missing data point.

N.D. indicates that the metabolite was not determined.

0 indicates that when the metabolite was undetectable, although it may have been produced and subsequently lost during the extraction, a value of 0 was used in the computation of the notional value.

Chloro-phenylalanine supplied ⁽²⁾	Metabolites produced (10 ⁻² nmoles /g ⁻¹ fresh weight) ⁽¹⁾					
	Chloro-phenylacetic acid		Chloro-benzoic acid		Chloro-cinnamic acid	
	replicate data	notional value ⁽³⁾	replicate data	notional value ⁽³⁾	replicate data	notional value ⁽³⁾
2-chloro-	0		514		0	
	0	0	0	200	0	0
	0		---		0	
3-chloro-	20		686		64	
	---	10	67	500	0	50
	15		1284		---	
4-chloro-	21		162		---	
	65	50	183	500	---	200
	27		1973		101	
2,3-dichloro-	33		25		N.D.	
	---	10	---	50	N.D.	N.D.
	0		20		N.D.	
2,4-dichloro-	65		32		N.D.	
	0	50	0	10	N.D.	N.D.
	0		0		N.D.	
2,5-dichloro-	31		52		N.D.	
	0	10	38	50	N.D.	N.D.
	0		0		N.D.	
2,6-dichloro-	21		1		0	
	27	10	8	10	97	50
	0		0		0	
3,4-dichloro-	32		757		N.D.	
	0	10	1813	500	N.D.	N.D.
	0		644		N.D.	
3,5-dichloro-	0		615		N.D.	
	42	10	776	500	N.D.	N.D.
	0		486		N.D.	

Cl-PAA synthesis

All of the dichloro-phenylalanines were metabolized to the corresponding dichloro-PAA's and dichloro-BZA's. With each of the 2,3-, 2,4-, 2,5- and 2,6-Cl₂-PHE treatments, formation of corresponding Cl₂-BZA occurred at about the same levels as the corresponding Cl₂-PAA. With 3,4- and 3,5-Cl₂-PHE's, however, metabolism to the corresponding Cl-BZA was much greater than to the corresponding Cl-PAA (Table 8).

Comparison of the nine chloro-phenylalanines for their degree of metabolism to the corresponding Cl-PAA shows that except for 2-Cl-PHE, all were metabolized in *Cirsium arvense* to the corresponding Cl-PAA, but only in low amounts (Table 8). Notional values for the Cl-PAA's ranged from 10×10^{-2} nmoles per g fresh weight for 3-, 2,3- 2,5- 2,6-, 3,4- and 3,5-Cl₂-PAA to 50×10^{-2} nmoles per g fresh weight for 4 and 2,4-Cl₂-PAA.

A similar comparison of the chloro-phenylalanines with respect to their metabolism to chloro-benzoic acids showed that there were some major differences in the extent to which each chloro-PHE was converted to this metabolite (Table 8). Amounts of Cl-BZA detected with each treatment ranged from very low notional amounts, such as 10×10^{-2} nmoles of 2,4- and 2,6-Cl₂-BZA's per g fresh weight from the corresponding dichloro-PHE treatment, to high notional amounts of 500×10^{-2} nmoles of 3,4-Cl-BZA per g fresh weight after feeding 3,4-Cl₂-PHE. Other chloro-phenylalanines that were metabolized in relatively high amounts to corresponding Cl-BZA's are the 3-, 4- and 3,5-Cl₂-

PHE's. 2-Cl-PHE produced an intermediate amount of 2-Cl-BZA with a notional value of 200×10^{-2} nmoles per g fresh weight, while the 2,3-, and 2,5-Cl₂-PHE treatments produced low levels of the corresponding Cl₂-BZA's with notional values of 50×10^{-2} nmoles per g fresh weight of tissue.

Metabolism of the chloro-phenylalanines to chloro-cinnamic acids was undetectable for 2-Cl-PHE and moderate for 4-Cl-PHE while 3-Cl- and 2,6-Cl₂-PHE produced low amounts (notional value of 50×10^{-2} nmoles per g fresh weight of tissue) of the corresponding Cl-CNA's (Table 8).

4.2.4 Metabolism of Chloro-Phenylalanines in the Weed Plant *Sinapis arvensis*.

Results from experiments on metabolism of the chloro-phenylalanines in excised shoots of *Sinapis arvensis* are summarized in Table 9. With the monochloro-phenylalanines, both 2-Cl- and 3-Cl-PHE gave rise to all three of the corresponding Cl-PAA, Cl-BZA and Cl-CNA. 4-Cl-PHE was metabolized to 4-Cl-PAA and 4-Cl-BZA but no 4-Cl-CNA was detected. 4-Cl-PAA was produced in much greater amounts than 4-Cl-BZA.

Each of the dichloro-phenylalanines were metabolized to the corresponding dichloro-PAA and dichloro-BZA. In all of the treatments, except for the 3,4-Cl-PHE one, the differences between the amounts of corresponding Cl₂-PAA and Cl₂-BZA produced were relatively small. 3,4-Cl-PHE yielded much more of the corresponding Cl-BZA than Cl-PAA. 2,6-Cl₂-PHE was also metabolized to 2,6-Cl₂-CNA which was found in greater amount than 2,6-Cl₂-PAA or 2,6-Cl₂-BZA.

When the nine chloro-phenylalanine treatments compared with respect to the

Table 9. Amounts of chloro-phenyl metabolites produced in excised shoots of *Sinapis arvensis* fed with 10^{-4} M solutions of different D,L-chloro-phenylalanines for 24 h.

(1) Amounts estimated from ratio of areas of molecular ion peaks (metabolite / internal standard) in the TIC scan of PFB-derivatized HPLC fractions, relative to ratios of known amounts of metabolite and internal standard.

(2) 50 g samples of *Sinapis arvensis* shoots were supplied with 10^{-4} M solutions of chloro-phenylalanines (50 ml) followed by distilled water for a total period of 24 h.

(3) Notional value represents the approximate average of the replicate data falling within the following range:

range	notional value
undetectable	0
1-19	10
20-99	50
100-299	200
300-1000	500

--- indicates a missing data point.

N.D. indicates that the metabolite was not determined.

0 indicates that when the metabolite was undetectable, although it may have been produced and subsequently lost during the extraction, a value of 0 was used in the computation of the notional value.

