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UNIVERSITY OF ALBERTA

Some effects of chlorsulfuron on assimilate translocation in a susceptible weed species, *Thlaspi arvense* L.

by

Andrea J.M. Lowther

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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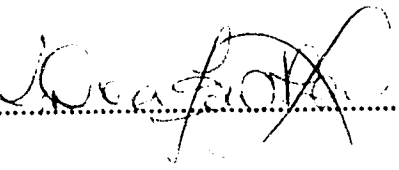
(signed) 

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **Some effects of chlorsulfuron on assimilate translocation in a susceptible weed species, *Thlaspi arvense* L.** submitted by Andrea J.M. Lowther in partial fulfilment of the requirements of the degree of Master of Science in Weed Science.

.....
W. H. Vanden Born

.....
J. P. Tewari

.....
H. D. Bestman

.....
J. J. Zwiazek

Date ....., 1990

This thesis is dedicated to my parents, May and Joseph Lowther, who supported and encouraged my desire to obtain a higher degree. Although we have been separated for over 3 years, I have never stopped receiving their support.

Thanks, Mum and Dad. I love you both so much.

Abstract

Chlorsulfuron is known to have an inhibitory effect on assimilate translocation in susceptible weed species. This study was conducted in order to examine the effect of chlorsulfuron on translocation in stinkweed (*Thlaspi arvense* L.) grown in nutrient culture. Exudation of ^{14}C-assimilates, amino acids, and sugars in excised leaves, and levels of starch, sucrose, glucose, and fructose and amino acids in intact leaves were measured. The herbicide caused reductions in exudation of ^{14}C-assimilates, amino acids, and sugars of 60%, 17%, and 55%, respectively, 34 hours after treatment. In intact leaves, chlorsulfuron caused a decrease in starch reserves to 32% of controls, and a large increase in amino acids, to 6 times that of control leaves, and an increase in sucrose, glucose, and fructose to 3, 10, and 13 times that of controls, respectively, 72 hours after treatment. The results of these experiments indicate that chlorsulfuron had a rapid effect on some aspect of phloem loading, as sucrose, the main component of phloem contents, is reduced in the exudate, and concomitantly, starch and sugars rise in the leaf. The treated leaf had lost the ability to export carbon asimilates to the same extent as control leaves. Addition of branched-chain amino acids to the nutrient solution brought about a partial reversal of the phytotoxic effects of chlorsulfuron both in excised leaves and intact plants.

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1. Introduction

An integral part of herbicide technology is understanding the mode of action of the herbicide in those species of plants against which it is toxic. This understanding provides information regarding plant responses to the herbicide, and also information regarding the most efficient way to apply the herbicide to achieve effective weed control.

Besides having an understanding of the mode of action of a particular herbicide, understanding the secondary, non-lethal effects, of the herbicide on plant metabolic processes provides information on both the chemistry of the herbicide and the interactions of that herbicide within the plant system. In the past, some herbicides have been used in biochemistry research as inhibitors of certain metabolic processes, to yield information about that process which otherwise could not have been discovered. An example is the use of diuron in the study of electron transfer in photosynthesis. It has been discovered by Bestman (1988) that chlorsulfuron has an effect on the translocation of assimilates in excised leaves of stinkweed seedlings. It is entirely possible that chlorsulfuron may have a place in the study of phloem translocation, a process that is not yet fully understood. The purpose of this study was to continue with Bestman's work and to shed more light on the processes that may contribute to the effect of chlorsulfuron on translocation in a susceptible weed species.

In this study, chlorsulfuron effects on translocation were examined in a susceptible weed species, stinkweed (*Thlaspi arvense* L.). The objectives were to (a) re-establish the magnitude by which translocation of major photosynthates was inhibited by chlorsulfuron, as a baseline for this work, (b) measure the levels of amino acids and sugars exuded by chlorsulfuron-treated leaves, as these are believed to be the major assimilates to be found in the

phloem sap, and (c) document the levels of starch, sugars (sucrose, glucose, and fructose, the major breakdown products of starch in plants), and amino acids over time in treated leaves for a more complete picture of the changing levels of these metabolites in response to treatment with chlorsulfuron. In conjunction with all these, objective (d) was to attempt to reverse or reduce the effects of chlorsulfuron with the use of added branched-chain amino acids, with both intact plants and excised treated leaves.

Stinkweed was chosen as the experimental species in this study as research in this particular area had already been started with it and the results that would emerge from this study would complement the work already reported by Bestman (1988). Enough seed was available to carry out all the experiments reported herein using the same collection batch.

2. Literature Review

2.1 Chlorsulfuron

Chlorsulfuron, (2-chloro-N-[[(4-methoxy-6-methyl-1,2,3-triazin-2-yl)amino]carbonyl]benzenesulfonamide), is a relatively new, selective herbicide belonging to the sulfonylurea group, a group noted for high potency in susceptible weed species and for the low application rates needed. This group of herbicides controls a very broad weed spectrum, and the consistency of this control, together with the low mammalian toxicity of the sulfonylureas, has resulted in increased popularity and use of these herbicides worldwide.

2.1.1 Commercial use

Chlorsulfuron is used for broadleaf weed control in cereals and may be applied both preemergence and postemergence (Levitt *et al.*, 1980; Palm *et al.*, 1980; Blair and Martin, 1988). Under the tradename of GLEAN[1] it is available as a 75% dry flowable formulation. It is registered for use in barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), and durum, spring, and winter wheat (*Triticum aestivum* L.), and on plants in non-crop areas. It is recommended for use at three different and increasing rates for weeds of increasing tolerance. Wild buckwheat (*Polygonum convolvulus* L.) and chickweed (*Stellaria media* [L.] Cyrill) require the highest rate of 9 g/ha active ingredient to achieve control. Flixweed (*Descurainia sophia* [L.] Webb) and lamb's quarters (*Chenopodium album* L.) can be controlled with the lowest rate of 4.5 g/ha. Chlorsulfuron is applied at the two-leaf to flag-leaf stages in barley, oats, and wheat and when the weeds are actively

[1]Registered trademark of DuPont Canada Inc.

growing. Chlorsulfuron is also registered for use in tank mixtures with a variety of herbicides used for grassy weed control, to increase the weed spectrum in a single spray operation (Guide to Crop Protection in Alberta, 1989. AGDEX 606-1). One of the surfactants Agral 90[2] , Citowett Plus[3] , or Triton XR[4] is recommended for use with chlorsulfuron.

2.1.2 Persistence in the soil.

Chlorsulfuron can persist in the soil, the residual period depending upon a number of factors, including soil pH, soil organic matter levels, the cation exchange capacity of the soil, soil moisture, soil temperature, and the dose of chlorsulfuron applied in previous seasons. Mersie and Foy (1985) found that, as pH increased beyond 5.6 (towards neutrality), chlorsulfuron was attracted to the soil colloids, thus decreasing the amount of herbicide available for uptake by plants. There was also increased degradation of the herbicide at the more acidic pH values, as less of the herbicide was attracted to soil colloids. Consequently, more was available for degradation by soil microorganisms. Conversely, as pH decreased towards neutrality, more herbicide was available for uptake and could elicit a more severe phytotoxic action due to increased dissociation and polarity (Fredrickson and Shea, 1986). Mersie and Foy (1985) found the optimum soil pH for chlorsulfuron phytotoxicity to be 6.9. As a result of the residual characteristic attributed to the herbicide, care has to be exercised when planning rotations that include the use of chlorsulfuron to ensure that sensitive crops are not in danger of being injured.

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2 Registered trademark of ICI Chipman
3 Registered trademark of BASF Canada Inc.
4 Registered trademark of Rohm & Haas Canada Inc.

2.1.3 Cropping practices

Minimum recropping intervals, the time between spraying chlorsulfuron and planting a sensitive crop, for some common Alberta crops can range from 2 to 48 months (Appendix 1). Chlorsulfuron carryover in soils can result in a reduction in emergence, reduced foliage weight, reduced yield, and in visual symptoms of injury in a susceptible crop species (Peterson and Arnold, 1985; Ritter *et al.*, 1988).

The manufacturers of GLEAN now recommend its use only once in every three years due to the appearance of resistant weed biotypes. Recently, there have been reports in Western Canada and the United States of some weed populations developing resistance after repeated use of the higher rates of chlorsulfuron (1987 - prickly lettuce (Mallory-Smith *et al.*, 1990); 1987 - kochia (Primiani *et al.*,1990); Russian thistle (Holt and LeBaron, 1990)).

2.1.4 Selectivity

Differential metabolism of the herbicide in tolerant and susceptible species is the basis for chlorsulfuron selectivity (Figure 1) (Hageman and Behrens, 1984a; Hutchinson *et al.*, 1984; Sweetser *et al.*, 1982). Hageman and Behrens (1984a) found that susceptible broadleaf species metabolized only 7% of the absorbed chlorsulfuron in 72 hours while tolerant species metabolized 81%. In the tolerant broadleaved species, chlorsulfuron was hydroxylated to metabolite B-1, which was then conjugated with a sugar, presumed to be glucose (Hutchinson *et al.*, 1984). Tolerant grasses, on the other hand,

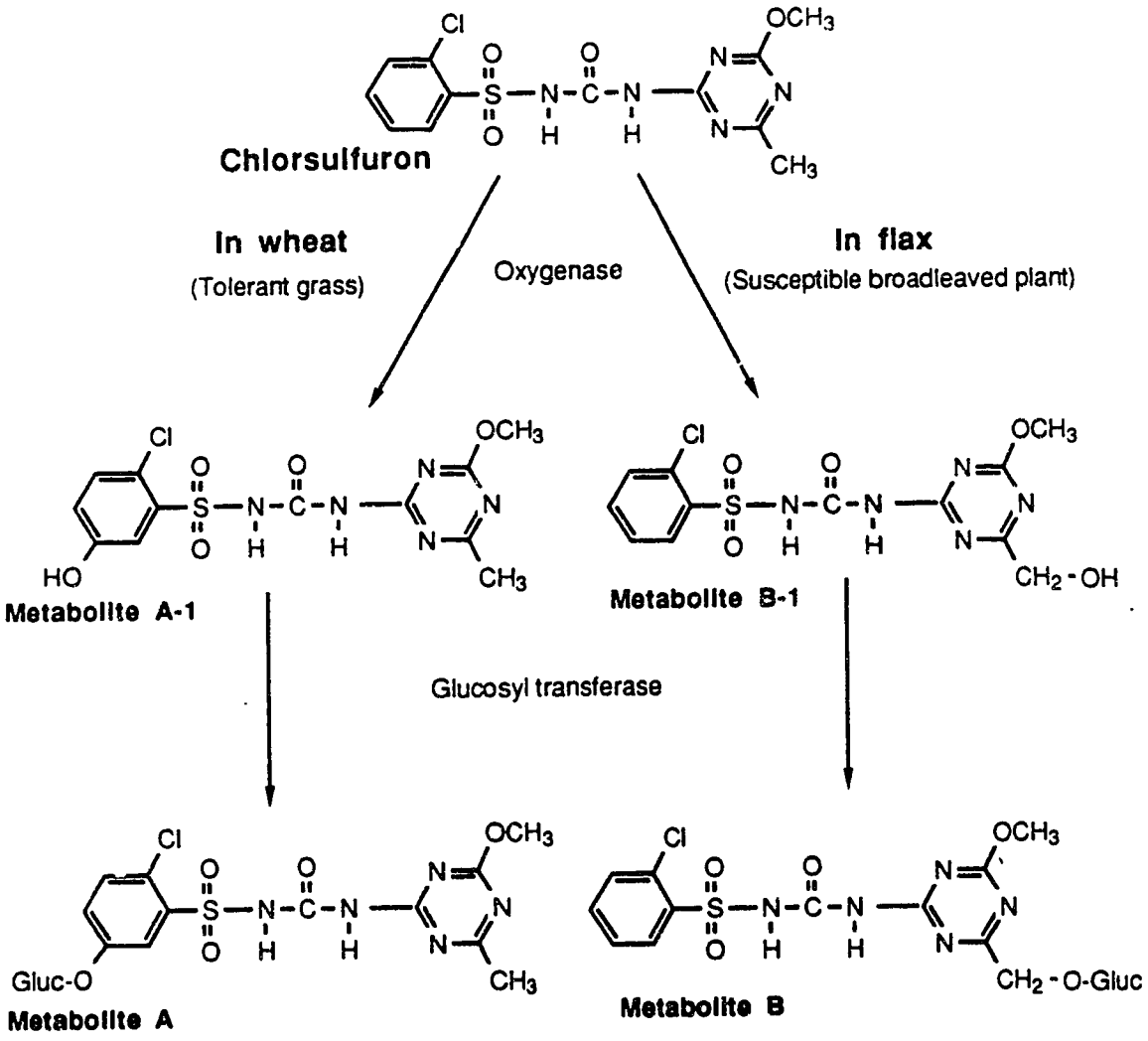

Figure 1. Proposed mechanism of chlorsulfuron breakdown in a tolerant grass,wheat,and a susceptible broadleaved plant, flax.

metabolized chlorsulfuron rapidly to an inactive polar product which resulted when the phenyl ring underwent hydroxylation to metabolite A-1, followed by conjugation with a carbohydrate moiety to metabolite A, also thought to be glucose (Sweetser *et al.*, 1982).

2.1.5 Absorption and translocation

Chlorsulfuron absorption by foliage and roots has been reported by a number of different authors but results are not consistent amongst them. A table is presented which allows for a clearer view of the recently published results (Table 1).

Sweetser *et al.* (1982) reported that 24 hours after the application of ^{14}C-chlorsulfuron, approximately 84% of the applied radioactivity had been absorbed by the treated leaves of sugarbeet, soybean, mustard, and cotton, all of which are susceptible species. The leaves of tolerant wheat, barley, and wild oats absorbed 64% of the applied radioactivity. However, the difference in absorption between tolerant and sensitive species, 20%, is not great enough, in their opinion, to account for tolerance of some species. The extent of absorption was not the same in all species, nor was it always as high as Sweetser *et al.* (1982) found. In eastern black nightshade (*Solanum ptycanthum* Dun.), a tolerant species, and velvetleaf (*Abutilon theophrasti* Medic.), a susceptible species, Hageman and Behrens (1984a) found that 40% of the applied ^{14}C-chlorsulfuron was absorbed by the leaves 24 hours after application, and that there was no difference in absorption between the two species.

Devine and Vanden Born (1985) examined the absorption of chlorsulfuron by leaves of Canada thistle (*Cirsium arvense* [L.] Scop.). Twenty-four hours after application of ^{14}C-chlorsulfuron to the fifth, sixth, or

Table 1. Percent chlorsulfuron absorbed over time in susceptible and tolerant species as reported by a number of different authors. (Papers listed by first author only).

