Effects of Chlorimuron-ethyl, Imazethapyr, and Propachlor on Free Amino Acids and Protein Formation in *Vicia faba* L.

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Decreases in protein and changes in the free amino acid profile were ascertained in *Vicia faba* L. treated with chlorimuron-ethyl, imazethapyr, or propachlor. Valine, leucine, isoleucine, and total N were decreased by chlorimuron-ethyl and imazethapyr, while total soluble N was increased by all treatments. These changes were accompanied by decreases of the activities of acetohydroxy acid synthase (AHAS; EC 4.1.3.18), nitrite reductase (NiR; EC 1.7.7.1), glutamine synthetase (GS; EC 6.3.1.2), and glutamate synthase (NADH-GOGAT; EC 1.4.1.14). During the experimental period, the inhibition of GS activity preceded that of NiR and followed that of AHAS in samples treated with chlorimuron-ethyl or imazethapyr, while a reverse sequence was apparent in samples treated with propachlor. Therefore, the reduction in protein formation and the disturbance in free amino acid profile appears to be due to inhibition of AHAS following treatment with chlorimuron-ethyl or imazethapyr and to inhibition of NiR following treatment with propachlor.

Keywords: *Herbicides; acetohydroxy acid synthase; nitrate reductase; nitrite reductase; glutamine synthetase; glutamate synthase*

INTRODUCTION

Chlorimuron-ethyl [ethyl 2-[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]sulfonyl]benzoate] and imazethapyr [(*R*,*S*)-5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid] are herbicide compounds belonging to the sulfonylurea and imidazolinone groups, respectively. They are characterized by a selective action in the control of a wide variety of grasses and broad-leaf weeds in leguminous fields. Both sulfonylureas and imidazolinones act by inhibiting acetohydroxy acid synthase (AHAS), the first enzyme common to the biosynthetic pathway of branched-chain amino acids: valine, leucine and isoleucine (Beyer et al., 1988; Moberg and Cross, 1990; Stidham and Singh, 1991). This inhibition would lead to a decrease in the levels of the three amino acids and hence to the depression of total protein content. Following treatment with AHAS inhibitors, reduction of protein formation has been observed by Scarponi et al. (1995) in *Glycine max* treated with imazethapyr and by Shaner and Reider (1986) and Martinetti et al. (1995) in *Zea mays* treated with imazapyr and rimsulfuron, respectively.

Propachlor (2-chloro-*N*-isopropylacetanilide), a compound belonging to the α-chloroacetanilide herbicides, is used for the control of several annual grasses and broad-leaf weeds in leguminous fields. Although the mode of action of α-chloroacetanilides is still unknown, inhibition of protein synthesis in response to treatment with these compounds has been observed (Sharp, 1988; LeBaron et al., 1988). It has been ascertained that propachlor inhibits leucine incorporation into protein in *Avena sativa* (Deal et al., 1980) and in *Glycine max* as well as in *Cucumus sativus* (Duke et al., 1975).

The disturbance in protein formation appears to be in correlation with the disturbance in amino acid profile in response to the two inhibitors of AHAS as well as to the α-chloroacetanilide herbicides. Therefore, the objectives of this study were to ascertain eventual interferences of chlorimuron-ethyl, imazethapyr, and propachlor on protein formation and on the free amino acid profile in *Vicia faba* L., which is a leguminous crop plant of economic importance in the Mediterranean area, and to designate the biochemical modality of these effects.

MATERIALS AND METHODS

Chemicals. Analytical grade chlorimuron-ethyl was supplied by DuPont, imazethapyr by Cyanamid, and propachlor by Monsanto, Italy. Standard amino acids, methylviologen, hydroxylamine hydrochloride, γ-glutamylhydroxamate, triethylamine, and phenyl isothiocyanate (PITC) were obtained from Sigma Chemical Co. (St. Louis, MO). Brilliant Blue G was obtained from Aldrich (Steinheim, Germany), and acetonitrile (HPLC grade) and water (HPLC grade) were purchased from BDH (Poole, England). Phenylmethanesulfonyl fluoride was obtained from ICN Biochemicals Inc. (Costa Mesa, CA). All other reagents were of ACS grade.