Chloro-phenylalanine supplied ⁽²⁾	Metabolites produced (10 ⁻² nmoles /g ⁻¹ fresh weight) ⁽¹⁾					
	Chloro-phenylacetic acid		Chloro-benzoic acid		Chloro-cinnamic acid	
	replicate data	notional value ⁽³⁾	replicate data	notional value ⁽³⁾	replicate data	notional value ⁽³⁾
2-chloro-	0		69		---	
	0	50	18	50	0	200
	111		87		310	
3-chloro-	0		94		---	
	0	50	108	200	---	200
	252		149		169	
4-chloro-	0		116		0	
	0	500*	156	200	0	0
	2944		107		---	
2,3-dichloro-	---		---		N.D.	
	---	50	---	50	N.D.	N.D.
	44		26		N.D.	
2,4-dichloro-	44		124		N.D.	
	0	50	0	50	N.D.	N.D.
	45		34		N.D.	
2,5-dichloro-	17		111		N.D.	
	61	50	0	50	N.D.	N.D.
	26		43		N.D.	
2,6-dichloro-	67		0		0	
	0	50	0	10	0	200
	23		3		308	
3,4-dichloro-	39		226		N.D.	
	12	50	47	200	N.D.	N.D.
	23		195		N.D.	
3,5-dichloro-	0		44		N.D.	
	19	10	60	50	N.D.	N.D.
	0		95		N.D.	

formation of corresponding Cl-PAA's, except for 4-Cl-PHE, they are not appreciably different from each other. The 4-Cl-PHE treatment gave a notional for 4-Cl-PAA of 500×10^{-2} nmoles per g fresh weight, whereas all of the other chloro-phenylalanines treatments gave notional values in the range of 10 to 50×10^{-2} nmoles per g fresh weight for the corresponding Cl-PAA's. It should be noted that although 4-Cl-PHE gave a high notional value for 4-Cl-PAA of 500×10^{-2} nmoles per g fresh weight and does appear to be metabolized to a greater extent than the other chloro-phenylalanines, in three repeat experiments, 4-Cl-PAA was detected only once in very high amounts. Further experiments would therefore be required to confirm the degree of conversion of 4-Cl-PHE to 4-Cl-PAA in this species.

When the different chloro-phenylalanines are compared with respect to their metabolism to Cl-BZA's in *Sinapis arvensis*, some differences are found (Table 9). Notional values for the Cl-BZA's detected ranged from very low (10×10^{-2} nmoles per g fresh weight) for 2,6-Cl₂-BZA, to moderately high (200×10^{-2} nmoles per g fresh weight) for 3-, 4- and 3,4-Cl₂-BZA's. The 2-, 2,3-, 2,4-, 2,5- and 3,5-Cl₂-PHE produced intermediate amounts of their corresponding chloro-BZA's.

Metabolism of the chloro-phenylalanines to corresponding Cl-CNA's in *S. arvensis* was appreciable in three of the four Cl-PHE's for which metabolism to chloro-CNA's was determined (Table 9). Although 4-Cl-CNA was not detected in the 4-Cl-PHE treatment, 2-Cl-, 3-Cl and 2,6-Cl₂-CNA were produced from the corresponding Cl-PHE's in amounts corresponding to notional values of 200×10^{-2}

nmoles per g fresh weight. Other chloro-CNA's may have been produced in the other Cl-PHE treatments, but these could not be detected due to the absence of the authentic standards.

4.2.5 Comparison of the Metabolism of each Chloro-Phenylalanine in the Three Crop and Weed Species

When comparing metabolism of the nine chloro-phenylalanines in the three species in relation to the possible synthesis of herbicidal metabolites, we will focus mainly on the chloro-phenylalanines whose corresponding Cl-PAA's, Cl-BZA's, and Cl-CNA's have been shown to possess high growth-regulating activity.

Some differences were observed between the three species in their capacity to metabolize 2-chlorophenylalanine. 2-Cl-PAA was produced in low amounts in both *Vicia faba* and *Sinapis arvensis*, but was not detected in *Cirsium arvense* (Table 10). The amount of 2-Cl-BZA produced was higher in *Cirsium arvense* than in the other two species whereas metabolism to 2-Cl-CNA was higher in *Sinapis arvensis* than in *Vicia faba* or *Cirsium arvense*.

Formation of the 3-chloro-metabolites following a feeding with 3-chlorophenylalanine did not follow the same pattern in all three species. 3-Cl-PAA was not detected in *Vicia faba* but was produced in both weeds, although only in very low amounts in *Cirsium arvense* (Table 10). Average amounts of 3-Cl-BZA detected were over 100×10^{-2} nmoles per g fresh weight of tissue in all three species, with *Cirsium arvense* producing very high amounts which were 2 times

Table 10. Comparative metabolism of each chloro-phenylalanine in *Vicia faba*,
Cirsium arvense and *Sinapis arvensis*.

(1) The notional values represent the approximate average of replicate data falling within the following range;

range	notional value
undetectable	0
1-19	10
20-99	50
100-299	200
300-1000	500

The notional values for the three species were taken from Tables 7, 8 and

- (2) Cl-PAA = chloro-phenylacetic acid
Cl-BZA = chloro-benzoic acid
Cl-CNA = chloro-cinnamic acid.

Chloro-phenylalanine supplied	Chloro-phenyl metabolite produced ⁽²⁾	Amount of metabolites produced Notional value ⁽¹⁾ (x 10 ⁻² nmoles * g fresh weight)		
		<i>Vicia faba</i>	<i>Cirsium arvense</i>	<i>Sinapis arvensis</i>
2-Cl-PHE	2-Cl-PAA	50	0	50
	2-Cl-BZA	50	200	50
	2-Cl-CNA	50	0	200
3-Cl-PHE	3-Cl-PAA	0	10	50
	3-Cl-BZA	200	500	200
	3-Cl-CNA	10	50	200
4-Cl-PHE	4-Cl-PAA	10	50	500
	4-Cl-BZA	200	500	200
	4-Cl-CNA	50	200	0
2,3-Cl-PHE	2,3-Cl-PAA	50	10	50
	2,3-Cl-BZA	10	50	50
2,4-Cl-PHE	2,4-Cl-PAA	200	50	50
	2,4-Cl-BZA	50	10	50
2,5-Cl-PHE	2,5-Cl-PAA	50	10	50
	2,5-Cl-BZA	50	50	50
2,6-Cl-PHE	2,6-Cl-PAA	0	10	50
	2,6-Cl-BZA	0	10	10
	2,6-Cl-CNA	200	50	200
3,4-Cl-PHE	3,4-Cl-PAA	0	10	50
	3,4-Cl-BZA	200	500	200
3,5-Cl-PHE	3,5-Cl-PAA	10	10	10
	3,5-Cl-BZA	500	500	50

greater than the amounts found in *V. faba* and *S. arvensis*. Production of 3-Cl-CNA was low in *Vicia faba* and *Cirsium arvense* but was considerably higher in *Sinapis arvensis*.

4-chlorophenylalanine was metabolized to the corresponding Cl-PAA in low amounts in *Vicia faba* and *Cirsium arvense* but was detected in very high amounts in one of the repeat experiments with *Sinapis arvensis* (Table 10). In *Cirsium arvense*, 4-Cl-PHE was metabolized to 4-Cl-BZA in high amounts which were more than two times greater than in *V. faba*, while production of this metabolite in *Sinapis arvensis* was moderate and comparable to that found in *V. faba*. Metabolism of 4-Cl-PHE to 4-Cl-CNA was low in *Sinapis arvensis* and *Vicia faba* but occurred at moderate levels in *Cirsium arvense*.

Metabolism of 2,3-dichlorophenylalanine to 2,3-Cl₂-PAA and 2,3-Cl₂-BZA was low (50×10^{-2} nmoles per g fresh weight tissue or less) and comparable in all three species (Table 10). *Vicia faba* showed the greatest capacity to metabolize 2,4-dichlorophenylalanine with 2,4-Cl₂-PAA being detected in moderate amounts in this species, whereas only low amounts were found in the two weed species (Table 10). 2,4-Cl₂-Cl-BZA was found in low amounts in the three species. The possible production of 2,3- and 2,4-Cl-CNA's by any of the species could not be determined due to the lack of the authentic standards.