Species	Susceptible (s) or Tolerant (t)	Chlorsulfuron absorption, % total applied Time after treatment, h 12	24	48	144	Author	Date
Sugarbeet	s						
Soybean	s						
Mustard	s						
Cotton	s		84			Sweetser	1982
Wheat	t						
Barley	t						
Wild Oats	t		64			Sweetser	1982
Nightshade	t		40				
Velvetleaf	s		40			Hageman	1984
Canada thistle	s		30		75	Devine	1985
Canada thistle	s			39		Peterson	1988
Stinkweed	s	8	25			Bestman	1988 *
Wild garlic	s		8		62	Leys	1988

*Absorption reported as a percentage of total ^{14}C recovered

seventh leaf, 30% of the applied radioactivity was absorbed from the leaf surface. Leys and Slife (1988) reported that wild garlic (*Allium vineale*) had absorbed 62% of the applied ^{14}C-chlorsulfuron after 144 hours. The results of Devine and Vanden Born (1985) are similar to those of Leys and Slife (1988), who found Canada thistle absorbed 62% of the applied radioactivity after 144 hours.

Bestman (1988) found that 12 and 24 hours after the application of ^{14}C-chlorsulfuron to the third or fourth true leaf of stinkweed, 8 and 25%, respectively, of the recovered activity had been absorbed. Physiological age of the treated leaf, or its location on the shoot, had no effect on the absorption pattern or distribution of the ^{14}C-label.

In wild garlic the absorption was slower, 8-9% after 24 hours (Leys and Slife, 1988) compared to 35-45% after 24 hours in eastern black nightshade and velvetleaf (Hageman and Behrens, 1984a). This shows that not only are the amounts absorbed different in different species, but also that the rate of absorption is highly variable.

The amount reported absorbed in Canada thistle by Devine and Vanden Born (1985) after 24 hours was much less than the results of Sweetser *et al.* (1982) and Hageman and Behrens (1984a). Bestman (1988) suggested that the differences between his results and those of Sweetser *et al.* (1982) and Hageman and Behrens (1984a) probably were due to the different species of weeds used and/or to the variability in the methods used to measure uptake. No explanation has been offered as to why there is so much variability among species.

Bestman (1988) also looked at root absorption of chlorsulfuron by stinkweed. He found that 12 hours after a root application of chlorsulfuron, 12 times more chlorsulfuron was found in the shoot than 12 hours after a leaf

application. After 48 hours, the roots had absorbed 33% of the applied ^{14}C-chlorsulfuron which was considerably more than was reported for the stinkweed leaves. No explanation was offered for the greater ability of the roots to absorb chlorsulfuron. One could speculate that perhaps the fact that the roots lack a cuticle may have an effect on the ability of chlorsulfuron to be absorbed. The cuticle is acknowledged to be the greatest barrier to any foreign substance in gaining entry to the plant through the leaf (Bukovac *et al.*, 1990; Hess and Falk, 1990)

Great variability in the extent of translocation of chlorsulfuron has been reported in the literature (Table 2). Sweetser *et al.* (1982), reported no differences in the quantity of chlorsulfuron, or metabolites of chlorsulfuron, translocated out of the leaves of tolerant or susceptible species. Twenty-four hours after application of ^{14}C-chlorsulfuron, 7% of the applied radioactivity had been translocated out of the treated leaves of sugarbeet, soybean, mustard, and cotton and out of the treated leaves of tolerant species such as wheat, barley, and wild oats.

Hageman and Behrens (1984a) found that eastern black nightshade, a tolerant species, translocated more chlorsulfuron out of the treated leaf than did the susceptible species velvetleaf, both after 24 and 48 hours (68 and 80%, respectively, for eastern black nightshade, and 49 and 57%, respectively, for velvetleaf). This may be a result of the effect chlorsulfuron has on inhibiting translocation (Vanden Born *et al.*, 1988). No statistical difference was found in the amount of chlorsulfuron translocated out of the eastern black nightshade or the velvetleaf over the first 12 hours after application (54 and 46%, respectively).

Devine and Vanden Born (1985) examined translocation of chlorsulfuron in Canada thistle. They found that after 144 hours only 21% of

Table 2. Percent chlorsulfuron translocated over time in susceptible and tolerant species as reported by a number of different authors. (Papers are listed by first author only).

Species	Susceptible (s) or Tolerant (t)	Chlorsulfuron translocation, % total applied — Time after treatment, h				Author	Date	
		12	24	48	144			
Sugarbeet	s							
Soybean	s							
Mustard	s							
Cotton	s		7			Sweetser	1982	
Wheat	t							
Barley	t							
Wild Oats	t		7			Sweetser	1982	
Nightshade	t		49	57				
Velvetleaf	s		68	80		Hageman	1984	*
Canada thist	s				21	Devine	1985	*
Canada thist	s			10		Peterson	1988	
Stinkweed	s	1.7				Bestman	1988	* *
Wild garlic	s				17	Leys	1988	

* Translocation reported as a percentage of total chlorsulfuron absorbed

**Translocation reported as a percentage of total chlorsulfuron recovered

the applied herbicide had been translocated out of the treated leaf to other plant parts, considerably less than that found by Hageman and Behrens (1984a) in the species they were working with. Leys and Slife (1988) found that 17% of the applied ^{14}C-chlorsulfuron was translocated out of the treated leaf of wild garlic after 144 hours. These results are similar to the results of Devine and Vanden Born (1985) and to those of Peterson and Swisher (1985), both of whom worked with Canada thistle.

Bestman (1988) reported that translocation of chlorsulfuron was much less extensive in stinkweed than was reported for other weeds. At 24 hours after application of ^{14}C-chlorsulfuron to the third true leaf, only 1.7% had been translocated out of the treated leaf to other parts of the plant. He postulated that (a) differences in the cuticles (and absorption) or in growing conditions may have been responsible for the large differences between stinkweed and the species studied by Sweetser *et al.* (1982) and Hageman and Behrens (1984a) and (b) chlorsulfuron inhibits its own translocation in susceptible species.

There was greater absorption of chlorsulfuron by the roots than by leaves, with a concomitant increase in the amount translocated. In the experiment in which the roots absorbed 33% of the chlorsulfuron applied (Bestman, 1988), 92% of the absorbed herbicide was recovered from the shoot, indicating greater translocation from the roots to the shoots than out of the leaves. Less chlorsulfuron was found in the third leaf of Canada thistle with 8-10 leaves after a root application than after a foliar application (Devine and Vanden Born, 1985). Also, when applied to a leaf of stinkweed, the chlorsulfuron accumulated in adjacent leaves, but with a root application, there was little difference in the amounts of chlorsulfuron found in the third and fourth true leaves, these being taken as the adjacent leaves. When

applied to the leaves, chlorsulfuron levels were barely detectable in the rest of the shoot, but when applied through the roots, 100-400 times more chlorsulfuron was detected in the shoot.

2.1.6 Mode of action

The first studies on the mode of action of chlorsulfuron showed it to be a fast and powerful inhibitor of cell division and plant growth (Ray, 1980). A concentration as low as 2.8 nM (1 ppb) chlorsulfuron affected growth negatively. Within 6 hours of application of 2.8×10^{-5} M chlorsulfuron to the leaves of corn, the growth rate of these leaves was only 50% of control. With wheat, a tolerant species, this treatment had no effect on the leaf growth rate. There was no direct effect on photosynthesis or respiration at the concentrations that had arrested growth (Ray, 1980; De Villiers *et al.*, 1980). At concentrations that inhibit corn root tip cell division by up to 90%, there was no effect on protein or RNA synthesis, suggesting that the mode of action of chlorsulfuron was very specific. Pea root tip cells were studied to elucidate if growth inhibition was a result of inhibition of cell elongation, or cell division, or perhaps both. Cell elongation was found not to be affected. However, the mitotic index[5] was greatly reduced in cells from treated root tips (0.9 compared to 6.4 in cells from untreated roots), indicating that cell division was inhibited. Ray (1982b) measured the effect of chlorsulfuron on the incorporation of ^{3}H-thymidine into DNA. This technique gives an indirect measure of the amount of DNA synthesized in cells which, in turn, yields information on the extent of cell division. One to two hours after treatment with 2.8 nM chlorsulfuron, inhibition of cell division was noticeable in corn

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[5] The number of dividing cells per 100 cells.

root tip cells. After 6 hours, the amount of thymidine incorporated into DNA in the treated root tip cells was reduced 80 - 90% below the control. However, total uptake of thymidine was unaffected, indicating that the inhibition was not a result of reduced concentrations of thymidine in the cell. Rost (1984) reported that chlorsulfuron was a preprophase[6] inhibitor and indirectly blocked cell division. This was inconsistent with the results of Ray (1982b), however, who reported rapid inhibition in the incorporation of thymidine into DNA. DNA replication occurs before the onset of preprophase in the dividing cell. Therefore, the effect of chlorsulfuron on DNA synthesis and growth was secondary and indirect as there was no inhibition of the enzymes DNA polymerase and thymidine kinase, and the addition of deoxyribonucleosides, DNA precursors, did not reverse the inhibition.

Chlorsulfuron acts directly upon the synthesis of the branched-chain amino acids valine, leucine, and isoleucine (Ray, 1984). The enzyme acetolactate synthase (ALS; EC 4.1.3.18) is common to the biosynthetic pathway of these amino acids and it is strongly inhibited by chlorsulfuron. As little as 28 nM chlorsulfuron significantly inhibited the activity of ALS in excised pea root cultures (Ray, 1984). With the addition of 100 µM valine, leucine, isoleucine, and alanine to the pea root cultures, the inhibitory effect of chlorsulfuron was completely alleviated. However, 100 µM valine and isoleucine in combination could protect roots from growth inhibition in the presence of up to 280 nM chlorsulfuron, which was at least 100 times the concentration usually needed to inhibit growth. Isoleucine, leucine, or alanine alone or in combination with each other did not protect pea root

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[6] DNA is replicated, but chromosomes are not yet visible as distinct structures.

cultures against the inhibitory effect of the herbicide, though valine alone could partially reverse the inhibitory effects. Matsunaka *et al.* (1985) found that a mixture of valine, leucine, and isoleucine at 400 µM would relieve growth inhibition in tobacco cell cultures treated with chlorsulfuron. Scheel and Casida (1985) reported that both valine and leucine, and combinations of isoleucine with valine and/or leucine alleviated chlorsulfuron effects in soybean cultures at concentrations of 380 µM. Rost and Reynolds (1985) found isoleucine and valine at 100 µM ineffective at protecting pea root cultures from chlorsulfuron damage. Robbins and Rost (1987) reported that isoleucine and valine at 50 µM overcame the effects of chlorsulfuron on cell cycling. Giardina *et al.* (1987) reported that valine and isoleucine were ineffective in alleviating the effects of chlorsulfuron as measured by potassium (K^+) uptake in washed maize roots. Chlorsulfuron had no effect on basal (no energy involved) K^+ uptake levels. They also reported that valine and isoleucine at 100 µM were ineffective in preventing growth inhibition in maize plants as measured by root elongation. *In vitro*, however, they found that maize ALS was inhibited to the same extent as pea ALS by chlorsulfuron, indicating the ALS enzymes in both these species are of the same nature. Rubin and Casida (1985) also found two genotypes of maize and one of pea that were not protected by valine and isoleucine. The ALS extracted from these plants was sensitive to chlorsulfuron *in vitro*, however, Giardina *et al.* (1987) suggested that, although ALS was inhibited *in vitro*, this did not provide sufficient evidence that this inhibition is the only or primary site of action. They postulated a second site of action to be on the plasma membrane, since fusicoccin, a phytotoxic promotor of K^+ uptake through the plasma membrane, did not overcome the inhibitory effect of chlorsulfuron (De Agazio and Giardina, 1987).

2.1.7 Secondary effects of chlorsulfuron

The first effect of chlorsulfuron on susceptible target plants is to cause a cessation of growth, for reasons explained above. There are, however, many other secondary inhibitory effects which are exerted upon the plant by this herbicide. DeVilliers *et al.* (1980) investigated the effects of chlorsulfuron on photosynthesis, respiration, RNA synthesis, protein synthesis, and lipid synthesis, using single leaf cells of *Phaseolus vulgaris* L. At concentrations of 10^{-4} M and 5 x 10^{-4} M chlorsulfuron, photosynthesis was inhibited by 23% and 91%, respectively, after 120 minutes. Respiration was inhibited 26% after 120 minutes when cells were treated with 5 x 10^{-4} M chlorsulfuron.

Ray (1982a) noticed no effect of 2.8 x 10^{-4} M chlorsulfuron on photosynthesis by isolated chloroplasts from pea leaves or respiration by isolated cells from spinach leaves. Lower concentrations (10^{-7} M, 10^{-6} M, and 10^{-5} M) had no noticeable effect on RNA synthesis, but at 10^{-4} M and 5 x 10^{-4} M there was 19% and 67% inhibition, respectively, after 120 minutes. Protein synthesis at these concentrations was inhibited 29% and 59%, respectively. Ray (1982b) found that at rates required to inhibit cell division there was no effect on RNA synthesis or protein synthesis. The effects of chlorsulfuron on lipid synthesis were interesting in that at lower concentrations synthesis was stimulated. At the higher concentrations, 10^{-4} M and 5 x 10^{-4} M, synthesis of lipids was inhibited 48% and 64%, respectively.

Suttle and Schreiner (1982) investigated the effects of chlorsulfuron on anthocyanin synthesis and ethylene production in soybean hypocotyls. Applications of 1 µg chlorsulfuron resulted in a 55% increase in anthocyanin

content. The amount of anthocyanin continued to increase with chlorsulfuron applications of up to 5 µg but no further increases occurred with applications up to 100 µg chlorsulfuron. Ethylene levels in plants treated with 10 µg chlorsulfuron rose after 4 days, levelled off after 7 days, and remained above control levels for the duration of the experiment. Ethylene has already been shown to increase anthocyanin levels in some plants (Craker, 1971; Craker and Wetherbee, 1973), and the possibility of a connection was investigated by Suttle and Schreiner (1982) in soybean, but none was found.

Hageman and Behrens (1984b) reported that chlorsulfuron induced leaf abscission in velvetleaf. Five days after treatment, leaf abscission had reached 94%. Chlorsulfuron had stimulated the production of ethylene in the leaf abscission zone. Ethylene levels reached a maximum after 36 hours. At that time the leaves exhibited chlorosis, epinasty, and a loss of nyctinasty, all of which have been shown to be typical symptoms of ethylene in plant systems. The leaf blade produced more ethylene than did the leaf abscission zone but this was attributed to the greater interception of herbicide by the leaf blade. The abscission was due to an increase in the endogenous ethylene levels which, in turn, was mediated by chlorsulfuron.

2.2 Phloem translocation

Phloem translocation has been studied by numerous researchers for many years, and certain aspects of the process still remain in question. The structure of the phloem has been described exhaustively (Wardlaw, 1974; Behnke, 1968; Eschrich, 1970; Weatherly and Johnson, 1968; Pathasarathy, 1975, and references therein; Delrot, 1987). Of particular interest in this

thesis was the nature of the phloem contents and how these contents are loaded and translocated from source to sink.