Apparatus. A Perkin-Elmer HPLC instrument was assembled from the following modular components: Series 410 LC pumps, a Rheodyne Model 7125-075 injector, a Model 235 diode array detector interfaced with an Omega 2 analytical chromatographic workstation (version 2.50 software) and an Omega 235 software upgrade kit (PE Nelson), and an LC-18 DB column Supelcosil, 25 cm × 4.6 mm i.d. (5 μm particle size), protected with a Supelguard precolumn (3 cm long) having exactly the same characteristics (Supelco Inc., Bellefonte, PA).

A Varian Model Cary 210 double-beam grating spectrophotometer was used.

Plant Material and Growth Conditions. A pure strain of broad bean (*Vicia faba* L. Giza 2) seeds was used. Seeds were surface sterilized by soaking in 70% (v/v) ethanol for 5 min followed by 10 min in a 3% (w/v) solution of sodium hypochlorite. The seeds were then thoroughly washed and maintained in distilled water for 8 h. The seeds were

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germinated in sand quartz (prewashed with hydrochloric acid) in plastic pots (40 × 20 × 10 cm). Each pot contained germinating seeds spaced 5 cm apart in 5 cm adjacent rows. The pots were kept in controlled conditions in the growth chamber at 24/16 °C day/night, with a 14-h photoperiod, 300 μeinstein m^{-2} s^{-1} photon flux density, and 75−80% relative humidity. Distilled water (50 mL) was applied daily to each pot.

When the seedlings were 10 days old, one-fourth strength Hoagland solution (Hoagland and Arnon, 1950) was used instead of distilled water and the pots were divided into four groups. One group was left as control and one for each treatment with chlorimuron-ethyl, imazethapyr, and propachlor at 30, 50 and 1500 g ha^{-1}, respectively. Of each herbicide, the required dose to be added to the surface area per pot was calculated in relation to the rate per hectare. Then the herbicide quantity was solubilized in a suitable amount of distilled water, containing Tween 20 (0.25% v/v) as a nonionic surfactant, and applied as foliar sprays to whole plants twice with a mechanical sprayer, in one direction and crosswise. Shoots were collected just before herbicide application (zero time) and 1, 2, 4, 8, 12, and 16 days after treatment. At harvest, the entire shoots were rinsed with water and then washed with copious amounts of tap water to get rid of any adsorbed herbicide and then dried by blotting with paper towels.

Protein Extraction and Determination. Entire shoots (15 g) were cut into pieces and homogenized by sonication with chilled acetone (75 mL) for 1 min. The crude homogenates were filtered through a Büchner funnel and washed with chilled acetone. The residues were spread on filter paper and allowed to dry at room temperature to obtain the acetone powders (Harborne, 1988). For the complete extraction of protein, dry weights of powders (0.5 g) were covered with buffer solution (25 mL; 0.05 M Tris-HCl, pH 9.0) and allowed to stand for 10 min at 4 °C, and then the mixtures were centrifuged at 48200*g* for 15 min at 4 °C. Total protein content of the supernatant was determined spectrophotometrically by reaction with Brilliant Blue G according to the Bradford (1976) procedure.

Analysis of Amino Acid Pools. Amino acids were extracted from entire shoots (5 g) with methyl alcohol (3 × 25 mL), and the extracts were collected for amino acid determination following the procedure of Rhodes et al. (1987).

A standard solution was prepared by dissolving all amino acids to a final concentration of 1.0 mM each in 0.1 M HCl and was stored at −30 °C until needed. The standard solution and the sample extracts were dried, and the amino acids were derivatized to their PITC derivatives according to the method of Bidlingmeyer et al. (1987). Subsequently, 500 μL of sodium phosphate (5 mM, pH 7.6), containing 5% acetonitrile, was added as diluent and 6.0 μL was injected into the HPLC system.

Determination of Nitrogenous Fractions. The nitrogenous constituents were extracted according to the method adapted by Yemm and Willis (1956). Total N was estimated in the dried ground plant tissue, while the total soluble N was estimated in the extracts by the conventional micro-Kjeldahl method (Pirie, 1955) as adapted by Younis et al. (1988).