Metabolism of 2,5-Cl₂-PHE to 2,5-Cl₂-PAA and 2,5-Cl₂-BZA was low and comparable in all three species (Table 10). Metabolism of 2,6-dichlorophenylalanine to 2,6-Cl₂-PAA showed some slight differences between the

species. 2,6-Cl-PAA was not detected in *Vicia faba* but was produced in low amounts in *S. arvensis* (notional value of 50×10^{-2} nmoles per g fresh weight. Production of 2,6-Cl₂-BZA was low and comparable in all three species (Table 10). 2,6-Cl₂-CNA was formed in moderate amounts in *Sinapis arvensis* and *Vicia faba* and in low amounts in *C. arvense*.

All of the above four dichloro-phenylalanines with a chlorine atom in the 2-position were metabolized in very low to moderate amounts in the three test species; the maximum notional values obtained for the production of the corresponding dichloro-phenyl metabolites were of 200×10^{-2} nmoles per g fresh weight of shoot tissue. The 3,4- and 3,5-dichlorophenylalanines, however, were metabolized more efficiently, at least when conversion to their corresponding dichloro-BZA is considered.

Metabolism of 3,4-Cl₂-PHE to 3,4-Cl-PAA was slightly higher in *Sinapis arvensis* than in *Vicia faba*, where this metabolite was not detected while in *S. arvensis* the average amount of 3,4-Cl₂-PAA produced corresponded to a notional value of 50×10^{-2} nmoles per g fresh weight of fed shoot tissue (Table 10). In *Cirsium arvense* 3,4-Cl₂-BZA was detected in very high amounts which were more than double those found in either *V. faba* or *S. arvensis*, which produced moderate and similar amounts of this metabolite.

Metabolism of 3,5-dichlorophenylalanine to 3,5-Cl₂-PAA was very low and comparable in all three species (Table 10). 3,5-Cl₂-BZA, however, was produced in very high amounts in *Cirsium arvense* but not in *Sinapis arvensis* or *Vicia faba*

where the production of this metabolite was low.

4.3 Structure-Metabolism Correlations.

In this final part of the study, the overall metabolism of the nine chloro-phenylalanines is compared using values obtained by combining the notional values for the Cl-PAA and Cl-BZA produced in each chloro-phenylalanine treatment as an indicator of the degree of metabolism of this chloro-phenylalanine. Cl-CNA results were not included since this metabolite was not determined in all treatments. The summation values for the metabolism of each chloro-phenylalanine in the three species is shown in Table 11 along with the average of the three species. Figures 10 to 14 show the relationship between metabolism of the chloro-phenylalanines, based on the average value for the 3 species, and each of the five dihedral angles in the chloro-phenylalanine molecule determined through computer modelling. Of the possible molecular structure parameters obtained through the computer modelling study, it was decided to use the dihedral angle because it is simple to work with, and because it gives an indication of the spatial relationship between certain functional groups of the molecule which could influence the way the molecule will fit into the active site of the enzymes involved in the metabolic pathways, and thus the extent to which they will be metabolized.

It is clear from the summation values presented in Table 11 that 3-Cl- and 4-Cl-PHE as well as 3,4- and 3,5-Cl₂-PHE are the chloro-phenylalanines which were metabolized to the greatest extent. From the scatter plots showing the

relationship between each of the five calculated dihedral angles in the chloro-phenylalanines molecules and the extent of their metabolism it can be seen that the above four compounds all have angle values of around 102° for angle 1 (Figure 10), 179° for angles 2 and 3 (Figure 11 and 12), over 61° for angle 4 (Figure 13) and close to or above 3° for angle 5 (Figure 14). In contrast, the chloro-phenylalanines which were metabolized to a much lesser extent, namely, 2-Cl-, 2,3-, 2,4-, 2,5-, and 2,6-Cl₂-PHE, have angle values of approximately $89-91^\circ$ for angle 1, under 175° for angle 2 and 3, under 60° for angle 4 and closer to $1-2^\circ$. Since neither the degree of metabolism or the angles values parameters were normally distributed, a regression analysis could not be performed on the results. However, the Kendall's coefficient of rank correlation (τ) was calculated and a significant correlation was found between degree of metabolism of the chloro-phenylalanines and each of the five dihedral angles calculated. The correlation coefficients are relatively low but these findings still suggest that there is a correlation between the steric configuration of a chloro-phenylalanine molecule and its ability to serve as a substrate for the enzymes involved in phenylalanine metabolism leading to PAA and BZA.

Table 11. Summation values indicating the degree of metabolism of each chloro-phenylalanine in the three test species.

For each chloro-phenylalanine, the degree of metabolism is estimated from the combined notional values for Cl-PAA and Cl-BZA produced as presented in Tables 7, 8 and 9.

Chloro- phenylalanine	Degree of metabolism (sum of notional value for Cl-PAA and Cl-BZA) $\times 10^{-2}$ nmoles / g fresh weight			
	<i>Vicia</i>	<i>Cirsium</i>	<i>Sinapis</i>	Average of three species
	<i>fab</i>	<i>arvense</i>	<i>arvensis</i>	
2-Cl-PHE	100	200	100	133
3-Cl-PHE	200	510	250	320
4-Cl-PHE	210	550	700	487
2,3-Cl-PHE	60	60	100	73
2,4-Cl-PHE	250	60	100	137
2,5-Cl-PHE	100	60	100	87
2,6-Cl-PHE	0	20	60	27
3,4-Cl-PHE	200	510	250	320
3,5-CL-PHE	510	510	60	360

Figure 10. Relationship between dihedral angle 1 and the degree of metabolism of chloro-phenylalanines.

Values for the metabolism of each chloro-phenylalanine were taken from Table 11.

Kendall's coefficient of rank correlation : 0.44
p value (under $H_0: \tau = 0$) : .003

Figure 11. Relationship between dihedral angle 2 and the degree of metabolism of chloro-phenylalanines.

Values for the metabolism of each chloro-phneylalanine were taken from Table 11.

Kendall's coefficient of rank correlation : 0.39
p value (under $H_0: \tau = 0$) : .009

Figure 12. Relationship between dihedral angle 3 and the degree of metabolism of chloro-phenylalanines.

Values for the metabolism of each chloro-phenylalanine were taken from Table 11.

Kendall's coefficient of rank correlation : 0.53
p value (under $H_0: \tau = 0$) : .0003

Figure 13. Relationship between dihedral angle 4 and the degree of metabolism of chloro-phenylalanines.

Values for the metabolism of each chloro-phenylalanine were taken from Table 11.

**Kendall's coefficient of rank correlation : 0.47
p value (under $H_0: \tau = 0$) : .001**

Figure 14. Relationship between dihedral angle ϕ and the degree of metabolism of chloro-phenylalanines.

Values for the metabolism of each chloro-phenylalanine were taken from Table 11.