2.2.1 Chemical composition of the phloem

The chemical composition of the phloem has been characterised extensively in *Ricinus communis* L. (Hall and Baker, 1972; Vreugdenhil and Koot-Gronsveld, 1989; Schobert and Komor, 1989; Kallarackal and Komor, 1989). The pH of the phloem contents was between 7.5 and 8.2 and the sucrose content averaged approximately 100 mg/ml (Hall and Baker, 1972; Vreugdenhil and Koot-Gronsveld, 1989). Potassium was present in high concentrations (0.33-4.4 mg/ml), calcium in very low concentrations (0.2-14.8 µg/ml). Correlations between potassium and sugar levels were investigated by a number of authors and were found to be positive, negative, or zero (Kallarackal *et al.*, 1989). No explanation of the significance of these ions in the phloem was offered by any of the authors.

Sucrose is the main translocated sugar in the phloem, with reducing sugars rarely appearing at any appreciable level. Kallarackal and Komor (1989) found that hexoses such as glucose and fructose occurred only in trace amounts in the phloem. They suggested that the low levels were due not to any inability of the phloem loading system to load the hexoses, but to the very low level of these sugars in the apoplast surrounding the phloem.

Schobert and Komor (1989) analysed the amino acid content of the phloem. Glutamine accounted for approximately one-third of the total amino acids present at a concentration of 50 mM, and valine, isoleucine, lysine and arginine were present at concentrations of 10-15 mM indicating that amino acids are second only to carbohydrates in quantity in the phloem. Housley *et al.* (1977) suggested that amino acids are not as readily taken into the

phloem as is sucrose. The amino acid spectrum of the phloem depended upon the amino acid supply, in that, when the endosperm was removed from cotyledons, the total amount of amino acids decreased and the composition changed (Housley *et al.*, 1977). Urquhart and Joy (1981) found a different amino acid composition in pea plants. Asparagine, homoserine, glutamate, aspartate, and serine were the predominant species found. Apparently, the amino acid spectrum of the phloem is specific to each species.

2.2.2 Phloem loading

The mechanism of phloem loading, defined by Geiger (1975) to be "the process by which the major translocated substances are selectively and actively delivered to the sieve tubes in the source region prior to translocation", has also been under investigation and a number of theories have been put forward. For a substance to be loaded into the phloem it must first be brought into close contact with the phloem tissue from the mesophyll cells. There is still debate as to whether this movement occurs in the apoplast, through the cell wall and other non-living parts of the plant, or in the symplast, via the cell and plasmodesmata[7] . The difficulty in studying this particular area is the inability to separate the phloem tissues from those of the rest of the leaf. It is known that the phloem accumulates sugars and the extent of the process suggests an active accumulation, i.e., it is an energy-requiring process. Many plant tissues have a sucrose/H^+-complex transport system (Buckhout, 1989) and this may also function in phloem loading. No direct evidence is yet available to corroborate this theory.

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[7] Plant cells are interconnected via protoplasmic strands called plasmodesmata.

2.2.2.1 Symplastic theory

Symplastic movement involves direct cell-to-cell transfer of molecules via the plasmodesmata and was first proposed by Cataldo (1974) and Ziegler (1974). It involves sucrose moving along concentration gradients within cells towards the phloem complex. At the boundary of the phloem proper and the mesophyll cell, there is a steep concentration gradient. Proponents of the symplastic theory propose that the assimilates are loaded into the phloem against this gradient as a result of the plasmodesmata acting as one-way valves or as part of an endoplasmic reticulum-mediated pumping mechanism. There is no concrete evidence for this model although it is generally agreed to be conceivable. There are, however, a number of points which the symplastic theory cannot answer at the present. Heyser (1980) and Wright and Fisher (1981) found that active sucrose loading into sieve tubes was closely associated with proton co-transport which would suggest that the loading process of sucrose is active, which seems unlikely if it is symplastic. Phloem loading is extremely selective and the capacity of the plasmodesmata to be as selective is questionable. Also, there are few symplastic connections between mesophyll symplast and the phloem tissues (Fisher and Evert, 1982). As the distance from the phloem increases, more plasmodesmata are found connecting cells than in the regions close to the phloem complex. These authors concluded that it is unlikely that the sucrose found in the phloem is coming in via the plasmodesmata as the capacity to load that much is not physically present in this manner. Van Bel *et al.* (1986) found that sieve tubes can take up sucrose from a surrounding solution faster than the mesophyll tissue. However, in the same paper Van Bel also found evidence to support the symplast-apoplast-symplast theory of

phloem loading. This theory proposes that the sucrose is released into the apoplast from the mesophyll-symplast prior to loading into the phloem.

2.2.2.2 Apoplastic theory

Apoplast loading into the phloem assumes that the assimilates have to be loaded actively as they are present in the free space around the phloem tissue and will not diffuse against the concentration gradient present in the conduit cells. Another implication is that the assimilate must be released by the mesophyll cells into the free space at some point. Many papers are available that demonstrate the presence of sucrose in the free space surrounding the phloem (for example, Giaquinta, (1977), Giaquinta, (1976), Sovonick *et al.*, (1974), Geiger *et al.*, (1974)). There is no experimental evidence to show that the sucrose is released in the vicinity of the phloem, although it is assumed to be so because the opposing flow of water in the xylem could pull the sucrose away in the transpiration stream (Evert *et al.*, 1985). It has been proven conclusively that the phloem can accumulate sucrose against a concentration gradient. However, this same sucrose from the free space may also be accumulated by the mesophyll and parenchyma cells (Geiger, 1975; Fondy and Geiger, 1977). Van Bel (1987) best concluded the status of knowledge in this area when he said "the apoplast concept of phloem loading has no universal validity".

2.2.3 Movement of agricultural chemicals into the phloem

It is necessary to have foliar-applied agricultural chemicals move in the phloem in order to distribute the chemical within the plant. This is particularly important with chemicals whose site of action is removed from the site of application. Two theories explain the phloem mobility of

xenobiotics, namely, the weak-acid theory and the intermediate permeability theory. Neither theory suggests a carrier-mediated system of any kind.

2.2.3.1 Weak-acid theory

This theory depends upon the chemical having a weak acid function as part of the molecule (Crisp and Look, 1979; Neumann *et al.*, 1985; Chamberlain *et al.*, 1986). The molecule enters the phloem passively as an undissociated acid, where the alkaline nature of the phloem contents (see section 2.2.1) causes the molecule to dissociate into an anion to which the membrane is relatively impermeable (Tyree *et al.*, 1979; Edgington, 1981; Briggs *et al.*, 1987). The ionized molecule becomes trapped in the phloem and concentrations 10- to 100-fold of that outside the phloem can accumulate (Briggs *et al.*, 1987). Lichtner (1986) found that, as the lipophilicity of the molecule is increased, the permeability of the dissociated molecule (ion) is increased and as a result it can more easily leave the phloem as it can more easily cross membranes.

2.2.3.2 Intermediate permeability theory

This theory was first presented by Tyree *et al.*, (1979). According to this hypothesis, a non-acidic biochemically stable chemical could diffuse passively into the phloem and be translocated. If the chemical has a high membrane permeability, it can pass out of the phloem and into the xylem and, as a consequence, display ambimobility. If the permeability of the chemical is low enough it can be transported long distances in the phloem as it will have a reduced tendency to diffuse back out across membranes. Bromilow *et al.* (1987) confirmed the theory, and also found that lipophilicity is an important factor in determining permeation rates of chemicals through

plant membranes. They tested a range of compounds with differing lipophilicities, ranging from log K_{ow} -0.87 to 2.27, and found that only the most polar compounds were retained in the phloem long enough to be translocated any distance.

The weak acid theory and the intermediate permeability theory have been recently unified into one model (Kleier, 1988; Hsu *et al.*, 1988; Hsu and Kleier, 1990). Their model suggests the weak acid and intermediate permeability theories are not mutually exclusive, but rather complementary to each other.

2.2.4 Chlorsulfuron and translocation

The mobility of chlorsulfuron was compared to that of clopyralid by Devine and Vanden Born (1985). The two herbicides are quite alike in their physico-chemical properties (Appendix 2), but chlorsulfuron was much less mobile than clopyralid. Devine *et al.* (1987) found that chlorsulfuron was absorbed into plant cells faster than clopyralid. This was accounted for by the greater lipophilicity of the chlorsulfuron which aided entry of the herbicide through the cuticle. They concluded that clopyralid is extremely mobile in the phloem, but that chlorsulfuron is not (Devine and Vanden Born, 1985). They suggested the possibility that chlorsulfuron prevents its own translocation in some plants by a still unknown process, perhaps by affecting the phloem directly.

Treatment of excised leaves and intact plants of stinkweed with 1 µg of chlorsulfuron inhibited translocation of assimilates from the leaf (Bestman *et al.*, 1990; Geiger and Bestman, 1990). The inhibition is thought to be a direct effect of the herbicide on the transport system itself, and is believed to inhibit the translocation of the herbicide itself.

Bestman (1988) postulated that translocation of chlorsulfuron out of the roots suggested movement in the xylem. Chlorsulfuron must also have been mobile in the phloem somewhat to allow movement out of the treated leaves. Transfer from the phloem to the xylem must also have been possible as there was accumulation in the adjacent leaves after application to the leaf.

3. Materials and Methods

3.1 General

3.1.1 Plant material

Stinkweed (*Thlaspi arvense* L.) seeds were pre-germinated in the dark under two different temperatur. regimes[8] . A sheet of Whatman No. 3 filter paper, 9 cm diameter, was placed in a petri dish and 5 ml 10 mM KNO_3 was added. Seed weighing 0.5 g was placed in the petri dish, and each dish was sealed with parafilm, covered with aluminum foil, and kept at 12 C for 4 days. The petri dishes were then exposed to daylight for 5-10 minutes, wrapped in foil again, and kept at room temperature for 2-3 more days or until the seed coat had split open.

Germinated stinkweed seeds were planted in horticultural grade vermiculite in 4 x 4 cm trays that were placed in a growth cabinet with 22 C/18 C day/night temperatures, 18-hour photoperiod, and a light intensity of 400 $\mu E.m^2.sec^{-1}$, provided by fluorescent and incandescent lamps. Relative humidity was 50%.

Plants were used at the 6 - 7 leaf stage in all experiments with the 3rd or 4th true leaf being treated with herbicide or with application solution only.

3.1.2 Nutrient solutions

The seedling trays were watered daily from underneath with half-strength Hoagland's solution (Hoagland and Arnon, 1950) with iron added to a concentration of 5 µg/ml. In the experiments where amino acids were

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[8] S. Goudy, personal communication

supplied in the nutrient solution, 1 mM concentrations of L-valine, L-isoleucine, and L-leucine were used.

3.1.3 Herbicide

Technical grade chlorsulfuron (95% pure) was stored as a 500 µg/ml stock solution in tetrahydrafuran at -20 C. It was applied in solvent containing 10% (v/v) tetrahydrafuran and 0.1% (v/v) surfactant (50% (w/v) octyl phenoxy polyethoxyethanol), in distilled water. A fresh solution was made up for each experiment by pipetting 100 µl of chlorsulfuron stock solution into a small glass vial, and evaporating the tetrahydrafuran in a stream of nitrogen gas, prior to adding 500 µl of application solution. The vial was then placed in a sonic bath for 1 minute to ensure good mixing. From this solution, 10 µl was applied to leaves with a micropipette[9] in such a way as to cover as much of the leaf as possible. Application solution without chlorsulfuron was used to treat control plants.

3.1.4 Extraction of leaf tissue

At harvest, treated and control leaves were weighed, quickly frozen in liquid nitrogen, and stored at -20 C until analysis. The leaves were ground in a mortar and pestle with approximately 1 ml MCW (methanol:chloroform:water (12:5:3, v/v/v)) extraction solution, or enough to dampen the leaf throughout. After grinding, 2 x 2.5 ml MCW was used to wash the ground leaf out of the mortar and into a 12 x 75 mm disposable culture tube. The tubes were centrifuged at 1,200 rpm for 10 minutes. The supernatant was removed and saved, and another 5 ml MCW was added to the pellet. The tubes were centrifuged until the supernatant was clear and

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[9] Wiretrol, Drummond Scientific Co., Broomhall, PA. USA.

all the pigment had been removed, usually three times. After the final centrifugation, the tubes were placed in an oven at 37 C overnight to dry the pellet. Starch analysis was carried out on the dried pellet as described in section 3.1.6.4.

The combined supernatants for each sample were subsequently separated thus: for every 5 ml supernatant, 3 ml water was added, and the tubes were centrifuged at 1,200 rpm for 5 minutes. This separated the chloroform phase, which contained the lipids and pigments, from the methanol-water phase which contained the reducing sugars and other polar compounds. After centrifuging, the supernatant and water mixture had divided into two distinct layers, with the clear methanol-water phase at the top and the green chloroform phase at the bottom. The top phase was removed under vacuum and collected in 100-ml boiling flasks. The colored chloroform phase was discarded. The contents of the boiling flasks were dried down on a rotary evaporator[10] at 50 C under vacuum and then taken up in 1 ml water. These fractions were stored at -20 C and saved for sugar and amino acid analysis as described in section 3.1.6.1, 3.1.6.2 and 3.1.6.3.

3.1.5 Preparation of amyloglucosidase enzyme

A column was prepared after the method described by Penefsky (1977). A porous polyethylene disk[11] was fitted into a 1 ml plastic tuberculin syringe[12] The syringe was then filled to the top with Sephadex G-50 fine, already swollen in 50 mM Tris/acetate, pH 7.5. The column was allowed to drain until no more liquid came out. It was subsequently washed with 10 ml

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10 Brinkman Instruments, Rexdale, Ontario.
11 Kontes Glassware/Instruments
12 Becton Dickson, Rutherford, New Jersey 07070. USA.

0.2 M sodium acetate buffer, pH 4.5, and then centrifuged in a clinical centrifuge for 2 minutes at the 4 setting (~100 rpm).

To prepare the enzyme, 100 µl of amyloglucosidase enzyme (EC 3.2.1.3.) as bought was pipetted onto the column, and centrifuged in a test-tube for 2 minutes in a clinical centrifuge at the 4 setting (~100 rpm). The enzyme collected in the test-tube was free of glucose as determined by the glucose assay described in section 3.1.6.5. Another 50 µl sodium acetate buffer was added to the enzyme, and this was the form in which it was used.

3.1.6 Assay procedures

3.1.6.1 Sugar analysis

An anthrone assay, according to the method of Spiro (1966), was used to estimate total sugars. To prepare the reagent solution, 400 mg of anthrone was combined with 16 ml 95% ethanol and 60 ml double distilled water. To this was added 200 ml concentrated H_2SO_4 while the flask was kept cool under running water. The solution was stored in the dark at 4 C in a dark glass bottle. A 50 µl aliquot of sample was combined with 2 ml of the anthrone reagent in a 6-ml culture tube which was then sealed with a lid and heated in an aluminum block at 95 C for 10 minutes, cooled on ice, and then the absorbance at 620 nm[13] was determined. Each time the assay was run, a standard curve was prepared, with 5.56 mM[14] glucose, to relate absorbance units to sugar concentration. The total amount of sugars was expressed as millimoles glucose equivalents.