Assay of Acetohydroxy Acid Synthase (AHAS) Activity. According to the procedures of Singh et al. (1988), entire shoots were collected and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The activity of AHAS was measured in 0.5 mL of reaction mixture containing 0.1 mL of enzyme extract by colorimetric estimation of acetolactate, after its conversion to acetoin by decarboxylation in acidic condition. Appropriate checks of direct acetoin formation during the enzyme assay were made by measuring and subtracting the absorbance in the absence of the enzyme.

Assay of NADH-Nitrate Reductase (NR) Activity. Extraction and assay procedures of NR were carried out according to the method of Nakagawa et al. (1985). All steps in the enzyme extraction were conducted at 0−4 °C. Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The enzyme activity was determined in 1 mL of reaction mixture containing 0.2 mL of enzyme extract by the reduction of nitrate to nitrite and subsequent colorimetric measurement of the nitrite produced.

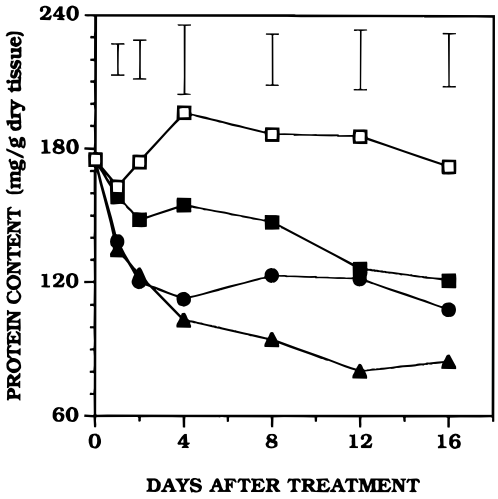

Figure 1. Protein content in *V. faba* L. seedlings: (□) control; (▲) chlorimuron-ethyl; (●) imazethapyr; and (■) propachlor. Each value is the mean of three determinations. Vertical bars represent LSD at $p < 0.05$.

Assay of Nitrite Reductase (NiR) Activity. The extraction procedure was carried out at 0−4 °C according to the method of Nagaoka et al. (1984). Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The activity of NiR was assayed according to the method of Wray and Filner (1970) in 1 mL of reaction mixture containing 0.1 mL of enzyme extract.

Assay of Glutamine Synthetase (GS) Activity. The procedures of extraction and assay of GS were performed according to the method of Lea et al. (1990). Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The determination of GS activity was measured in 0.5 mL of reaction mixture containing 0.2 mL of enzyme extract by colorimetric estimation of γ-glutamylhydroxamate formed after reaction of glutamate (50 mM) with hydroxylamine hydrochloride (5 mM). "Blank" tubes comprising the enzyme extract and all of the reagents except ATP were also incubated.

Assay of Glutamate Synthase (NADH-GOGAT) Activity. Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction according to the procedure of Marquez et al. (1988). According to the procedure of Hecht et al. (1988), the assay of NADH-GOGAT activity was performed in 1 mL of reaction mixture containing 0.2 mL of enzyme extract by continuous spectrophotometric monitoring of the consumption of NADH. For each measurement, two controls were performed with glutamine and 2-oxoglutarate being left out of the reaction medium in turn.

Each plant extraction procedure and subsequent determination of amino acids, protein content, and enzyme activities were replicated three times. All data were statistically analyzed using the least significant differences (LSD) method (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

To evaluate the effect of chlorimuron-ethyl, imazethapyr, and propachlor on the formation of protein in broad bean, its content in treated and untreated shoots was determined (Figure 1). The data show that treatment with chlorimuron-ethyl, imazethapyr, and propachlor significantly reduced the protein content below the control levels except for treatment with

propachlor during the first day interval. The magnitude of reduction amounted to 50% with chlorimuron-ethyl, 37% with imazethapyr, and 30% with propachlor on the 16th day after treatment.