Kendall's coefficient of rank correlation : 0.53
p value (under $H_0: \tau = 0$) : .0003

5. Discussion

5.1 Metabolism of D,L-phenylalanine in the three Crop and Weed Species.

Endogenous amounts of PAA and BZA found in the three plant species used in this study are similar to the amounts reported previously in other studies on higher plants. Wightman and Lighty (1982), using gas chromatography (GC) of methyl esters as the analytical method, reported endogenous levels ranging from 51 to 161 $\times 10^{-2}$ nmoles of PAA per g fresh weight in shoot tissues of the crop plants, tomato, pea, tobacco, sunflower, barley and corn. Schneider *et al.* (1985), using GC-MS and an internal deuterated standard for quantitative analysis, found that pea epicotyls contained about 42 $\times 10^{-2}$ nmoles of PAA per g fresh weight. Taylor *et al.* (1988) studied six crop and weed species using GC-MS for quantitative determination and reported values between 3 and 62 $\times 10^{-2}$ nmoles per g fresh weight of shoot tissue for PAA, and values of between 9 and 550 $\times 10^{-2}$ nmoles per g fresh weight for BZA. The variation in PAA content between the species probably reflects natural interspecific variations. For example, Schneider and Wightman (1986) found that endogenous PAA levels were much higher in the ostrich fern, *Matteucia struthiopteris*, than in Angiosperm plants. Part of the variation, however, might be due to the fact that the plants used in the various studies were not all of the same age, or at the same physiological stage, and PAA and IAA levels are known to change in a plant organ as the tissues develop from young to the mature state (Wightman 1977). Schneider and Wightman (1986)

similarly found that levels of IAA and PAA in ostrich fern were higher in young fronds than in the vegetative tissue of mature fronds.

In the present study, a 3.5-fold increase in PAA levels over endogenous amounts was observed in PHE-fed shoots in *Vicia faba*. This increase is comparable to the 3-4-fold increases found by Taylor *et al.* (1988) in similar experiments with other crop and weed plants. The increase in PAA levels on feeding PHE to shoots of *Vicia faba* suggests that a metabolic pathway leading from phenylalanine to PAA, such as the one suggested by Wightman (1973) and shown in Figure 5, is operating in this plant.

In *Cirsium arvense* and *Sinapis arvensis*, feeding of excised shoots with PHE did not lead to the detection of amounts of PAA greater than the endogenous level found in unfed shoots. In both species, however, PHE feeding led to an appreciable increase in the level of BZA. Taylor *et al.* (1988) also found similar increases in BZA levels following a PHE-feeding in the six species they studied. It is known that IAA and PAA arise from their respective amino acids precursors, tryptophan and phenylalanine, through similar metabolic pathways (Wightman 1973, Frégeau and Wightman 1983, Camirand *et al.* 1983). Should IAA and PAA also show similar catabolic pathways, similar for example to the one that leads from IAA to indole-3-carboxylic acid (ICA) (Sembdner *et al.* 1980), then the equivalent of ICA derived from IAA would be benzoic acid from PAA. The existence of such a pathway might be the reason why no increase in levels of PAA, but a high increase in the levels of BZA, was found in PHE-fed shoots of *Sinapis*

arvensis and *Cirsium arvense*, since PAA levels may not accumulate above a certain low concentration in these species because of its ready catabolism to BZA.

Cinnamic acid arises from phenylalanine through the action of the enzyme, phenylammonialyase (PAL) (Goodwin and Mercer 1983). Deamination of L-phenylalanine by PAL to yield cinnamic acid is ubiquitous in the plant kingdom and in view of this, it would seem surprising that CNA was not found in unfed shoots of any of the three species studied, and only in the shoots of one species following D,L-phenylalanine feeding. Similar results were obtained by Taylor *et al.* (1988) who did not find any cinnamic acid in two of the six species they studied and found it in only very low amounts, compared to BZA and PAA, in the other four species. In the species in which CNA was detected by these authors, the levels of this compound did not appear to follow any specific pattern following a water or phenylalanine feeding. Since cinnamic acid is a key intermediate in the formation of many secondary metabolites (Goodwin and Mercer 1983, Zenk 1979) it is possible that it does not accumulate in detectable amounts in most plants but is quickly metabolized to other secondary compounds including benzoic acid.

Another possible explanation for the lack of detection of CNA would be if this metabolite was subject to greater losses during the extraction procedure than PAA or BZA. Taylor *et al.* (1988) determined the recovery percentage of PAA, BZA and CNA in their study and found that the recovery rate of CNA was similar to that of PAA. Greater losses of cinnamic acid during extraction are therefore

not likely to be the reason for its apparent absence from most samples since the extraction procedure used in the present study was similar to the one employed by Taylor *et al.* (1988). In the course of this study, however, it was noticed that when identical amounts of authentic CNA, PAA and BZA were derivatized and analysed by GC-MS, the CNA standard repeatedly gave a lower peak area in the total ion current scan relative to identical amounts of the PAA and BZA standards. It might be, therefore, that the PFBr derivatization reaction is less efficient with cinnamic acids than with phenylacetic and benzoic acids, or that the PFBr esters of cinnamic acids do not ionize as strongly as the PAA or BZA esters resulting in a weaker signal in the TIC scan. Whatever the cause, it results in a higher minimum amount of cinnamic acid that must be present to ensure detection and thus there is a greater chance of not detecting this metabolite when it is present in small amounts.

In general, in excised shoots that were fed with water for 24h, the amounts of PAA and BZA were intermediate between those detected in unfed and PHE-fed shoots. Thus, excision of the shoots followed by water-feeding appears to lead to a certain increase in the levels of PAA and BZA, but not to the extent of the increase observed in PHE-fed shoots. This supports the view that the increase in phenyl metabolites seen in PHE-fed shoots is not due entirely to the experimental procedure, but the result of active synthesis of PAA and/or BZA from PHE. Taylor *et al.* (1988) when studying phenylalanine metabolism in shoots of other species also found an increase in PAA and/or BZA upon excision and water

feeding. The increase in these two metabolites is possibly part of a general stimulation in the production of secondary metabolites in response to the physical and water-related stresses resulting from excision and experimental manipulation. In this connection, the transcription of plant genes coding for the enzyme phenylammonia-lyase has been shown to be induced by tissue wounding (Shaw *et al.* 1990, Lois *et al.*, 1989, Bevan *et al.* 1989).

5.2 Differential Metabolism of the nine Chloro-phenylalanines in each Test Species.

All nine chloro-phenylalanines were found to be metabolized, to different degrees, in *Vicia faba* (Table 7). With the monochloro-PHE's, both 2-Cl- and 4-Cl-compounds gave rise to small or moderate amounts of the corresponding Cl-PAA, Cl-BZA and Cl-CNA, whereas 3-Cl-PHE gave no 3-Cl-PAA but produced detectable amounts of 3-Cl-BZA and 3-Cl-CNA. As was noticed with the control treatments where unsubstituted benzoic acid was the dominant metabolite, Cl-BZA's appear to be the dominant metabolites produced from the monochloro-phenylalanines. It is interesting to note that unsubstituted benzoic acid as well as most of the chlorinated benzoic acids do not exhibit growth-regulating activity and that therefore their accumulation to high levels in the plant would not cause any disruption. Unsubstituted PAA and CNA as well as most of the Cl-PAA's and Cl-CNA's however show growth regulating activity. It might be that plants have evolved a way of insuring that free PAA and CNA (or Cl-PAA's and Cl-CNA's) levels are kept low but do not exert such control over BZA (or Cl-BZA's) levels.