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[13] Bausch & Lomb Spectronic Model 601.

[14] Glucose solution supplied by Sigma Diagnostics in Glucose Kit.

3.1.6.2 Amino acid analysis

A ninhydrin assay after the method of Moore (1968) was used to estimate total amino acids. A 40 µl aliquot of sample was combined with 360 µl ninhydrin reagent in 6-ml disposable culture tubes. The tubes were capped and heated in a aluminum block at 90 C for 10 minutes. After this, the tubes were cooled on ice, 1.6 ml water: 1-propanol (1:1, v/v) was added and the tubes were vortexed to ensure good mixing. The absorbance at 570 nm was determined, and the amino acid concentration present was expressed as leucine equivalents. Each time the assay was run, a standard curve was prepared using 2 mM leucine to relate absorbance units to amino acid concentration.

3.1.6.3 Glucose, fructose, and sucrose analysis

Concentrations of the sugars were determined using a combined enzymatic-UV-method[15] . In this assay, D-glucose was determined before and after the hydrolysis of sucrose. D-fructose was determined after the second D-glucose absorbance reading was taken. Absorbance was measured at 334 nm.

The first absorbance reading gave the free D-glucose in the solution. When sucrose was then hydrolysed, it released equal amounts of glucose and fructose. The second glucose absorbance reading then was taken. This value minus the first gave the amount of glucose released by the sucrose upon hydrolysis, and this was also equal to the amount of fructose released by the sucrose. The final fructose absorbance reading gave the total fructose in solution, which, minus the amount calculated to be released

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[15] Boehringer Mannheim GMBH Biochemica.

by the sucrose on hydrolysis, gave the amount of free fructose in the original solution.

Any free glucose-6-phosphate (G-6-P) or fructose-6-phosphate (F-6-P) present in the exudate will also be measured in this assay. The basic reactions for the assay are the conversions of glucose and fructose to G-6-Pand F-6-P, respectively, by hexokinase, and then the conversion of G-6-P and F-6-P to gluconate-6-phosphate and G-6-P, respectively. Both G-6-P and F-6-P, however, are presumed to be present at very low concentrations in the exudate.

3.1.6.4 Starch analysis

The weight of the dried pellet was recorded after extraction of the leaf and 2 ml water was added to the 6-ml culture tube it was in. The tube was capped, a pinhole was made in the cap, and the contents were boiled vigorously in an aluminum block for 60 minutes to gelatinize the starch. The tubes were removed from the block and cooled on ice before the addition of 10 µl prepared amyloglucosidase enzyme (section 3.1.5.). Incubation for 24 hours at 37 C followed to hydrolyse the starch to glucose. After incubation, a weighed 10 µl aliquot was used for a glucose determination by the method described in section 3.1.6.5.

3.1.6.5 Starch-derived glucose analysis

An enzymatic[16] , quantitative determination of glucose concentration was used to estimate the concentration of starch-derived glucose. A 10 µl weighed sample was added to a mixture of glucose oxidase, peroxidase,

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16 Sigma Diagnostics, P.O. Box 14508, St. Louis, MO 63178. USA.

and *o*-dianisidine. The reaction proceeded to completion in approximately 30 minutes at 37 C, and the absorbance at 470 nm was recorded. A standard curve was prepared with 2.78 mM glucose each time the assay was run to relate absorbance units to concentration.

3.1.5.6 Liquid scintillation spectrometry

All radioactivity was determined using liquid scintillation spectrometry (LSS)[17]. Standard scintillation vials were used. Aquasol-2[18] or Ready-Gel[19] liquid scintillation cocktail was used for counting aqueous samples.

3.1.6.7 Gas chromatography

CO_2 determinations were made using a gas chromatograph (GC)[20] with a 12.70 x 0.32 cm column packed with HayeSep T[21]. Running conditions for the column were as follows: helium gas flow, 30 cm^3/min; oven temperature, 32 C; TCD detector; sample size, 1 ml.

3.2 Assimilate translocation

The translocation of assimilates out of excised stinkweed leaves was studied using a method developed by Bestman (1988). The experiment was carried out twice, once with plants that, before excision of the leaves, had excess branched-chain amino acids, valine, leucine, and isoleucine, present in the nutrient solution at 1 mM concentration, and once without

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17 United Technologies Packard. Minaxiβ Tricarb 4000 series
18 New England Nuclear, 549 Albany St., Boston, MA 02118. USA.
19 Beckman Instruments Inc., 2500 Harbour Boulevard, Fullerton, CA 92634-3100. USA.
20 Hewlett Packard. Model 5890.
21 Supelco Canada Ltd., 46-220 Wyecroft Rd., Oakville, Ont. Canada.

supplementing with amino acids. Each experiment consisted of two runs with six leaves per run.

3.2.1 Experimental protocol

In the ^{14}C-assimilate translocation experiments the 3rd or 4th true leaf was treated with 1 µg chlorsulfuron in 10 µl application solution, or with the application solution alone, using a micropipette. The droplet thus applied was then spread over the entire surface of the leaf using the side of the micropipette. After 24 hours, the leaves were excised and immediately placed in a petri dish containing fresh exudate solution (5 mM phosphate buffer (pH 6.0) with 0.5 mM EDTA). The petioles were recut below the solution surface and quickly transferred into 1.5-ml microfuge tubes containing exudation solution already in place in the inner compartment of the assimilation chamber (only the inner compartment of the assimilation chamber was used for experiments on the excised leaves). Once all the leaves were in place, the chamber was sealed and the gas flow was started to maintain an atmosphere of appromimately 500 µl/L CO_2 (the flow was adjusted manually, and it was not exactly the same for every run in the chamber). Prior to $^{14}CO_2$ exposure as described in section 3.2.2, the gas flow was monitored for an hour and the amount of CO_2 fixed by the leaves was calculated from GC analysis of samples as described earlier (section 3.1.6.7).

Every 2 hours, for 10 hours, the exudate in the microfuge tubes was taken out and replaced with fresh exudation solution. Each exudate sample was divided into four equal, weighed aliquots. Three were lyophilized, and stored at -20 C, until they were analyzed for total sugars and amino acids. One aliquot was immediately assayed by LSS for radioactivity present.

3.2.2 $^{14}CO_2$ exposure procedure

$^{14}CO_2$ was introduced to the inner compartment with a peristaltic pump system separate from the pump system used to maintain the atmosphere for the excised leaves in the absence of $^{14}CO_2$. The $^{14}CO_2$ was generated by injecting (^{14}C)-$NaHCO_3$ solution into lactic acid. The system was closed and the excess $^{14}CO_2$ was collected in 15% (w/v) KOH traps. The traps were changed after every second run. The level of CO_2 in the inner compartment was maintained by pumping $NaHCO_3$ into the lactic acid at a rate equivalent to that of the predetermined CO_2 incorporation by the leaves. The labelling period was 30 minutes, after which the system was returned to the non-radioactive gas flow, and the experiment ran for 10 hours.

3.2.3 Exudation by leaves of seedlings grown in amino acid supplemented nutrient solution

The experiment described in section 3.2.1 was repeated but with the plants receiving nutrient solution with added branched-chain amino acids (L-valine, L-leucine, and L-isoleucine) as described in section 3.1.2, 24 hours prior to and for the duration of the experiment after treatment with chlorsulfuron.

3.3 Effect of chlorsulfuron on metabolite levels in leaves of intact plants

The purpose of this experiment was to monitor the levels of starch, amino acids, sucrose, glucose, and fructose in treated leaves over a period of three days.

3.3.1 Experimental procedure

Plants were grown as previously described in section 3.1.1, and treated with 1 µg chlorsulfuron applied from a micropipette. Six, 12, 24, 36, 48, or 72 hours after treatment, the treated and control leaves were harvested, weighed, and frozen in liquid nitrogen. The experiment was carried out twice, with three leaves per treatment. The leaves were ground and extracted as described previously. The amount of starch in the pellet was determined, and amino acid, glucose, fructose, and sucrose assays were carried out on the supernatant using the procedures for these assays that have already been described.

3.4 Effect of exogenously supplied amino acids on metabolite levels in leaves of intact plants treated with chlorsulfuron

The time-course experiment described above was repeated with plants in nutrient solution supplemented with branched-chain amino acids (1mM concentrations each of L-leucine, L-isoleucine, and L-valine), to determine if the addition of these amino acids could alleviate the chlorsulfuron effect in leaves of intact plants. This ability of the amino acids to protect excised leaves or cell cultures against the effects of chlorsulfuron has been demonstrated by many authors (Bestman, 1988; Matsunaka *et al.*, 1985; Scheel and Casida, 1985; Ray, 1984).

3.4.1 Experimental procedure

The experiment was carried out according to the procedures in section 3.3.1, except that only three leaf harvest times were included, 24, 36, and 48 hours. From the previous experiment it was concluded that most of the important changes in metabolites occurred around these times. Starch, amino acid, sucrose, glucose, and fructose levels were measured.

4.1 Effect of chlorsulfuron on exudation of selected assimilates from excised leaves of stinkweed seedlings

4.1.1 Total ^{14}C-activity exuded from excised leaves

The application of 1 µg chlorsulfuron to the third true leaf of stinkweed seedlings 24 hours prior to exposure of the excised, treated leaves of these seedlings to $^{14}CO_2$, caused a reduction in total ^{14}C-activity exuded from these excised leaves (Figure 2).

Two hours after exposure to $^{14}CO_2$, total ^{14}C-activity exuded by chlorsulfuron-treated leaves was 25% that of control leaves (Table 3). Over the 10-hour duration of the experiments, treated leaves exuded 40% of the amounts exuded by control leaves, 491 and 1216 dpm/mg fresh weight of leaves, respectively. These results are in accord with observations reported by Bestman (1988) and confirm the effect chlorsulfuron has in reducing the ability of the leaf petioles to exude ^{14}C-labelled metabolites.

$^{14}CO_2$ is known to be assimilated by leaves in the same manner as CO_2. Therefore, $^{14}CO_2$ is incorporated into metabolites as a result of being fixed in photosynthesis. Chlorsulfuron does not appear to have any effect on $^{14}CO_2$-fixation by photosynthetic processes within 24 hours after treatment (Bestman, 1988) and, accordingly, a reduction in total ^{14}C-activity in leaf exudates indicates a decrease in the total amounts of metabolites exuded by treated leaves.

When plants were placed in nutrient solution with added branched-chain amino acids (L-leucine, L-isoleucine, and L-valine), 24 hours prior to herbicide application, and the experiment was repeated, there was a

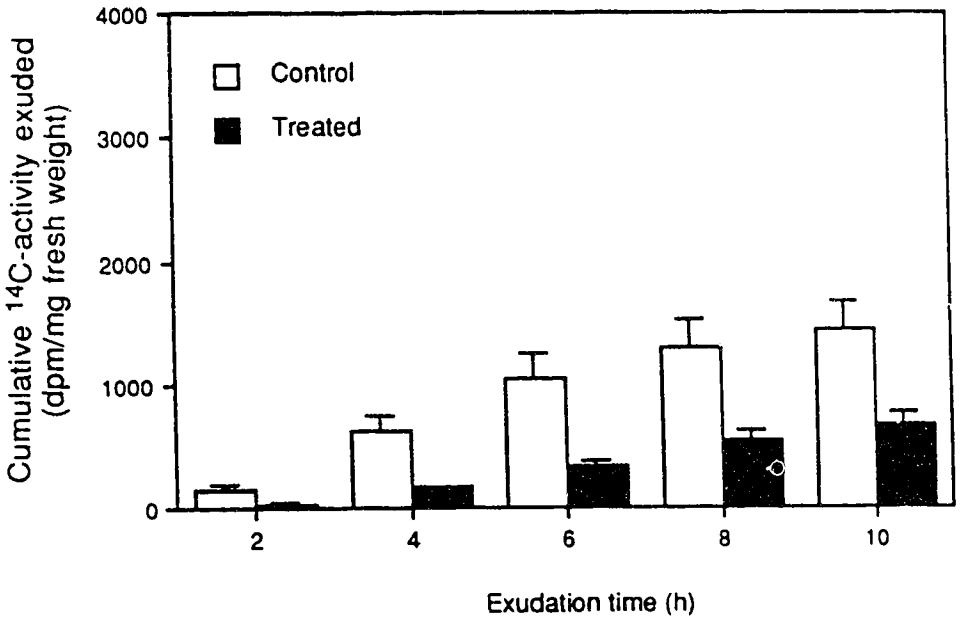

Figure 2. Exudation profiles of ^{14}C-activity for single, excised leaves of stinkweed seedlings, following application of 0 (control) or 1 µg (treated) chlorsulfuron 24 h prior to excision and exposure to $^{14}CO_2$ for 30 min. The data, means and standard errors, are the results of three runs with six leaves (three controls, three treated) per run.

Table 3. The effect of 0 (control) or 1 µg chlorsulfuron on ^{14}C-activity exuded by single, excised leaves of stinkweed seedlings. Leaves were exposed to $^{14}CO_2$ for 30 min 24 h after treatment with herbicide. The data, means with standard errors in parentheses, are the results of three runs with three leaves per treatment per run.

Time	Cumulative ^{14}C-activity exuded		
	Control	Treated	% of Control
(h)	(dpm/mg FW*)	(dpm/mg FW*)	(%)
2	134 [45.5]	34 [7.0]	25
4	498 [126.1]	138 [22.1]	28
6	802 [197.5]	229 [55.5]	29
8	1044 [254.8]	386 [65.0]	37
10	1216 [296.4]	491 [92.5]	40

*Fresh weight of leaves

treated leaves, but the percentage reduction attributable to chlorsulfuron was much less (Figure 3). After 2 hours, total ^{14}C-activity exuded by treated leaves was the same as by control leaves (Table 4). By 10 hours, treated leaves had exuded 64% of the radioactivity exuded by control leaves (Table 4). These data indicate that the addition of the amino acids to the nutrient solution in some way caused, or stimulated, the leaves to exude more ^{14}C-assimilate in the presence, and absence, of chlorsulfuron than without the addition of amino acids to the nutrient solution.

4.1.2 Total sugars exuded from excised leaves

The application of chlorsulfuron to the third true leaf of stinkweed seedlings had an inhibitory effect on the exudation of sugars from excised leaves (Figure 4). Ten hours after the start of the experiment, cumulative sugar amounts exuded by treated leaves were approximately 45% of controls, 4.6 and 10 nmoles/mg fresh weight, respectively (Table 5). This trend is similar to the effect chlorsulfuron had on exudation of total ^{14}C-activity from treated leaves.