The inhibition induced by chlorimuron-ethyl and imazethapyr was expected since previous studies showed that AHAS inhibitors can result in a decrease in protein as a consequence of the reduced formation of valine, leucine, and isoleucine (Shaner and Reider, 1986; Scarponi et al., 1995; Martinetti et al., 1995). Treatment with propachlor also resulted in a reduction of protein content but to a lesser magnitude. The target site of α-chloroacetanilide herbicides is still unclear, yet losses of protein content have been observed in *G. max* and *A. sativa* treated with propachlor (Gruenhagen and Moreland, 1971; Deal et al., 1980). Also, similar observations have been found in *Z. mays* and *G. max* treated with other α-chloroacetanilides, namely alachlor and metolachlor (Scarponi et al., 1992; Mansour et al., 1991).

To verify if the disturbance in protein formation resulted from a disturbance in the levels of formation of free amino acids and/or a reduction of their incorporation into protein, the content of free amino acids was also checked during the experimental period (Table 1). The data show that all of the employed herbicides induced certain fluctuations in the contents of free amino acids. The obtained significant changes appeared to extend throughout the experimental period in samples treated with chlorimuron-ethyl and imazethapyr, whereas a lack in such changes was apparent during the first 2 days from treatment with propachlor and was maintained more frequently in the subsequent period. However, increases in certain free amino acids appeared to be matched by corresponding decreases in other free amino acids; therefore, nonsignificant differences were apparent for the summation of the pool size of total free amino acids. Increases were observed more frequently in the content of lysine, histidine, threonine, and alanine. In contrast, decreases were found more frequently in the content of tryptophan, valine, leucine, isoleucine, tyrosine, and phenylalanine. Decreases in valine, leucine, and isoleucine were observed only in the chlorimuron-ethyl- and imazethapyr-treated samples.

In accord with the present observations, Shaner and Reider (1986) and Rhodes et al. (1987) observed decreases in valine, leucine, and isoleucine associated with increases in some other free amino acids in *Z. mays* and in *Lemna minor* treated with other AHAS inhibitors, namely imazapyr and chlorsulfuron, respectively. Therefore, they hypothesized that the increases in free amino acid levels were due to degradation of pre-existing proteins, rather than to new amino acid synthesis. This hypothesis seems applicable to explain the behavior of protein and free amino acids observed in *V. faba* treated with chlorimuron-ethyl or imazethapyr.

In propachlor-treated samples the changes in free amino acids content did not result in a lack of valine, leucine, and isoleucine, or of any specific amino acid, during the entire experimental period. Therefore, the reduced protein content does not appear to be directly related to the disturbance in the formation of any specific amino acid.

To support the above interpretations, the activity of the extractable AHAS was checked and the obtained data are depicted in Figure 2 together with the trend of valine, leucine, and isoleucine contents. The results show an abrupt and severe inhibition of AHAS activity in samples treated with chlorimuron-ethyl and imazethapyr in relation to the untreated control. The magnitude of inhibition was maintained at an almost steady level of 65% during the entire experimental period. The reduction of extractable AHAS activity was to be expected given the mode of action of sulfonylureas and imidazolinones, which are known to act on the same site on AHAS, though the enzyme-binding mechanisms are probably different (Shaner et al., 1990; Schloss et al., 1988). The AHAS activity appeared to be significantly affected by propachlor too, but it started from