All of the dichloro-phenylalanines were metabolized to a limited extent in *Vicia faba*. Only 2,4-Cl₂-PHE gave rise to moderate amounts of the dichloro-PAA; all the other dichloro-PHE's produced low or undetectable levels of the corresponding dichloro-PAA metabolite. Again, the dichloro-BZA's were generally found in greatest amount and the levels of the corresponding dichloro-CNA's could not be determined, except for 2,6-Cl₂-CNA, because of the lack of authentic standards required for GC-MS determination.

In *Cirsium arvense*, the nine chloro-phenylalanines were again found to be metabolized to varying degrees, depending on the substitution pattern of the Cl-PHE under investigation (Table 8). With the monochloro-PHE's, 2-Cl-PHE did not yield detectable amounts of 2-Cl-PAA but the 3-Cl- and 4-Cl-PHE's produced low amounts of the corresponding Cl-PAA. The monochloro-BZA's were the metabolites produced in greatest amount in this weed and as with the Cl-PAA's the 3- and 4-Cl-BZA's were produced in greater amounts than the 2-Cl-substituted metabolite. The corresponding Cl-CNA's, except 2-Cl-CNA, were found in low to moderate amounts which in all cases were greater than the amounts of Cl-PAA's produced. As was postulated in the hypothesis, differential metabolism of the chloro-phenylalanines thus appear to occur according to the substitution pattern and the 3- and 4-substitution position seem more favourable to metabolism than the 2-position. With the dichloro-phenylalanines, all were metabolized to a small extent to the corresponding dichloro-PAA in *Cirsium arvense*. The expected dichloro-BZA's were also found although the amounts of

2,3-, 2,4-, 2,5- and 2,6-Cl₂-BZA's were quite low. This, however, was not the case with the 3,4- and 3,5-Cl₂-BZA's which were produced in very high amounts in this weed from the corresponding dichloro-PHE's. It therefore seems that the same pattern of low metabolism of the 2-chloro-substituted compounds is also present with the dichloro-phenylalanines when production of Cl₂-BZA's is considered. Of the possible dichloro-CNA metabolites, 2,6-Cl₂-CNA was detected in moderate amounts and other dichloro-CNA may have been produced but could not be detected because of the lack of authentic standards. The capacity of *Cirsium arvense* to produce high amounts of 3- and 4-Cl-BZA, and of 3,4- and 3,5-Cl₂-BZA from the corresponding chloro-phenylalanines is interesting but not important in the context of herbicidal activity, since none of these chloro-benzoic acids exhibit growth-regulating activity (Table 12).

As postulated initially, in *Sinapis arvensis*, as in the other two species, all the chloro-phenylalanines were metabolized, to a certain degree, to the corresponding chloro-PAA and chloro-BZA (Table 9). The corresponding chloro-CNA metabolites were also produced from at least four of the chloro-phenylalanines, these being the only ones for which authentic chloro-CNA's were available for GC-MS determination. Metabolism of the monochloro-PHE's to the corresponding Cl-PAA's was low in this weed species except with 4-Cl-PHE, yielding very high levels of 4-Cl-PAA. All the monochloro-PHE's also gave rise to moderate amounts of the corresponding Cl-BZA and Cl-CNA metabolites although surprisingly, 4-Cl-PHE did not appear to produce detectable amounts of

Table 12. Summary of growth-regulating activity of chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids.

Results taken from Pybus *et al.* (1959 a and b) and Ugochukwu and Wain (1968).

Activity as compared to a water control:

- *** = very high activity,
- ** = high activity,
- * = low to moderate activity,
- = very low or no activity.

Chloro-substitution pattern	Relative activity of chloro-phenyl metabolites		
	Phenylacetic acid	Benzoic acid	Cinnamic acid
2-chloro-	* * *	-	* * *
3-chloro-	* * *	-	*
4-chloro-	*	-	* * *
2,3-chloro-	* * *	* *	-
2,4-chloro-	*	-	* *
2,5-chloro-	* *	* * *	-
2,6-chloro-	* * *	*	* * *
3,4-chloro-	* *	-	*
3,5-chloro-	-	-	-

4-Cl-CNA. A higher production of 4-Cl-CNA would have been expected since as in *Vicia faba*, the 4-Cl-PHE produced higher amounts of its corresponding Cl-PAA and Cl-BZA than did the 2-Cl-PHE and seemed a more favourable substitution position for metabolism than the 2- position.

All of the dichloro-PHE's were found to be metabolized in *Sinapis arvensis* to yield only small amounts of the corresponding dichloro-PAA and dichloro-BZA, the exception being 3,4-Cl₂-PHE which gave rise to moderate amounts of 3,4-Cl₂-BZA. Although the 2,3- and 2,5-Cl₂-PHE's produced only small amounts of the corresponding dichloro-PAA and dichloro-BZA, each of these metabolites is a highly active growth regulator with herbicidal activity (Table 12), and so the combined amounts of each pair of metabolites produced from these dichloro-PHE's could well be sufficient to exert herbicidal action in this weed. Similarly the combined amounts of 2,6-Cl₂-PAA and 2,6-Cl₂-CNA produced from 2,6-Cl₂-PHE (Table 9) is also probably sufficient to exert herbicidal action especially since both are very highly active growth regulators showing strong herbicidal activity (Table 12). Other potentially active dichloro-CNA metabolites may have been present in extracts of shoots fed with the other dichloro-PHE's, but their GC-MS determination was not possible. It would be interesting to examine further the potential herbicidal activity of the combined amounts of 2,3-, 2,5- and 2,6-dichloro-metabolites produced in this and other weed species.

5.3 Comparison of the Metabolism of each Chloro-Phenylalanine in the Three Test Species

As mentioned in the Results section 4.2.5, few major differences were observed between the three plant species with respect to their metabolism of the nine chloro-phenylalanines. Although there appeared to be differences in early experiments, the high variation in the results from repeat experiments made these differences not significant. Possible reasons why such high variations were found in the results from repeat experiments are discussed later in section 5.5. Meanwhile, the implications of the apparent differences observed will be considered in the present section in relation to the possible use of certain chloro-phenylalanines as precursors of herbicidal compounds. Further experiments are needed, however, to confirm in a decisive way the present differences.

Metabolism of 2-Cl-PHE to 2-Cl-PAA, 2-Cl-BZA and 2-Cl-CNA was low in *Vicia faba* (Table 10). Taylor *et al.* (1988) found a similar low rate of metabolism of this monochloro-phenylalanine in the two leguminous crop plants they studied; *Phaseolus vulgaris* and *Glycine max*. This is of some interest since both 2-Cl-PAA and 2-Cl-CNA are very active growth-regulating substances (Table 12). It is of greater interest when contrasted to the fact that in one of the weeds studied here, *Sinapis arvensis*, metabolism of 2-Cl-PHE to 2-Cl-CNA appeared higher than in the crop plant. In the other weed, *Cirsium arvense*, metabolism of 2-Cl-PHE to 2-Cl-BZA was higher than in *Vicia faba*, but this finding is not so interesting since 2-Cl-BZA does not exhibit growth-regulating activity (Table 12). High metabolism

of 2-Cl-PHE is not common to all weeds, however, since Taylor *et al.* (1988) found that in the three weeds they studied, *Setaria faberii*, *Amaranthus retroflexus* and *Chenopodium album*, metabolism of this chloro-phenylalanine was relatively low.