The addition of the amino acids L-leucine, L-isoleucine, and L-valine to the nutrient solution prior to treatment with chlorsulfuron did nothing to restore exudation of sugars from treated leaves to the levels for control leaves (Figure 5). Indeed, the decrease in exudation of sugars by treated leaves was greater than in the case of treated leaves in the absence of amino acids in the nutrient solution. At 2 hours, treated leaves had exuded only 2% of controls, 0.2 and 8.4 nmoles/mg fresh weight, respectively (Table 6). By 10 hours, treated leaves had exuded 35% of

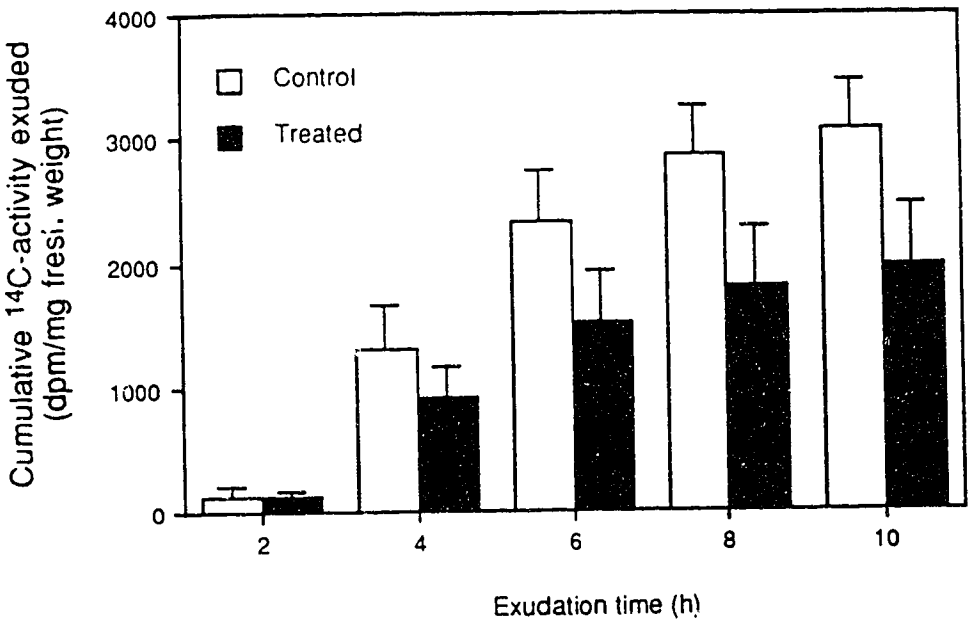

Figure 3. Exudation profiles of ^{14}C-activity for single, excised leaves of stinkweed seedlings, following application of 0 (control) or 1 µg (treated) chlorsulfuron 24 h prior to excision and exposure to $^{14}CO_2$ for 30 min. L-leucine, L-isoleucine, and L-valine were present in the nutrient solution 24 h prior to treatment with herbicide. The data, means and standard errors, are the results of three runs with six leaves (three controls, three treated) per run.

Table 4. The effect of 0 (control) or 1 µg chlorsulfuron on ^{14}C-activity exuded by excised leaves of stinkweed seedlings. Leaves were exposed to $^{14}CO_2$ for 30 min 24 h after treatment with herbicide. Branched-chain amino acids were added to the nutrient solution 24 h prior to treatment with the herbicide. The data, means with standard errors in parentheses, are the results of two runs with three leaves per treatment per run.

Time	Cumulative ^{14}C-activity exuded		
	Control	Treated	% of Control
(h)	(dpm/mg FW*)	(dpm/mg FW*)	(%)
2	134 [66.4]	130 [37.1]	97
4	1307 [358.4]	906 [253.4]	69
6	2330 [400.8]	1514 [416.1]	65
8	2867 [394.6]	1812 [474.9]	63
10	3060 [392.9]	1961 [499.2]	64

*Fresh weight of leaves

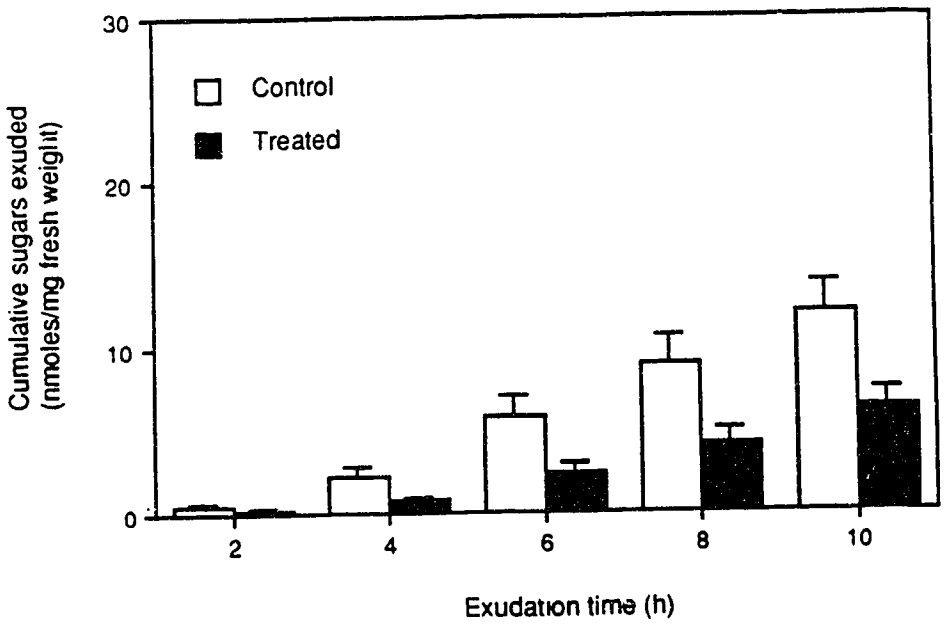

Figure 4. Exudation profiles of sugars for single, excised leaves of stinkweed seedlings, following application of 0 (control) or 1 µg (treated) chlorsulfuron 24 h prior to excision and exposure to $^{14}CO_2$ for 30 min. The data, means and standard errors, are the results of three runs with six leaves (three controls, three treated) per run.

Table 5. The effect of 0 (control) or 1 µg chlorsulfuron on sugars exuded by excised leaves of stinkweed seedlings. Leaves were exposed to $^{14}CO_2$ for 30 min 24 h after treatment with herbicide. The data, means with standard errors in parentheses, are the results of three runs with three leaves per treatment per run.

Time	Cumulative sugars exuded		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
2	0.47 [0.19]	0.21 [0.07]	45
4	1.63 [0.54]	0.61 [0.23]	37
6	3.88 [1.22]	1.57 [0.43]	41
8	6.61 [1.97]	2.54 [0.74]	38
10	10.03 [2.70]	4.56 [1.18]	45

*Fresh weight of leaves

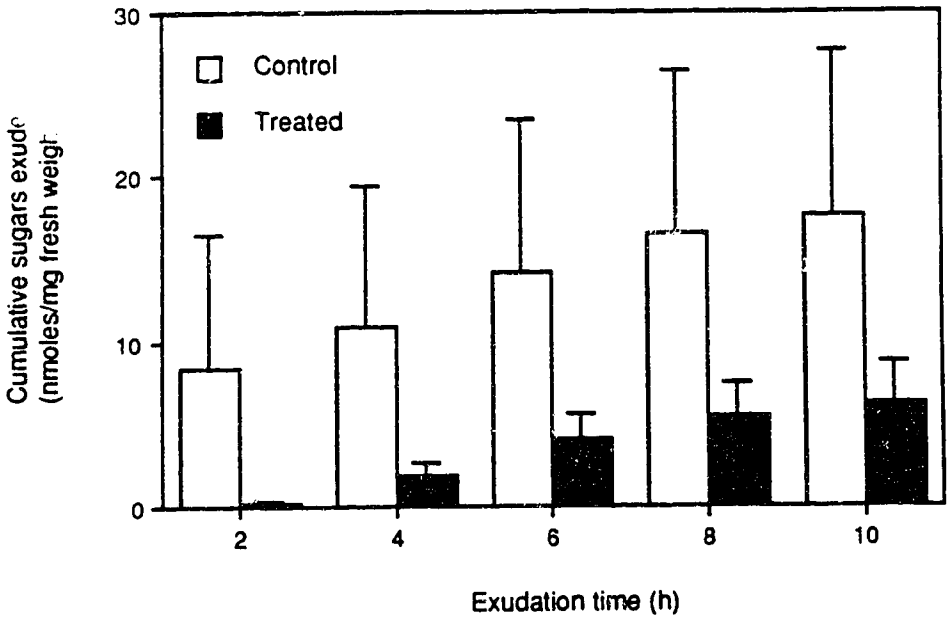

Figure 5. Exudation profiles of sugars for single, excised leaves of stinkweed seedlings, following application of 0 (control) or 1 μg (treated) chlorsulfuron 24 h prior to excision and exposure to $^{14}CO_2$ for 30 min. L-leucine, L-isoleucine, and L-valine were present in the nutrient solution 24 h prior to treatment with herbicide. The data, means and standard errors, are the results of two runs with six leaves (three controls, three treated) per run.

Table 6. The effect of 0 (control) or 1 µg chlorsulfuron on sugars exuded by excised leaves of stinkweed seedlings. Leaves were exposed to $^{14}CO_2$ for 30 min 24 h after treatment with herbicide. Branched-chain amino acids were added to the nutrient solution 24 h prior to treatment with the herbicide. The data, means with standard errors in parentheses, are the results of three runs with three leaves per treatment per run.

Time	Cumulative sugars exuded		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
2	8.4 [8.1]	0.2 [0.1]	2
4	10.9 [8.6]	1.8 [0.9]	17
6	14.2 [9.3]	4.0 [1.6]	28
8	16.5 [9.9]	5.4 [2.1]	33
10	17.5 [10.1]	6.2 [2.5]	35

*Fresh weight of leaves

controls, 6.2 and 17.5 nmoles/mg fresh weight, respectively (Table 6). These data disagree with the results obtained by Bestman (1988), who demonstrated a complete recovery of the ability by the leaf to exude sugars with the addition of amino acids to the nutrient solution.

As was the case with the exudation of ^{14}C-activity, exudation of sugars in control leaves was stimulated with the addition of branched-chain amino acids to the nutrient solution. At 2 hours, control leaves from this experiment had exuded almost 18 times more sugars than control leaves from the last experiment, 8.4 and 0.47 nmoles/mg fresh weight, respectively. By 10 hours the gap between the two was reduced 16-fold, with values of 17.5 and 10 nmoles/mg fresh weight, respectively (Tables 6 and 5, respectively).

4.1.3 Total soluble amino acids exuded from excised leaves

Chlorsulfuron had a relatively small effect on the exudation profiles of soluble amino acids by treated leaves (Figure 6). Two hours after the commencement of the experiment there was no observable effect. By 10 hours, however, treated leaves had exuded 17% less than control leaves, 3.4 and 4.0 nmoles/mg fresh weight, respectively (Table 7).

With amino acid supplementation of the nutrient solution, exudation of amino acids in control leaves was apparently enhanced or stimulated slightly. At 10 hours, controls from the unsupplemented and supplemented experiments had exuded 4 and 6 nmoles/mg fresh weight, respectively. At 2 hours, treated leaves were exuding 86% of controls, and by 10 hours this had decreased to 68% of controls (Table 8). The chlorsulfuron effect on amino acid exudation was not alleviated (Figure 7); rather, even less, as a

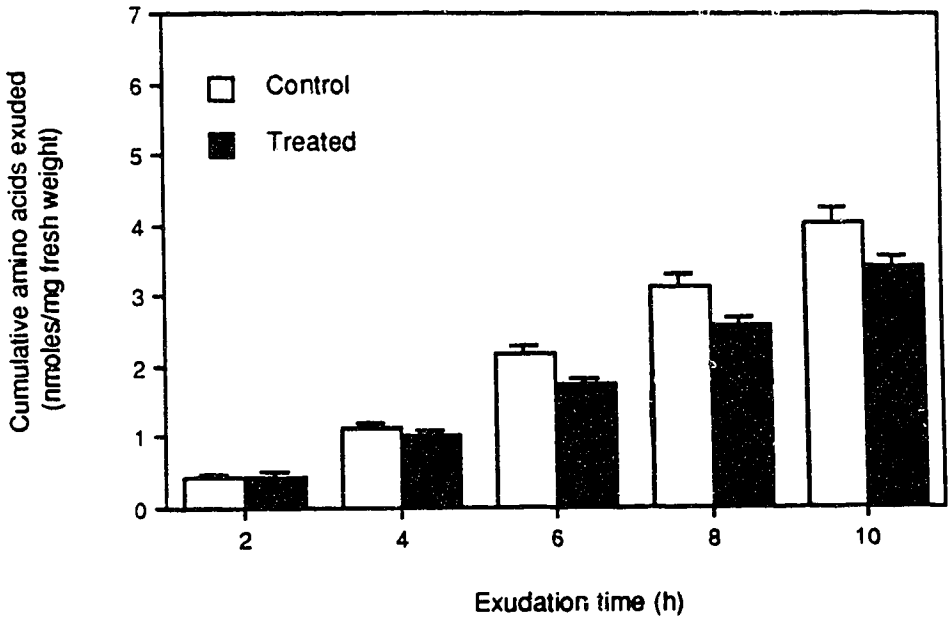

Figure 6. Exudation profiles of amino acids for single, excised leaves of stinkweed seedlings, following application of 0 (control) or 1 μg (treated) chlorsulfuron 24 h prior to excision and exposure to $^{14}CO_2$ for 30 min. The data, means and standard errors, are the results of three runs with six leaves (three controls, three treated) per run.

Table 7. The effect of 0 (control) or 1 μg chlorsulfuron on amino acids exuded by excised leaves of stinkweed seedlings. Leaves were exposed to $^{14}CO_2$ for 30 min 24 h after treatment with herbicide. The data, means with standard errors in parentheses, are the results of three runs with three leaves per treatment per run.

Time	Cumulative soluble amino acids exuded		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
2	0.49 [0.04]	0.52 [0.05]	106
4	1.15 [0.08]	1.10 [0.09]	96
6	2.08 [0.17]	1.78 [0.11]	86
8	3.04 [0.24]	2.52 [0.16]	83
10	4.05 [0.38]	3.36 [0.23]	83

*Fresh weight of leaves

Table 8. The effect of 0 (control) or 1 µg chlorsulfuron on amino acids exuded by excised leaves of stinkweed seedlings. Leaves were exposed to $^{14}CO_2$ for 30 min 24 h after treatment with herbicide. Branched-chain amino acids were added to the nutrient solution 24 h prior to treatment with the herbicide. The data, means with standard errors in parentheses, are the results of three runs with three leaves per treatment per run.