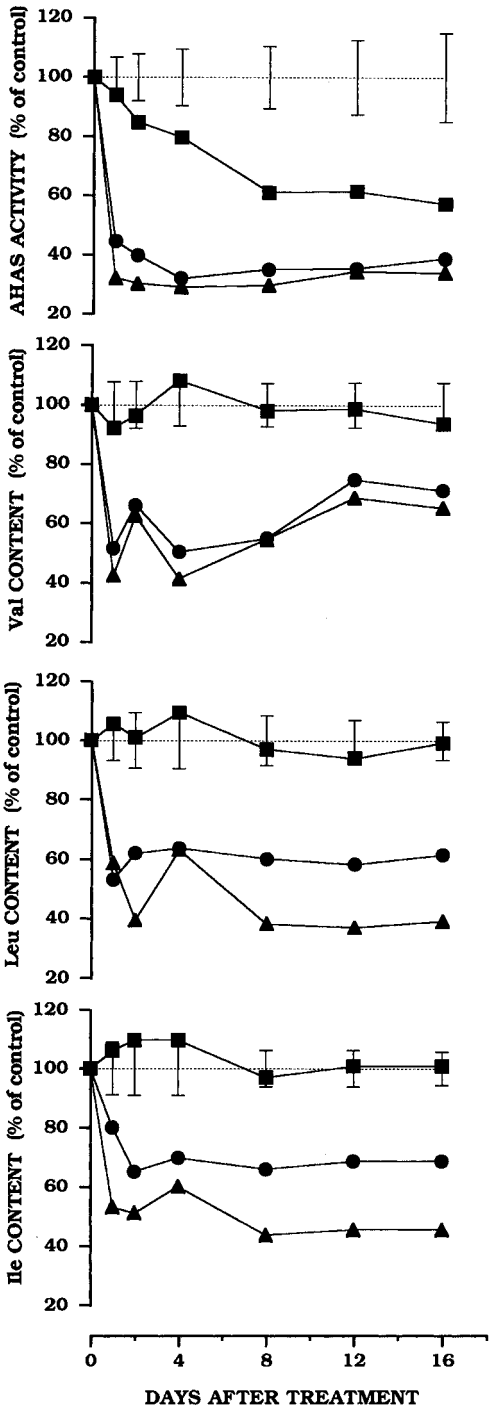

Figure 2. Activity of acetohydroxy acid synthase and content of valine, leucine, and isoleucine in *V. faba* L. seedlings as percent of control, (▲) chlorimuron-ethyl, (●) imazethapyr, and (■) propachlor. Each value is the mean of three determinations. Vertical bars represent LSD at $p < 0.05$. [Minimum and maximum values of AHAS activity in the controls during the experimental period were 160 and 171 nmol of acetolactate formed h^{-1} (mg of protein)$^{-1}$, respectively.]

Table 1. Changes in the Free Amino Acids in the Shoots of Treated *V. faba* L. (Nanomoles per Gram of Dry Weight)[a]