Metabolism of 3-Cl-PHE to 3-Cl-PAA and 3-Cl-CNA was also low in *Vicia faba* (Table 10). 3-Cl-PAA and 3-Cl-CNA are active growth-regulating metabolites with 3-Cl-PAA in particular, exhibiting very high activity (Table 12). The low rate of conversion of 3-Cl-PHE to these two metabolites in *Vicia faba* is thus a good situation. *Vicia faba*, however, did produce high amounts of 3-Cl-BZA, but since this metabolite is inactive, its high production in this crop plant is probably innocuous. Taylor *et al.* (1988) found moderate metabolism of 3-Cl-PHE to its metabolite, 3-Cl-PAA, in the two leguminous crop species, *Phaseolus vulgaris* and *Glycine max*. So contrary to what has been seen with 2-Cl-PHE, these crop species do not resemble *Vicia faba* in their ability to metabolize 3-Cl-PHE. In the two weeds studied here, overall metabolism of 3-Cl-PHE was higher than in *Vicia faba*. *Cirsium arvense* formed very high amounts of 3-Cl-BZA and low amounts of 3-Cl-PAA and 3-Cl-CNA, while moderate amounts of 3-Cl-BZA and 3-Cl-CNA were found in *Sinapis arvensis*. The inactivity of 3-Cl-BZA, however, makes the higher production of this acid in *Cirsium arvense* unimportant with respect to possible herbicidal action. 3-Cl-PAA and 3-Cl-CNA, on the other hand, show very high and moderate growth-regulating activity, respectively, and their dual production could lead to herbicidal action in *Sinapis arvensis*. In contrast, Taylor *et al.* (1988) reported that the metabolism of 3-Cl-PHE was low in the three

weeds they studied.

Metabolism of 4-Cl-PHE to 4-Cl-PAA was low in *Vicia faba* but very high in *Sinapis arvensis* (Table 10). The high production of 4-Cl-PAA in *Sinapis arvensis* is worth investigating further since 4-Cl-PAA is a growth-regulating compound, although not as potent as 2-Cl- or 3-Cl-PAA (Table 12). *Cirsium arvense* produced very high amounts of 4-Cl-BZA, while moderate amounts of this metabolite were found in *Vicia faba* and *Sinapis arvensis*. 4-Cl-BZA, however, does not exhibit any growth-regulating activity (Table 14) so its high production in *Cirsium arvense* and its moderate production in *Sinapis arvensis* are not of interest with respect to possible herbicidal action. The fact that it was also produced in moderate amounts in *Vicia faba* would probably not be a problem to this plant. Moderate to high formation of 4-Cl-BZA was also observed by Taylor *et al.* (1988) in all but one of the plant species they studied.

With the dichloro-phenylalanines, metabolism of 2,3-Cl₂-PHE and 2,5-Cl₂-PHE was low in all three species studied (Table 10). Similar results were obtained by Taylor *et al.* (1988) who found that metabolism of these two dichloro-phenylalanines was very low or could not be detected at all in the six species they studied. 2,3-Cl₂-PHE and 2,5-Cl₂-PHE are two of the more interesting chloro-phenylalanines since for each of them, the corresponding Cl₂-PAA and Cl₂-BZA show growth-regulating activity, with 2,3-Cl₂-PAA and 2,5-Cl₂-BZA being very active (Table 12). The low metabolism of these two dichloro-phenylalanines in *Vicia faba* and in the three crop plants studied by Taylor *et al.* (1988) is fortunate

while a similar low level of metabolism in *Cirsium arvense* and *Sinapis arvensis*, and in the three weeds studied by Taylor *et al.* (1988) may be deceiving since other weeds might show a higher capacity to metabolize those two potent chloro-phenylalanines.

Metabolism of 2,4-Cl₂-PHE was low in *Cirsium arvense* and *Sinapis arvensis* (Table 10). Taylor *et al.* (1988) also found that this dichloro-phenylalanine was poorly metabolized in all the plants they studied. In *Vicia faba*, however, moderate amounts of 2,4-Cl₂-PAA were produced. 2,4-Cl₂-PAA shows moderate growth-regulating activity and consequently, the conversion of 2,4-Cl₂-PHE to 2,4-Cl₂-PAA in *Vicia faba* would make this chloro-phenylalanine unsuitable for use on weeds associated with this crop, even if the weeds were found to metabolize 2,4-Cl₂-PHE to high levels of 2,4-Cl-PAA.

The negligible conversion of 2,6-Cl₂-PHE to the corresponding dichloro-PAA and dichloro-BZA from 2,6-Cl₂-PHE in *Vicia faba* is interesting, but is counter-balanced by the formation of some 2,6-Cl₂-CNA, which is a very active growth-regulator and is produced in amounts corresponding to a notional value of 200×10^{-3} nmoles per g fresh weight (Table 10). None of the crop plants studied by Taylor *et al.* (1988) were found to metabolize 2,6-Cl₂-PHE. In contrast, *Sinapis arvensis* produced moderate amounts of 2,6-Cl₂-CNA and this production would be worth investigating further since 2,6-Cl₂-CNA exhibits very high growth-regulating activity (Table 12). Of the weeds examined by Taylor *et al.* (1988), *Amaranthus retroflexus* and *Chenopodium album* were found to metabolize 2,6-

dichlorophenylalanine. Metabolism was low in *Amaranthus retroflexus* but high amounts of 2,6-Cl₂-PAA were produced in *Chenopodium album*.

Both 3,4-Cl₂-PHE and 3,5-Cl₂-PHE were metabolized to the corresponding dichloro-benzoic acids in very high amounts in *Cirsium arvense* (Table 10). It is unfortunate that 3,4-Cl₂-BZA and 3,5-Cl₂-BZA are devoid of growth-regulating activity thus making the high production of these acids in *Cirsium arvense* of no special interest in relation to the use of 3,4-Cl₂-PHE or 3,5-Cl₂-PHE as herbicides precursors. In contrast, the metabolism of 3,4-Cl-PHE to 3,4-Cl-BZA occurred only moderately in *Vicia faba*. Taylor *et al.* (1988) reported moderate conversion of 3,4-Cl₂-PHE to 3,4-Cl₂-PAA in the three crop plants they studied while metabolism of this dichloro-phenylalanine to the three corresponding dichloro-acids was very low in the weed species they examined. Metabolism of 3,5-Cl-PHE to the corresponding dichloro-acids was low or non-existent in all of the species tested by these authors.

5.4 Structure-Metabolism Relationships

As mentioned in Results section 4.4, when the results from the three species are collectively examined using the sum of the notional values for the corresponding Cl-PAA and Cl-BZA produced in each treatment as an indicator of the degree of metabolism of the supplied chloro-phenylalanine, it is found that 3-Cl-, 4-Cl-, 3,4-Cl₂- and 3,5-Cl₂-PHE are the chloro-phenylalanines that are metabolized to the greatest extent, with 4-Cl-PHE showing the highest degree of metabolism (Table 11). A similar treatment of the data from the six crop and

weed species examined by Taylor *et al.* (1988) shows that in their study too, 3-, 4-, 3,4- and 3,5-Cl₂-PHE were metabolized to a greater extent than the other chloro-phenylalanines, with 4-CL-PHE again showing the highest level of metabolism.