Time	Cumulative soluble amino acids exuded		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
2	0.7 [0.1]	0.6 [0.1]	86
4	2.0 [0.3]	1.6 [0.4]	80
6	3.6 [0.4]	2.6 [0.6]	72
8	5.1 [0.3]	3.5 [0.5]	69
10	5.9 [0.2]	4.0 [0.5]	68

*Fresh weight of leaves

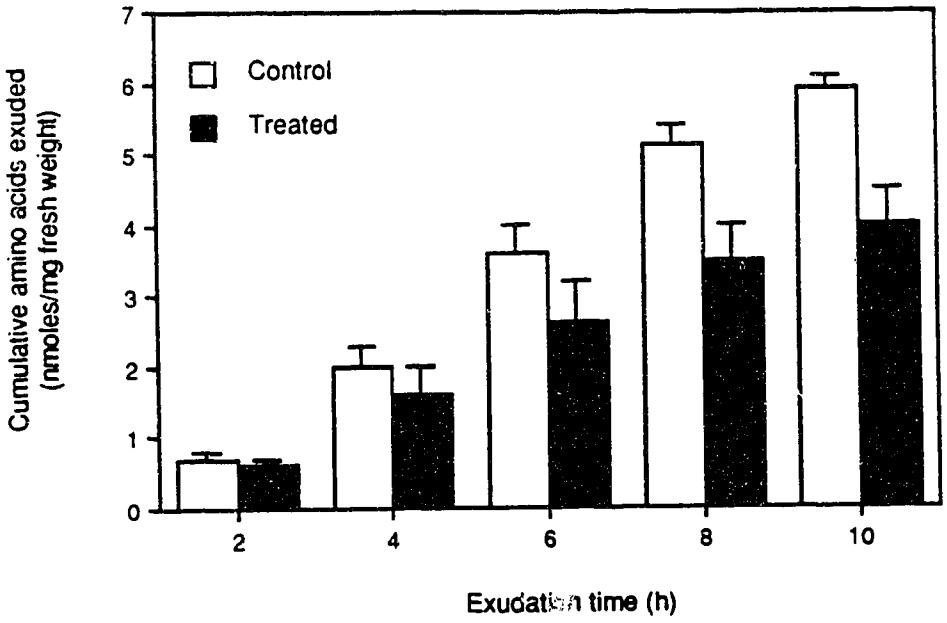

Figure 7. Exudation profiles of amino acids for single, excised leaves of stinkweed seedlings, following application of 0 (control) or 1 µg (treated) chlorsulfuron 24 h prior to excision and exposure to $^{14}CO_2$ for 30 min. L-leucine, L-isoleucine, and L-valine were present in the nutrient solution 24 h prior to treatment with herbicide. The data, means and standard errors, are the results of two runs with six leaves (three controls, three treated) per run.

percentage of controls, was exuded. These results are similar those obtained with sugars.

4.1.4 General discussion on exudation experiments

Chlorsulfuron caused a reduction in the exudation of ^{14}C-activity from excised leaves (Figure 2). In these experiments ^{14}C-activity was believed to represent mainly the exudation of sucrose from the cut ends of phloem tissue in excised leaves. Sucrose is known to make up the bulk of the phloem contents in most plants (Kallarackal and Komor, 1989; Hall and Baker, 1972). The selectivity with which sucrose is loaded into the phloem is believed to reside at the site of loading, the membrane (Humphreys, 1988). After herbicide treatment there was an effect on phloem tissue, or on the active phloem loading process of sucrose, that altered the profile of exudation of ^{14}C-activity from treated leaves. A reduction in sucrose exudation should be reflected as a decrease in exudation of ^{14}C-activity (Figure 2). The reduction observed agrees with data presented by Bestman (1988) in a similar experiment.

With the addition of branched-chain amino acids to the nutrient solution available for plant growth, the magnitude of the chlorsulfuron effect on exudation of ^{14}C-activity was reduced considerably (Figure 3), but it was not completely eliminated as was the case in a similar experiment reported by Bestman (1988). The supplementation of the nutrient solution induced a stimulation in exudation from control leaves (Figures 2 and 3). Bestman (1988) obtained a complete reversal of the chlorsulfuron effect on excised leaves with the addition of amino acids to the nutrient solution and he reported no stimulation in exudation of ^{14}C-activity by control leaves as a

result of the addition of the amino acids to the nutrient solution. However, in a more recent publication (Bestman *et al.*, 1990), he describes an unexplained increase in ^{3}H-activity (labelled sucrose applied to the leaf surface) exuded by excised leaves in the presence of an exogenous supply of branched-chain amino acids. He concluded that the ability of branched-chain amino acids in the nutrient solution to overcome the reduction in exudation of ^{14}C-activity suggests that the observed reduction was related to the mechanism of action of chlorsulfuron. In the data presented here, there is partial alleviation of the chlorsulfuron effect on exudation but it was incomplete and there was also the stimulation of exudation by the control leaves noted above to be explained. It is possible that this may be accounted for by variations and differences of individual leaves but, given the size of the difference, that is unlikely. Another explanation may be that the control leaves were receiving amino acids from the nutrient solution through the xylem and, having no requirement for them, would export them immediately in the phloem. There is a precedent for this explanation in that it is generally believed that leaves maintain an equilibrium with regard to internal nitrogen content, exporting any excess as amino acids in the phloem (Humphreys, 1988 and references therein).

Exudation of sugars was adversely affected by treatment with chlorsulfuron Reducing sugars are known to be almost completely excluded from the phloem, with non-reducing sugars being loaded exclusively into this tissue for translocation around the plant. Sugars in the phloem are thought to be made up of sucrose predominately. It is possible that the reducing sugars that were present in the exudate originated from the cut end of the petiole, or that they were the breakdown products of sucrose that had already been exuded from the petioles. Kallarackal and Komor (1989) did

find trace amounts of reducing sugars in the sieve tube sap of *Ricinus communis* L., but the amounts were negligible in comparison to the amounts of sucrose they measured. Supplementation of the nutrient solution with branched-chain amino acids prior to treatment with the herbicide did not completely alleviate the effects of chlorsulfuron on sugar exudation In control leaves the exudation was stimulated, but in treated leaves the exudation was still less than controls (Tables 5 and 6).

Humphreys (1988) in his review article noted that mature leaves maintain a constant level of nitrogen, the excess being translocated from the leaf in the phloem. Control leaves that had been supplemented with branched-chain amino acids exuded about 1.5 times more amino acids than those that received no additions (Tables 7 and 8). In chlorsulfuron-treated leaves, the excess L-leucine, L-isoleucine, and L-valine supplied via the nutrient solution are almost certainly being used to synthesise proteins, but in the control leaves, there ought to be no demand for these amino acids as there is no deficiency and, therefore, they are probably exported as excess in the phloem in order to maintain nitrogen equilibrium in the leaf.

The difference, described below, in the exposure of the plants to the modified nutrient solution may also have had an effect on how much of the branched-chain amino acids was absorbed into the plant and this, in turn, could have affected the amount of ^{14}C-activity, and other metabolites, exuded. In these experiments the plants were supplied with nutrient solution by watering from below in exactly the same manner as watering with the unmodified nutrient solution. In Bestman's work, the plants were removed from the vermiculite and placed in small jars containing the modified nutrient solution. It is possible, then, that more amino acids were absorbed with his method, and that more amino acids reached the leaves (all leaves on the

plant). This may serve to explain why he achieved complete reversal of the chlorsulfuron effect on exudation with the addition of amino acids to the nutrient solution.

The inhibition of exudation of amino acids by the herbicide was considerably less than the inhibition for total ^{14}C-activity and sugars. This may, in part, be a reflection of the small proportion of amino acids in the phloem contents compared to sugars (Schobert and Komor, 1989; Hall and Baker, 1972), or it may be an indication that chlorsulfuron had an effect primarily on sucrose and, consequently, on ^{14}C-activity and total sugars in the exudate.

The stimulatory effects of the supplemented nutrient solution on ^{14}C-assimilate, amino acid, and sugar exudation may have contributed to the stimulation in ^{14}C-activity exuded by control leaves. Given the stimulation is accounted for in this manner, there is still not complete alleviation of the chlorsulfuron effect on a percent of control basis (Table 4), as was demonstrated by Bestman (1988).

4.2 Effect of chlorsulfuron on selected metabolite levels in intact leaves.

4.2.1 Starch (starch-derived glucose) levels

Chlorsulfuron treatment of stinkweed seedlings resulted in visual injury symptoms 36 to 48 hours after application. At this time, a cessation of growth in comparison to untreated plants became obvious, along with chlorosis of the treated leaf and the apical region. By 72 hours after

application, the treated leaf was limp and 100% chlorotic, with the rest of the plant rapidly becoming chlorotic.

The application of 1 µg chlorsulfuron to the third true leaf of stinkweed seedlings produced changes in the levels of starch-derived glucose (starch)[22] (Figure 8). Control leaves demonstrate the peculiar fluctuation that starch levels exhibit to day/night conditions. In Figure 8, the bar across the top of the graph shows light and dark periods in relation to harvest times. At the 24-hour harvest, the leaves had been in darkness for 6 hours, and starch reserves were low, compared to the 36-hour harvest when the leaves had been illuminated for 14 hours, and starch reserves were elevated. At the 48 and 72-hour harvests, after another 6 hours in darkness, respectively, starch levels were again low. If leaves had been harvested at 60 hours, the graph should have had at that time an elevated level of starch reserves similar to that seen at 36 hours. The fluctuation is steady, with leaves accumulating and exporting the same amounts over the 3 days duration of the experiment (Table 9). At 12 and 36 hours, starch levels had accumulated to 103 and 98 nmoles/mg fresh weight, respectively (Table 9). After the night period at 24, 48, and 72 hours, the starch levels were 63, 71, and 56 nmoles/mg fresh weight, respectively.

In chlorsulfruon-treated leaves, the trend of accumulation and depletion of starch reserves was different. At 6 and 12 hours, there was no difference between treated and control leaves (Figure 8). At 24 hours, starch levels in treated leaves did not decline to the same level reached by control leaves, 87 and 63 nmoles/mg fresh weight, respectively (Table 9). At 36 hours there was no increase in the level of starch in treated leaves as in

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[22] Quantitative starch levels in the text are expressed as nmoles starch-derived glucose per milligram fresh weight of tissue.

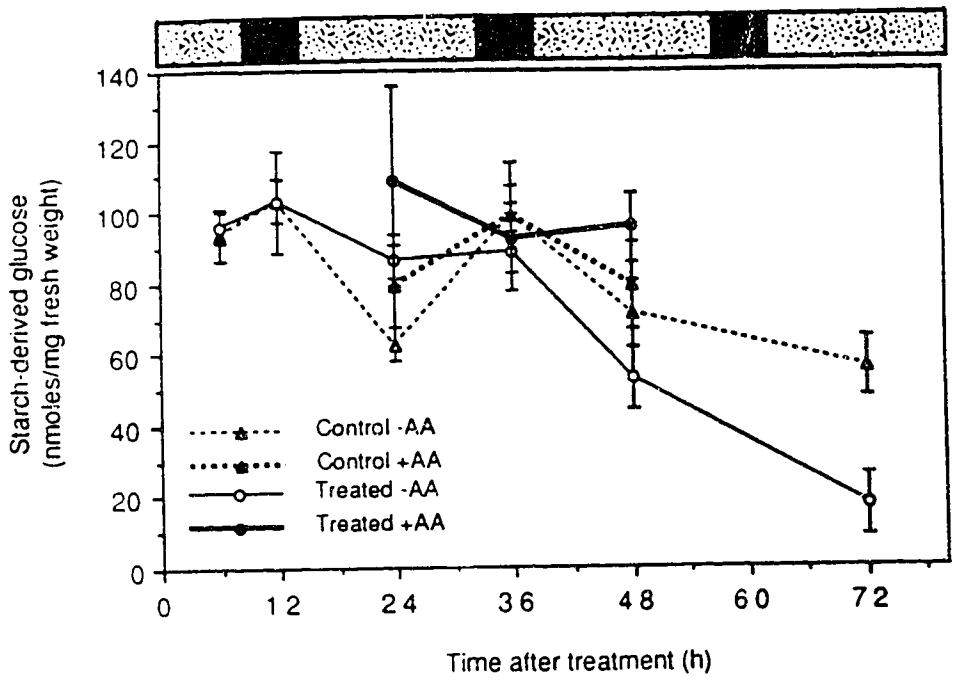

Figure 8. The effect of 1 µg chlorsulfuron applied to the third true leaf of stinkweed seedlings on starch-derived glucose levels. The data, means and standard errors, are the results of two runs with three leaves (seedlings) per run. The bar at the top of the graph represents light (shaded area) and dark (solid area) periods during the experiment. AA represents the branched-chain amino acids that were (+AA) or were not (-AA) added to the nutrient solution prior to treatment with the herbicide.

Table 9. The effect of 1 µg chlorsulfuron on starch-derived glucose levels in intact leaves of stinkweed seedlings. The data, means with standard errors in parentheses, are the results of two runs with three leaves per run.

Time	Starch-derived glucose		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
6	93.6 [7.1]	95.9 [3.3]	103
12	102.8 [14.6]	103.1 [8.9]	104
24	62.7 [4.4]	86.5 [5.3]	138
36	98.3 [4.1]	88.3 [7.8]	93
48	71.1 [9.7]	52.6 [5.5]	74
72	56.2 [8.0]	17.3 [8.7]	32

*Fresh weight of leaves

control leaves (Figure 8) and levels declined rapidly after 48 hours, to 17 nmoles/mg fresh weight at 72 hours (Table 9), only 32% of controls.

From 24 to 36 hours, a 12-hour period of daylight, treated leaves accumulated little starch compared to control leaves. Control leaves at 24 and 36 hours had 62.7 and 98.3 nmoles/mg fresh weight, respectively, an increase of 35.6 nmoles/mg fresh weight starch. In treated leaves, by comparison, the level of starch at 24 and 36 hours was 86.5 and 88.3 nmoles/mg fresh weight, respectively, an increase of only 1.8 nmoles/mg fresh weight starch (Table 9). After 36 hours, starch levels in treated leaves declined steadily regardless of day/night conditions, whereas control leaves appeared also to decline by considerably less.

The experiment was repeated with the addition of branched-chain amino acids (L-leucine, L-isoleucine, and L-valine) to the nutrient solution 24 hours prior to treatment with chlorsulfuron and until the end of the experiment. The purpose of the inclusion of these amino acids in the nutrient solution was to attempt to overcome the effect that chlorsulfuron had on the levels of starch in the treated leaf.

The levels of starch in the treated leaf were significantly greater than those of control leaves at 24 hours (Figure 8), indicating that the treated leaf had not recovered the ability to export excess starch during the night as sucrose in the phloem. At 36 hours after treatment, there was no difference between the treated and control leaves. At 48 hours, there was a significant difference between the amounts of starch in treated and control leaves, 96 and 79 nmoles/mg fresh weight, respectively (Table 10).

Table 10. The effect of 1 µg chlorsulfuron on starch-derived glucose levels in intact leaves of stinkweed seedlings. Branched chain amino acids were added to the nutrient solution 24 h prior to treatment with the herbicide. The data, means with standard errors in parentheses, are the results of two runs with three leaves per run.

Time	Starch-derived glucose		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
24	79.0 [11.7]	109 [27.1]	129
36	98.3 [15.5]	92.2 [14.9]	97
48	79.0 [12.6]	95.5 [10.0]	123

*Fresh weight of leaves

4.2.2 Soluble amino acids levels

The levels of soluble amino acids in control leaves over the course of this experiment remained relatively constant (Figure 9). The soluble amino acid level appeared to be less dependent on day/night changes than were starch levels. One thing to note in these experiments is that the average soluble amino acid level in control leaves from the first experiment was approximately 20% greater than that in the second experiment where amino acid supplementation of the nutrient solution occurred. There is no explanation for this reduction bar natural variation of biological systems, but the trends for controls in both experiments were the same (Figure 9).