In their study of the ability of mono-substituted phenylalanines to serve as substrates for the aspartate-aromatic aminotransferase isozymes I and II isolated from bushbean shoots, Taylor and Wightman (1987c) found that of the three possible ring-substitution positions, substitution in the 4- position with hydroxy-, chloro- or amino-substituents yielded molecules with the greatest ability to serve as substrates for the two isozymes. Substitution in the 3- position gave a phenylalanine showing intermediate activity as a substrate, while substitution in the 2- position resulted in compounds with only low ability to serve as substrates for isozyme II and with no ability to react with isozyme I in the case of a halogen substituted compound. Based on the assumption that the active site of aspartate-aromatic aminotransferase from a plant source is similar to that of the same enzyme from an animal source, Taylor and Wightman (1987c) explained the effect of ring-substituents on the ability of phenylalanine to serve as a substrate for the aminotransferase by drawing an analogy between the ion pairing between the aromatic ring and a positively charged region at the enzyme's active site, and an electrophilic aromatic substitution reaction. An electrophilic Arg residue of the active site "adds" to the electron rich aromatic ring of the substrate molecule. It is known that substituents on an aromatic ring, through their inductive or resonance effects, will affect a subsequent substitution reaction on the ring in two ways; they

can be either activating or deactivating toward a subsequent electrophilic substitution reaction, and they also have a positional directing effect (McMurray 1984). All of substituents that lead to high activity of the substituted phenylalanine as a substrate with isozyme II, (ie. halogen atoms, amino and hydroxyl groups), have in common that they develop a positive charge by resonance and thus leave an induced distal negative charge on the ring. They are also *ortho-para* directing, meaning they will favor an aromatic substitution in a position *ortho*- or *para*- to themselves. In consequence, ion pairing between the substituted phenylalanine and the arginine residue of the active site of the aminotransferase will be strongest when the induced negative charge, *ortho*- to the substituent, is able to line up with the positive charge on the enzyme (Taylor and Wightman 1987c).

The active site of aspartate-aromatic aminotransferase also presents some steric constraints. Dicarboxylate substrates, such as aspartate or glutamate, adjust conformationally to fit a 6.2Å wide active site and bind with their two carboxylate groups in a cisoid conformation (Taylor and Wightman 1987c). Presumably, unsubstituted phenylalanine must also adjust to the active site in a similar fashion. Taking into consideration these two conditions, namely, steric constraints of the active site and electronic effects of the substituent, Taylor and Wightman (1987c) showed that only when in the 4-position will a negative-charge-inducing and *ortho-para*-directing substituent yield a molecule where the α -carboxylate and distal induced negative charges are 6.2Å apart, and in a cisoid position in terms of their

approach to the active site. It is interesting to note that in the present study it was found that the chloro-phenylalanines which, like 4-Cl-PHE show a high degree of metabolism, namely, 3-, 3,4- and 3,5-Cl-PHE, have in common with 4-Cl-PHE a given combination of dihedral angles in their molecular structure. All the other chloro-phenylalanines which are metabolized to a lesser extent (i.e. the ones containing a chlorine atom in the 2- position) share a set of dihedral angles which is different from that of 4-Cl-PHE. The 3-, 3,4- and 3,5-Cl₂-PHE's will have their distal induced charge in a different position to that of 4-Cl-PHE and they will also present different steric hindrance, yet in the value of their dihedral angles and their degree of metabolism they more resemble 4-Cl-PHE than they do the other chloro-phenylalanines substituted in the 2- position. This suggests that some of these angles, or their combination, are important in relation to the ability of a given chloro-phenylalanine to fit into the active site of the aspartate-aromatic aminotransferase and so undergo transamination, and that substitution in the 2-position is not favorable to attaining that optimum structure.

Although there is a possibility that benzoic acid is formed through further metabolism of PAA (Taylor *et al.* 1988), it is generally considered that it is produced from cinnamic acid. The production of chloro-cinnamic acids was not determined in all of the chloro-phenylalanine treatments, but if benzoic acid is mainly derived from cinnamic acid, a high production of a chloro-benzoic acid would imply a high production of the corresponding chloro-cinnamic acid as the first intermediate in the catabolic pathway. Hanson and Havir (1981), in their

discussion of the catalytic mechanism of phenylammonia lyase, the enzyme responsible for the formation of cinnamic acid from phenylalanine, suggested that phenylalanine fits the active site with an angle of 180° between its $-NH_3$ and pro-3-S hydrogen groups. In 3-, 4-, 3,4- and 3,5- Cl_2 -PHE, for which the sum of chloro-PAA and chloro-BZA produced was greatest, the calculated value for this angle (angle 3) was close to 180° , while in the chloro-phenylalanines showing a lesser degree of metabolism to the corresponding chloro-metabolites, namely all the 2-Cl-substituted compounds, angle 3 was closer to 171° . If the production of chloro-cinnamic acids is considered directly, it is found that in four of the six species studied by Taylor *et al.* (1988) production of 3- and 4-Cl-CNA was higher than that of either 2- or 2,6-Cl-CNA, but in the three species examined in the present study the correlation does not hold very well, since only in *V. faba* were both 3- and 4-Cl-CNA produced in higher quantities than 2- and 2,6-Cl-CNA (Tables 7, 8 and 9). It would have been interesting to have had the authentic standards for all the Cl-CNA's and thus have been able to determine their possible formation from the nine chloro-phenylalanines, since of all the three metabolites studied, cinnamic acid is the only one which is produced from phenylalanine through the action of a single enzyme. Consequently, it would be easier to relate structure and metabolism in a more decisive manner for the production of this metabolite.

In the case of the phenylacetic acid metabolites, much is known about the first enzyme of the catabolic pathway (i.e. the aspartate-aromatic aminotransferase), but there are other enzymes involved before PAA is obtained

from PHE (Wightman 1973, Frégeau and Wightman 1983, Taylor and Wightman 1987a,b,c). Certain chloro-substitution patterns could yield molecules that may readily serve as substrates for the first enzyme but not necessarily for the other enzymes in the pathway, and the reverse may be true. As for the benzoic acid metabolites, the exact enzymatic pathway for their formation is still unknown. A complete structure-metabolism study of the production of these metabolites would require more information about the enzymes involved, and this would first require verification of each intermediate produced in the pathways. Also, for a precise study to be carried out, one would need to work with purified enzymes, and not with the whole plant, to avoid the metabolites being further metabolized or conjugated with other plant constituents.

5.5 General Comments.

As was seen in Results section 4.2, high variations were often observed between the results from repeat experiments. In some chloro-phenylalanine treatments, a high quantity of a given metabolite would be found in one experiment and none or only low quantities of the same metabolite would be detected in repeat experiments. For example, if one considers the production of 2-Cl-metabolites in *Vicia faba* (Table 7), it can be seen that in the first experiment, intermediate amounts of 2-Cl-PAA and 2-Cl-CNA were found, while in the second and third experiments, these two metabolites were not detected at all. When the values for the three experiments were averaged and the standard error calculated, this last parameter was high and the average value might not

represent the actual capacity of the plant to metabolize the chloro-phenylalanine in question, since it is doubtful that if a species had the ability to metabolize a given chloro-phenylalanine in one experiment, this capacity would subsequently disappear. For this and other reasons it was decided not to present an average value for each set of replicate data but to present a notional value based on the range in which the arithmetic average of each set of replicate data fell.

The first, and most obvious, possible explanation to account for the absence of phenyl metabolites from repeat experiments after they were previously found to be produced, is that a high proportion of the metabolites was lost during the extraction procedure so that by the time the sample was subjected to GC-MS, there were not enough metabolite molecules left for detection thus making it look as if none had been produced. The extraction procedure employed is long and involves many steps at which losses can occur. One such step is the purification step before HPLC using C₁₈ mini-columns. Guinn *et al.* (1986), in a study of the methods of purification and measurement of IAA by HPLC, mentioned that recovery rates were higher when this step was omitted. They cite overloading of the mini-column by other substances in the extract which would cause incomplete retention of IAA as one probable reason for the losses. Another reason they mention is incomplete elution of the metabolites from the mini-column. The pre-cleaning step with the C₁₈ mini-columns is, however, necessary because without it the HPLC column, which is an expensive item, would get overloaded and destroyed too fast, even in the presence of a guard column in the HPLC system.

Another step at which it was noticed that considerable losses of phenyl metabolites could occur is the derivatization step prior to GC-MS analysis. With repeat 20 μ g samples of 4-Cl- or 2,6-Cl₂-PAA, -BZA and -CNA standards placed in a vial, derivatized and sent to the GC-MS for analysis, it was found that sometimes none of the compounds was detected in the TIC scan. This occurred more often for Cl-CNA standards than for the Cl-PAA and Cl-BZA standards and the results were unpredictable. The time period allowed for the reaction with the PFB_r was increased from 1 to 1.5 hr, but that only brought a small improvement. Greater care was taken to let the samples cool after the reaction period but no differences were observed following this change of procedure.

A second possible explanation, for high variations in the results from three repeat experiments, (which is also related to the extraction and quantification method) would be a mistake in measuring the amount of internal standard added to a sample. this could either lead to over- or under-estimation of the amount of metabolite present in a given sample.

The variations observed in the amounts of chloro-phenyl metabolites produced in repeat experiments could also be linked to the experimental material. For example, variation in the activities of the enzymes involved in the production and/or further metabolism of the metabolites studied, due to experimental stress on the test plants. As mentioned in Section 4.1, feeding of the shoots with the phenylalanine or chloro-phenylalanines solutions lead to a certain amount of wilting. When the wilting was too severe (more than 8 to 10% loss of fresh weight

of the shoots) the samples were discarded and the experiment redone. Some degree of wilting was, however, almost always observed in the phenylalanine and chloro-phenylalanine treatments, and there is a possibility that this may have affected enzyme activity. Only the free acid forms of the chloro-phenylacetic, chloro-benzoic and chloro-cinnamic acids metabolites were determined in the present study. In most plants IAA can be found conjugated with glucose and aspartic acid (Bandurski and Nonhebel 1984) and conjugation is an important route for the detoxification of herbicidal compounds such as 2,4-D (White *et al.* 1989). IAA conjugation along with IAA catabolism is thought to play an important role in regulating the amounts of free (active) IAA present in plant tissues. Since PAA is also a natural auxin of plants (Wightman and Lighty 1982), there likely exists a means of controlling the amounts of active PAA in shoot tissue, and conjugation of PAA with glucose or aspartic acid is likely one of them. In this connection, N-benzoyl- and N-phenylacetyl-aspartate conjugates have been found in pea seeds (Gianfgna and Davies 1980). There is thus a strong possibility that some of the chloro-phenylacetic, chloro-benzoic acid and chloro-cinnamic acids produced in these experiments would have been converted *in vivo* to their corresponding glucosyl or aspartyl esters. If this was the case, it is possible that the enzyme involved in the conjugation processes may have been sensitive to the wilting of the plants resulting in high variation in the amounts of free chloro-phenyl metabolites found in the tissues.

6. Conclusions

As was initially hypothesized, the present study has shown that excised shoots of the crop and of the two weeds investigated do have the ability to metabolize most of the chloro-phenylalanines to the corresponding chloro-PAA, chloro-BZA and chloro-CNA metabolites. It was seen that the extent of metabolism varied with the chloro-substitution pattern with, in general, substitution in the 3- and 4- position being more favourable to metabolism than substitution in the 2- position. Some differences between the species in their degree of metabolism of the chloro-phenylalanines were also found and with regards to the possible use of chloro-phenylalanines as precursors for the lethal synthesis of herbicidal products, the most interesting findings are the moderate production of 4-Cl-CNA from 4-Cl-PHE in *Cirsium arvense*, as well as the production of 2- and 3-Cl-CNA, 4-Cl-PAA and 2,6-Cl-CNA from the corresponding Cl-PHE's in *Sinapis arvensis* while all but one of these compounds are only produced in low amounts in *Vicia faba*.

The actual physiological effects of these potentially potent chloro-phenylalanines when applied through foliar spray on whole plants of the above weed species, however, has not yet been tested, since the quantities of chloro-phenylalanine compounds available were not sufficient to allow for such a study. An investigation aimed at testing the herbicidal potential of some of the chloro-phenylalanines under greenhouse and field conditions would be the next logical

step.

Taking into consideration the metabolism of 4-Cl-PHE to 4-Cl-PAA and the conversion of 2-Cl, 3-Cl and 2,6-Cl₂-PHE to the corresponding Cl-CNA's in *Sinapis arvensis*, it would be interesting to investigate the metabolism of these chloro-phenylalanines in the crop species canola (*Brassica napus*), since it is an important Canadian crop which is closely related to *Sinapis arvensis* and in which *Sinapis arvensis* is a common weed. Usually the more closely related a crop plant is to its associated weeds, the more difficult it becomes to kill the weeds without affecting the crop. Should *Brassica napus* prove to have a lower capacity than *Sinapis arvensis* to metabolize the 2-Cl, 3-Cl, 4-Cl- or 2,6-Cl₂-PHE, and these chloro-phenylalanines are actually shown to kill weeds when applied under field conditions, this type of lethal synthesis could provide a means of selectively controlling weeds in this crop.

When the relationship between structure of the chloro-phenylalanines and their degree of metabolism is considered, it appears that chloro-substitution in the 3- or 4-position, except if combined with substitution in the 2-position, leads to high rates of metabolism. All of the highly metabolized chloro-phenylalanines shared a similar combination of dihedral angles in their molecular structure which was different from that shared by the chloro-phenylalanines which were less efficiently metabolized, namely, the 2-Cl-substituted compounds. Therefore, a relationship between structure and metabolism of the chloro-phenylalanines was indeed found. It is interesting to note that the ring-substitution positions favoring

metabolism of the phenylalanine molecule are different from those favoring high physiological activity of the end-product molecules. For example, 2-Cl-substitution leads to poor metabolism of the chloro-phenylalanine, whereas two of the corresponding possible metabolites, 2-Cl-PAA and 2-Cl-CNA, are highly active compounds.

Finally, should further investigations be made on the metabolism of chloro-phenylalanines in other crop and weed plants, it would be advisable to concentrate on a restricted number of chloro-phenylalanines, thus allowing for the use of deuterated standards and for carrying out a greater number of repeat experiments. Feeding the chloro-phenylalanines to excised shoots at a lower concentrations would also be worth trying to see if it reduces the transitory wilting of the shoots, while still yielding a high enough level of metabolites to make their detection possible. Also, a further effort should be made to improve the PFB_r derivatization step and so obtain more consistent GC-MS results.

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