At 6 hours after application of the chlorsulfuron, there was no difference in the absolute amounts found in control leaves and in treated leaves (Table 11). By 12 hours, however, there was a near doubling in soluble amino acid levels in treated leaves compared to control leaves, 39 and 21 nmoles/mg fresh weight, respectively. The increase continued steadily until, by 48 hours, treated leaves had 3.5 times more soluble amino acids than control leaves. Over the next 24 hours, the amount doubled again, to six times that of control leaves, 130 and 21 nmoles/mg fresh weight, respectively.

When the seedlings were supplied with 1 mM concentrations of L-leucine, L-isoleucine, and L-valine in the nutrient solution, 24 hours prior to treatment with chlorsulfuron in the usual way, a partial reduction in the buildup in soluble amino acid levels occurred (Figure 9). At 48 hours, there was 39 nmoles/mg fresh weight in treated leaves and 16 nmoles/mg fresh weight in control leaves (Table 12). A comparison between the treated

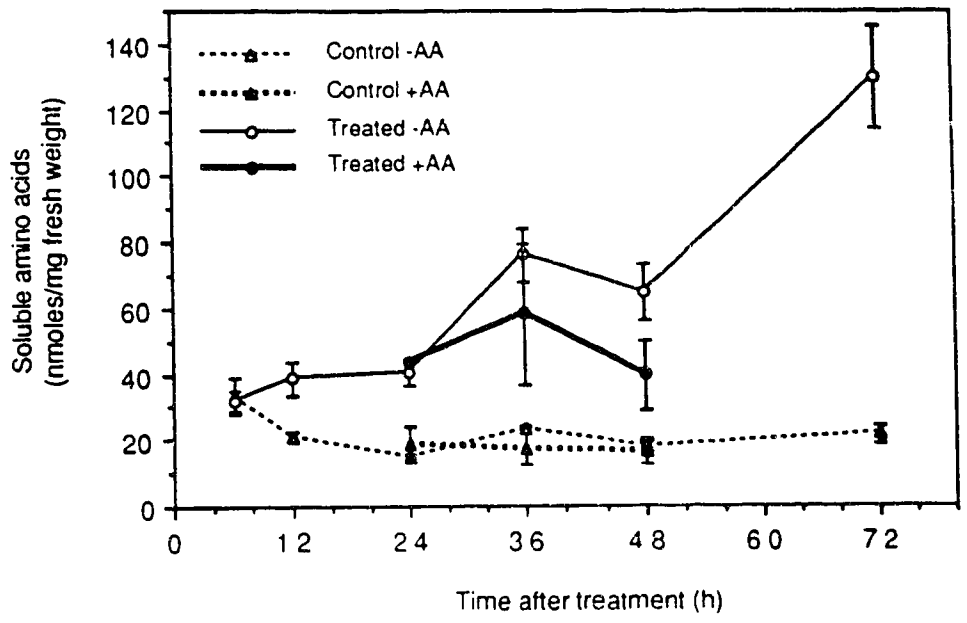

Figure 9. The effect of 1 µg chlorsulfuron applied to the third true leaf of stinkweed seedlings on soluble amino acid levels. The data, means and standard errors, are the results of two runs with three or four leaves (seedlings) per run. AA represents the branched-chain amino acids that were (+AA) or were not (-AA) added to the nutrient solution prior to treatment with the herbicide.

Table 11. The effect of 1 µg chlorsulfuron on soluble amino acid levels in intact leaves of stinkweed seedlings. The data, means with standard errors in parentheses, are the results of two runs with three or four leaves per run.

Time	Soluble amino acids		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
6	34 [5.4]	32 [3.1]	101
12	21 [1.6]	39 [5.2]	178
24	15 [1.1]	40 [3.3]	271
36	23 [1.0]	76 [8.1]	330
48	18 [1.9]	65 [8.4]	352
72	21 [2.5]	130 [15.5]	627

*Fresh weight of leaves

Table 12. The effect of 1 µg chlorsulfuron on soluble amino acid levels in intact leaves of stinkweed seedlings. Branched chain amino acids were added to the nutrient solution 24 h prior to treatment with the herbicide. The data, means with standard errors in parentheses, are the results of two runs with three or four leaves per run.

Time	Soluble amino acids		
	Control	Treated	% of Control
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(%)
24	19 [5.4]	44 [0.50]	211
36	17 [4.5]	58 [21.4]	309
48	16 [3.6]	39 [10.7]	226

*Fresh weight of leaves

leaves in this experiment and those in the experiment that had no supplementation of the nutrient solution shows the considerable reduction in soluble amino acid pools with supplementation of the nutrient solution at 48 hours (Figure 9).

4.2.3 Sucrose, glucose, and fructose levels

The application of chlorsulfuron to intact third true leaves of stinkweed seedlings resulted in an effect on pools of sucrose, glucose, and fructose. Indeed, glucose and fructose level appeared to be more sensitive to the application of chlorsulfuron to the intact plant than those of the other metabolites that were measured.

Sucrose levels in control leaves were relatively steady from 6 to 24 hours (Figure 10). Sucrose levels in treated leaves followed the trend observed in control leaves, but were elevated considerably from 12 hours to the end of the experiment. The levels of sucrose in control and treated leaves at 12 hours were 4.4 and 8.2 nmoles/mg fresh weight, respectively (Table 13). By 48 hours, treated leaves had over 2.5 times the amount of sucrose present in control leaves. At 36 hours, sucrose levels in herbicide-treated leaves reached a maximum of 16.7 nmoles/mg fresh weight (Table 13). With the addition of branched-chain amino acids to the nutrient solution prior to treatment with chlorsulfuron, the effect of the herbicide on sucrose levels was reduced somewhat (Figure 10), but only at 36 hours, 10.8 and 16.7 nmoles/mg fresh weight, respectively, in supplemented and unsupplemented plants (Tables 14 and 13). The mean of sucrose concentrations in supplemented and unsupplemented treated leaves over 24 to 48 hours was 12.4 nmoles/mg fresh weight and 12.6 nmoles/mg fresh

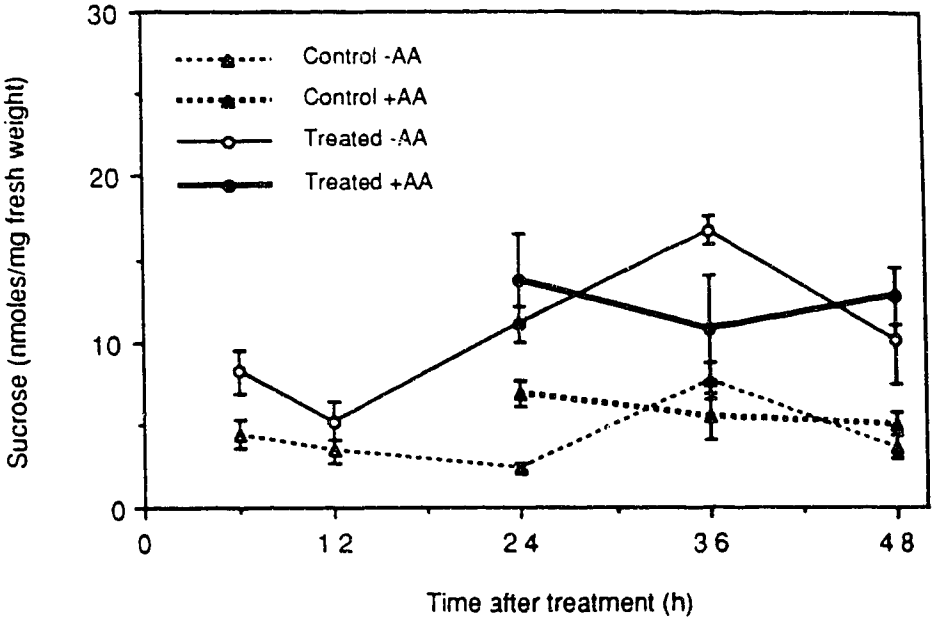

Figure 10. The effect of 1 µg chlorsulfuron applied to the third true leaf of stinkweed seedlings on sucrose levels. The data, means and standard errors are the results of two runs with three or four leaves (seedlings) per run. AA represents the branched-chain amino acids that were (+AA) or were not (-AA, added to the nutrient solution prior to treatment with the herbicide.

Table 13. The effect of 1 µg chlorsulfuron on sucrose, glucose, and fructose levels in intact leaves of stinkweed seedlings. The data, means with standard errors in parentheses, are the results of two runs with three of four leaves per run.

Time	Sucrose, glucose, and fructose					
	Sucrose		Glucose		Fructose	
	Control	Treated	Control	Treated	Control	Treated
(h)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)
6	4.4 [0.9]	8.2 [1.3]	11.8 [2.2]	19.6 [3.7]	2.4 [0.7]	6.2 [1.9]
12	3.4 [0.7]	5.2 [1.1]	4.7 [0.6]	13.7 [2.7]	0.7 [0.7]	3.4 [1.0]
24	2.4 [0.2]	11.0 [1.1]	5.2 [0.6]	28.1 [2.9]	1.7 [0.2]	7.8 [1.1]
36	7.6 [1.1]	16.7 [0.9]	9.7 [1.8]	47.3 [4.2]	1.9 [0.7]	14.3 [1.4]
48	3.6 [0.7]	10.1 [2.6]	7.0 [1.5]	72.0 [9.4]	2.2 [0.6]	27.9 [3.7]

*Fresh weight of leaves

Table 14. The effect of 1 µg chlorsulfuron on sucrose, glucose, and fructose levels in intact leaves of stinkweed seedlings. Branched chain amino acids were present in the nutrient solution prior to treatment with herbicide. The data, means with standard errors in parentheses, are the results of two runs with three leaves per run.

Time	Sucrose, glucose and fructose					
	Sucrose		Glucose		Fructose	
	Control	Treated	Control	Treated	Control	Treated
(n)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)	(nmoles/mg FW*)
24	6.8 [0.8]	13.7 [2.7]	12.1 [2.0]	26.9 [2.7]	3.8 [0.5]	6.7 [1.3]
36	5.5 [1.4]	10.8 [3.2]	7.5 [1.4]	34.9 [9.9]	1.3 [0.5]	7.1 [2.1]
48	5.0 [0.7]	12.8 [1.7]	9.1 [1.8]	32.2 [4.5]	2.5 [0.4]	11.0 [2.7]

*Fresh weight of leaves

weight, respectively (calculated from the data in Tables 14 and 13, respectively). These data suggest that the addition of the amino acids to the nutrient solution had no effect on sucrose pools in treated leaves.

Glucose levels in treated leaves unsupplemented with amino acids in the nutrient solution showed a large increase from 6 to 48 hours (Figure 11). At 6 hours, there was 60% more glucose in treated leaves compared to control leaves, 20 and 12 nmoles/mg fresh weight, respectively (Table 13). By 24 hours, there was more than five times as much glucose in treated leaves as in control leaves, 28 and 5 nmoles/mg fresh weight, respectively. At 48 hours, glucose levels in treated leaves were about 10 times those found in control leaves, 72 and 7 nmoles/mg fresh weight, respectively.

With the addition of L-leucine, L-isoleucine, L-valine to the nutrient solution prior to treatment with chlorsulfuron, glucose levels in treated leaves did not reach levels as high as in unsupplemented treated leaves, but they still were three times as high as in control leaves. At 24 hours after application of chlorsulfuron in the supplemented plants, the level of glucose was almost the same as that reached by similarly treated leaves of unsupplemented plants. By 48 hours, treated leaves of unsupplemented plants had twice as much glucose as the treated leaves of supplemented plants (Tables 13 and 14).

Of all the sugars measured, fructose levels were the most severely affected by treatment with chorsulfuron (Figure 12). At 6 hours after treatment, fructose levels were 2.5 times those of control plants, and by 48 hours this ratio had increased to almost 13 times (Table 13). Absolute amounts of fructose were less than those of glucose, 27.9 and 72 nmoles/mg fresh weight, respectively, in treated leaves at 48 hours (Table 13). This rise in fructose levels is concomitant with both the rise in glucose and sucrose,

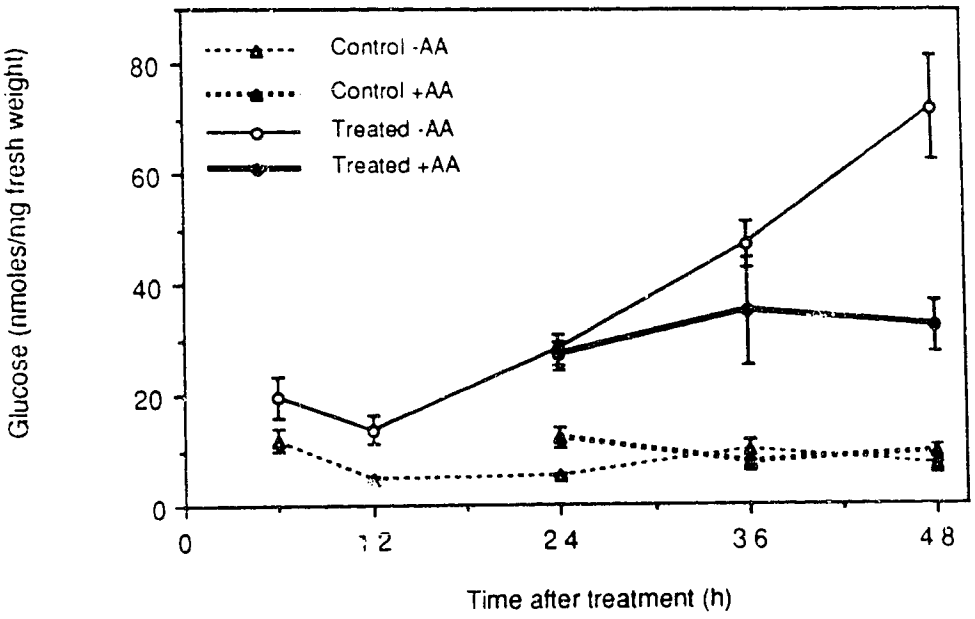

Figure 11. The effect of 1 µg chlorsulfuron applied to the third true leaf of stinkweed seedlings on glucose levels. The data, means and standard errors, are the results of two runs with three or four leaves (seedlings) per run. AA represents the branched-chain amino acids that were (+AA) or were not (-AA) added to the nutrient solution prior to treatment with the herbicide.

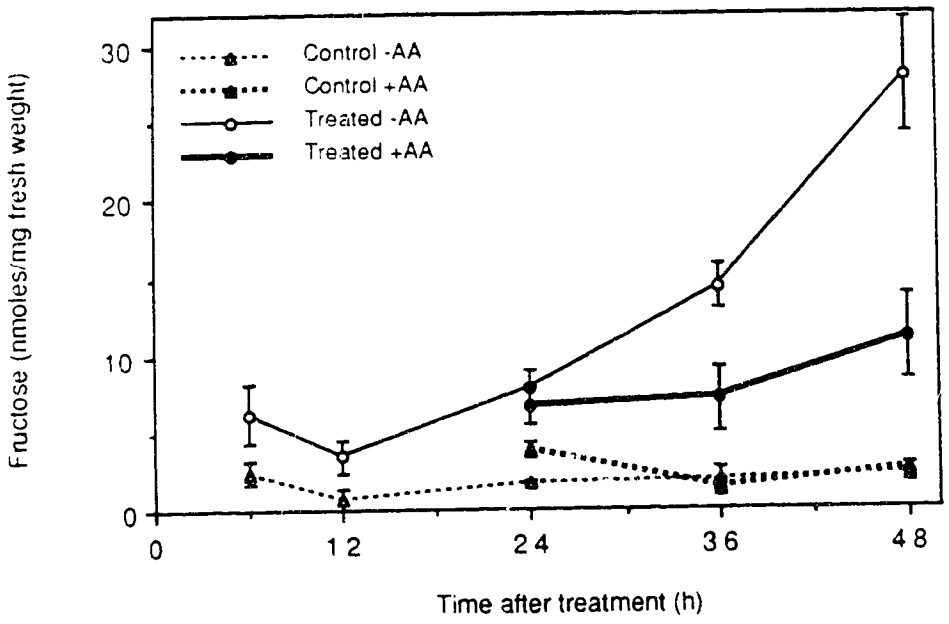

Figure 12. The effect of 1 µg chlorsulfuron applied to the third true leaf of stinkweed seedlings on fructose levels. The data, means and standard errors, are the results of two runs with three or four leaves (seedlings) per run. AA represents the branched-chain amino acids that were (+AA) or were not (-AA) added to the nutrient solution prior to treatment with the herbicide.

and starch levels.

With the addition of amino acids to the nutrient solution prior to treatment with chlorsulfuron, fructose levels did not get as high as they were in the absence of amino acid supplementation. At 24 and 48 hours, the levels were the same in both experiments (Figure 12). At 48 hours, the level of fructose was considerably lower in the supplemented leaves than in the unsupplemented leaves, 11 and 28 nmoles/mg fresh weight, respectively (Tables 14 and 13).

4.2.4 General discussion on time-course experiments

In actively photosynthesising leaves, starch levels tend to fluctuate over any 24-hour period having day and night. During the day, the leaf assimilates carbon through its photosynthetic apparatus. Assimilated carbon that cannot be exported immediately from the leaf is stored as starch grains in the chloroplasts (Salisbury and Ross, 1985). Consequently, over the course of the day, the level of starch rises in the leaf. During the night, starch in the leaf is exported as sucrose in the phloem and the starch level drops in the leaf. This fluctuation can be seen in the control leaves in Figure 9. At 6 and 12 and 36 hour harvests the plant had been illuminated for 9 and 15 and 15 hours, respectively. At the 24 and 48 and 72 hour harvests the plant had been illuminated only for 3 hours after a 6-hour night period, so these leaves would have less of a reserve of starch built up than those leaves illuminated for longer periods. The levels of starch in treated leaves followed closely the amount and trend of control leaves at 6 and 12 hours. At 24 hours, treated leaves had considerably more starch than control leaves (Figure 8). This makes intuitive sense. If, as is suggested earlier,

sucrose cannot be exported from treated leaves as efficiently as from control leaves, then less starch will be broken down and exported as sucrose from treated leaves over the night period. The data indicate a failure in the abilty of the treated leaf to export the starch, as sucrose, accumulated during the day. In Figure 8, the greater amount of starch at 24 hours in treated leaves represents, therefore, the inability of the treated leaf to export sucrose to the same extent as the control leaf can in the dark.

Why did the level of starch decline in treated plants? When chlorsulfuron is applied to a susceptible plant, the ALS enzyme is affected within six hours (Ray, 1980). Exudation of sucrose was affected by two hours (Table 5 and 6). It is assumed that no disruption occurs within the leaf as a result of a lack of branched-chain amino acids until after 24 hours (DeVilliers *et al.*, 1980; Ray, 1980). After this time, photosynthesis presumably is affected as photosynthesis becomes evident in the treated leaf. As a result, starch stored within the leaf becomes more important as an energy source. Consequently, reserves of starch are depleted to only 32% of that in control leaves by 72 hours (Table 9).

The treated leaves grown in nutrient solution with added branched-chain amino acids did not display a decrease in starch as great as that observed in the treated leaf grown without any additions to the nutrient solution, 96 and 53 nmoles/mg fresh weight, respectively, at 48 hours (Table 9 and 10). The amino acids, therefore, partially alleviated the chlorsulfuron effect. Bestman (1988) reported complete reversal of the chlorsulfuron effects in stinkweed seedlings every time he used amino acid-supplemented nutrient solutions. The method of application of herbicide in his work and in the work carried out here was fundamentally different. He applied 6-8 discrete drops of herbicide solution, 10 µl total volume, on the leaf surface.

In the work reported here, a 10 µl drop was applied to the leaf, and then spread evenly over the entire surface. Chlorsulfuron is known to have a very localized effect on plant tissue[23], because it does not spread evenly throughout a leaf upon application (Devine *et al.*, 1990). Therefore, droplets will not expose the entire leaf to the effects of the herbicide and, consequently, the branched-chain amino acids alleviate the chlorsulfuron effects. If the herbicide is applied as a film to the surface of the leaf, then the whole leaf is exposed to chlorsulfuron and the branched-chain amino acids reaching the leaf may not be enough to effect a complete reversal, only a partial reversal. If this is the case, complete alleviation may have been possible in Bestman's work, but not in the work reported here.

The effects of these added branched-chain amino acids to cell cultures and other systems has also been reported by other authors (Bestman, 1988; Matsunaka, 1985: Ray, 1984). Not all systems are protected by the amino acids to the same extent, and some get no protection whatsoever. Of the three authors mentioned above, only Bestman (1988) treated whole plants, but he experimented only with the excised treated leaves.

A natural occurrence in all biological systems is the constant turnover of proteins. Those proteins that are most important in metabolic regulation have particularly short half-lives. This allows their concentration and activity to be changed or modified rapidly as conditions require (Stryer, 1988). The mode of action of chlorsulfuron is to prevent the biosynthesis of L-leucine, L-isoleucine, and L-valine, three essential branched-chain amino acids required in protein synthesis (Ray, 1984). Chlorsulfuron, as was noted

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[23] L. Hall, personal communication

earlier, causes pools of soluble amino acids to build up to very high levels in treated leaves. It would appear from the results obtained here that proteins are broken down in the treated leaf as part of the normal turnover process, but they are not resynthesised because of a shortage of three essential amino acids that make up part of every protein in the plant. Consequently, soluble amino acid pools build up.

A shortage of three amino acids would become evident rapidly in ever increasing soluble amino acid pools because of the protein turnover phenomenon. The results of this experiment support this hypothesis. Only 12 hours after treatment with chlorsulfuron, there was 78% more soluble amino acids present in the treated leaves than in control leaves. As protein continued to be broken down, more and more soluble amino acids occurred in treated leaves. The addition of amino acids to the nutrient solution caused a partial reduction in soluble amino acid levels. If the full complement of amino acids is present in the leaf, then protein synthesis can go ahead. In this case, enough L-leucine, L-isoleucine, and L-valine apparently entered the treated leaves to cause a partial reduction in soluble amino acids levels.

Figure 13 shows the relationship of sucrose, glucose, and fructose to each other in treated leaves of plants grown in nutrient solution with no added amino acids. Glucose levels rose more dramatically than sucrose and fructose levels. Wilson and Lucas (1987) also demonstrated a rise in sucrose, glucose, fructose, and starch if translocation out of the leaf was prevented. In their case, they prevented translocation by heat-girdling the petiole, thereby killing the phloem tissue. At 34 hours, in their experiments, glucose increased considerably more than sucrose and/or fructose. A possible explanation for this increase in glucose over both sucrose and

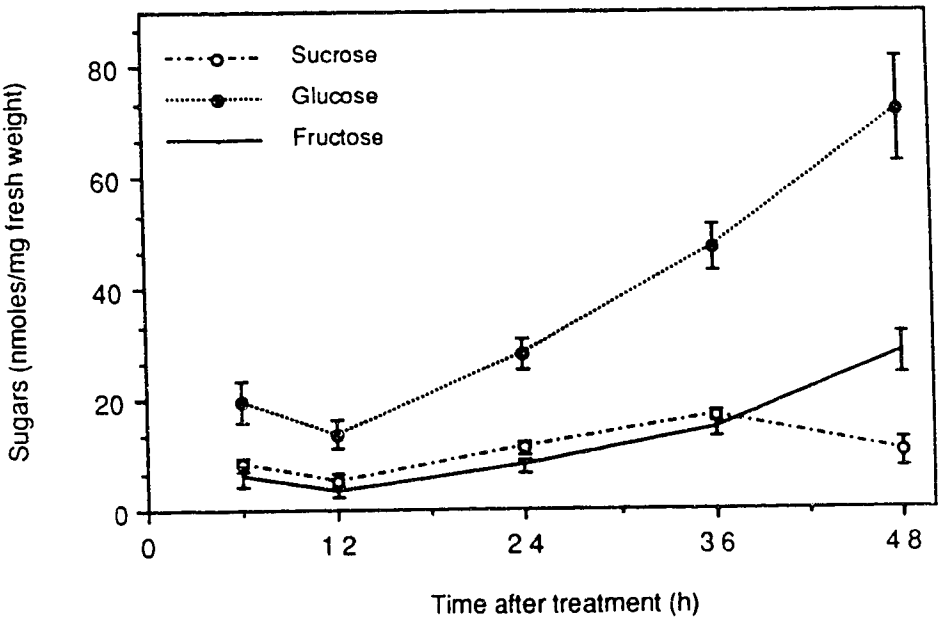

Figure 13. The effect of 1 µg chlorsulfuron applied to the third true leaf of stinkweed seedlings on sucrose, glucose, and fructose levels. The data, means and standard errors, are the results of two runs with three or four leaves (seedlings) per run.

fructose may be found in the biochemical origins of glucose within the leaf. When starch is broken down, sucrose and glucose are released. When sucrose is broken down, glucose and fructose are released. Therefore, glucose is being derived from two sources, starch and sucrose, whereas sucrose and fructose are each derived from only one source, starch and sucrose, respectivly.

Summary and Conclusions

When chlorsulfuron enters the leaf of a stinkweed plant, or any susceptible species, the enzyme acetolactate synthase is inhibited very rapidly (Ray, 1984). A direct result of this is a reduction in the synthesis, *de novo*, of L-leucine, L-isoleucine, and L-valine by the treated leaf. This, in turn, causes a reduction in protein synthesis which was found to be manifested in the treated leaf initially as a very large rise in the amount of soluble amino acids in the leaf. In this study, soluble amino acids were found to rise in treated leaves (Figure 9). It is assumed that the amino acids that build up in the treated leaves are derived mainly from protein turnover in the leaf.

Chlorsulfuron caused a reduction in exudation of ^{14}C-metabolites, reducing sugars, and amino acids. There is evidence for the existence of carrier proteins to load sugars and amino acids into the phloem (Delrot, 1989, and references therein). These carrier proteins must turn over like any other protein in the leaf. Consequently, treatment with chlorsulfuron could cause a reduction in the levels of carrier proteins in treated leaves, specifically those carriers that are responsible for loading sucrose and amino acids into the phloem. If this is the case, the phloem loading of sugars and amino acids will be reduced in the exudate as was found in this study.

In intact treated leaves, sucrose, glucose, and fructose increased over time. It can be assumed that the proteins involved in respiration are also becoming depleted and this, together with the reduction in export of sugars, will cause an increase in sugars in the treated leaf.

The only partial alleviation of the effects of chlorsulfuron on exudation in the first experiment and on pools of metabolites in the second experiment disagrees with conclusions drawn by Bestman (1988). However, the method of application of chlorsulfuron in this study may have resulted in a more extensive effect of the herbicide on the entire leaf and the amino acids provided in the nutrient solution were not present in sufficient quantities for a complete reversal in this case.

More work is required to further illuminate the complete picture of the chlorsulfuron effect on phloem translocation. In particular the method of application of chlorsulfuron, whether droplets or even application in a film, needs to be studied with a view to how it interacts with the amino acids provided in the nutrient solution.

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Appendix 1

MINIMUM RECROPPING INTERVALS (MONTHS)

Soil pH	Barley	Oats	Wheat (Durum)	Wheat (Spring,Winter)
7.0 or lower	10	10	10	2
7.1 to 7.5	22	22	10	2

Recropping to crops other than cereals

	Flax	Lentils	Peas	Canola
7.0 or less (Black or Grey Wooded(OM>5%))	48	48	34	22
7.0 or less (Brown or Dark Brown Soil)	NRR*	NRR	NRR	34
7.1 to 7.5 (Black or Grey Wooded(OM>5%)	NRR	NRR	34	34

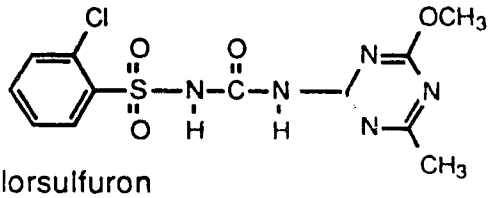
*NRR - No Recommendation Registered.

Table adapted from Guide to Crop Protection in Alberta, 1989, AGDEX 606-1

Appendix 2

(1) Physicochemical properties of chlorsulfuron

Common name and structural formula:

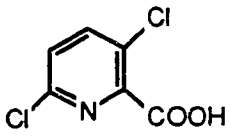


Chlorsulfuron

Molecular formula:		$C_{12}H_{12}ClN_5O_4S$	
Molecular weight:		357.8	
Physical state and color:		Crystalline solid, odorless, white	
Melting point:		174-178 C	
Decomposition temperature:		192 C	
Vapor pressure:		4.6×10^{-6} mm Hg at 25 C	
Solubility:	*Solvent*	*Temp., C*	*Solubility g/100ml*
	Acetone	22	5.7
	Hexane	22	0.001
	Methanol	22	1.4
	Methylene chloride	22	10.2
	Toluene	22	0.3
	Water (pH 5)	25	0.03
	Water (pH 7)	25	2.8
pKa:		4.6	
1-Octanol/water partition coefficient:		pH	Log P
		4.5	1.05
		12.0	-0.67

(2) Physicochemical properties of clopyralid

Common name and structural formula:



Clopyralid

Molecular formula:	$C_6H_3Cl_2NO_2$	
Molecular weight:	192	
Physical state and color:	Crystalline solid, odorless, white	
Melting point:	151-152 C	
Vapor pressure:	1.3×10^{-5} mm Hg at 25 C	
Solubility at 25 C:	*Solvent*	*g/100ml solvent*
	Acetone	>25.0
	Methanol	>15.0
	Xylene	<0.5
	Water	0.1
pKa:	2.3	
1-Octanol/water partition coefficient:	pH	Log P
	5.4	0.0018