	Asp	Glu	Asn	Ser	Gln	Cys	Gly	His	Thr	Ala	Pro	Tyr	Val	Ile	Leu	Phe	Try	Lys	sum
at zero time	1.51	1.17	1.29	0.91	1.55	0.74	0.37	0.86	0.25	0.70	0.25	0.38	0.49	0.71	0.71	0.50	0.83	0.29	13.52
1 day after treatment																			
control	1.52	1.21	1.27	0.90	1.58	0.77	0.33	0.88	0.28	0.70	0.22	0.41	0.50	0.68	0.69	0.52	0.81	0.31	13.60
propachlor	1.46	1.14	1.19	0.82	1.45	0.68	0.29	0.93	0.32	0.69	0.19	0.44	0.46	0.72	0.73	0.47	0.79	0.32	13.09
imazethapyr	1.66	1.24	1.45	0.98	1.59	**0.90**	0.33	0.96	0.31	**0.80**	0.19	0.35	**0.26**	**0.54**	**0.37**	0.45	**0.68**	**0.44**	13.51
chlorimuron-ethyl	1.67	1.19	1.44	0.86	1.54	0.80	0.30	0.78	**0.35**	0.63	0.19	**0.30**	**0.21**	**0.36**	**0.41**	**0.39**	0.73	0.36	12.51
LSD at 5%	0.16	0.13	0.16	0.11	0.18	0.10	0.04	0.12	0.06	0.09	0.04	0.07	0.08	0.12	0.09	0.07	0.11	0.06	1.57
2 days after treatment																			
control	1.57	1.24	1.32	0.97	1.64	0.80	0.34	0.90	0.31	0.70	0.26	0.46	0.51	0.70	0.73	0.51	0.86	0.29	14.11
propachlor	1.59	1.34	1.41	0.93	1.78	**1.04**	0.29	0.93	0.32	0.76	0.30	0.42	0.49	0.77	0.74	0.58	0.79	**0.44**	14.93
imazethapyr	1.66	1.34	1.45	**1.09**	**1.85**	**1.09**	0.30	**1.07**	0.34	0.73	**0.31**	**0.35**	**0.34**	**0.46**	**0.45**	0.43	**0.68**	0.33	14.25
chlorimuron-ethyl	1.67	1.19	1.44	1.00	1.77	0.84	0.30	1.00	0.35	0.74	**0.22**	0.40	**0.32**	**0.36**	**0.29**	**0.39**	**0.73**	0.29	13.28
LSD at 5%	0.19	0.13	0.16	0.10	0.18	0.10	0.05	0.10	0.05	0.09	0.04	0.07	0.08	0.13	0.15	0.10	0.11	0.05	1.55
4 days after treatment																			
control	1.65	1.36	1.47	1.04	1.74	0.93	0.37	0.99	0.34	0.80	0.23	0.48	0.51	0.78	0.68	0.48	0.85	0.25	14.94
propachlor	1.72	**1.70**	**1.75**	1.10	1.88	0.98	0.29	**1.14**	0.32	**0.93**	0.19	0.41	0.55	0.85	0.74	0.53	0.79	0.28	16.17
imazethapyr	1.75	**1.68**	1.58	**1.23**	1.78	0.97	**0.26**	1.08	0.31	**0.93**	0.22	**0.35**	**0.26**	**0.54**	**0.43**	**0.29**	0.79	0.22	14.67
chlorimuron-ethyl	1.67	**1.64**	1.44	**1.19**	1.89	0.84	0.30	1.00	0.35	**0.91**	0.27	**0.30**	**0.21**	**0.47**	**0.43**	**0.28**	**0.73**	0.28	14.19
LSD at 5%	0.20	0.16	0.18	0.13	0.18	0.11	0.08	0.11	0.06	0.11	0.06	0.09	0.07	0.14	0.13	0.09	0.10	0.06	1.62
8 days after treatment																			
control	1.61	1.44	1.37	1.08	1.73	0.93	0.41	0.93	0.47	0.78	0.24	0.47	0.47	0.82	0.76	0.58	0.74	0.26	15.07
propachlor	1.57	1.46	1.41	1.15	1.77	**1.08**	0.44	1.04	**0.55**	**0.86**	0.29	0.49	0.46	0.79	0.73	0.58	**0.57**	**0.30**	15.57
imazethapyr	1.66	1.55	1.45	1.09	1.70	1.00	0.33	0.96	**0.57**	0.84	0.22	0.46	**0.26**	**0.54**	**0.45**	**0.45**	**0.57**	**0.33**	14.44
chlorimuron-ethyl	1.67	1.37	1.44	1.00	1.78	0.95	0.42	1.00	0.46	0.81	0.22	0.41	**0.25**	**0.36**	**0.29**	**0.44**	**0.62**	0.28	13.77
LSD at 5%	0.20	0.18	0.14	0.14	0.20	0.12	0.08	0.12	0.07	0.08	0.04	0.08	0.07	0.10	0.13	0.09	0.07	0.04	1.96
12 days after treatment																			
control	1.63	1.41	1.39	1.15	1.75	0.92	0.43	0.94	0.46	0.76	0.23	0.51	0.48	0.82	0.81	0.59	0.69	0.23	15.20
propachlor	**1.87**	1.53	**1.59**	1.20	1.95	1.01	0.42	**1.09**	**0.56**	0.72	0.22	**0.39**	0.47	0.83	0.76	0.60	**0.59**	**0.34**	16.15
imazethapyr	1.73	1.52	1.50	1.25	1.77	1.01	0.43	**1.11**	0.51	0.72	0.26	0.48	**0.36**	**0.56**	**0.47**	0.51	**0.59**	0.26	15.05
chlorimuron-ethyl	1.73	1.47	1.54	1.25	1.84	0.98	0.44	1.04	0.47	0.77	0.23	**0.43**	**0.33**	**0.37**	**0.30**	**0.41**	**0.58**	0.30	14.47
LSD at 5%	0.19	0.18	0.15	0.15	0.21	0.11	0.07	0.14	0.07	0.09	0.06	0.07	0.07	0.10	0.11	0.10	0.07	0.04	1.53
16 days after treatment																			
control	1.68	1.46	1.43	1.19	1.80	0.95	0.44	0.97	0.48	0.78	0.27	0.53	0.52	0.84	0.79	0.61	0.72	0.24	15.68
propachlor	**1.92**	1.57	**1.63**	1.24	**2.01**	1.04	0.43	**1.12**	**0.58**	0.74	0.23	**0.41**	0.49	0.85	0.79	0.62	**0.61**	**0.35**	16.63
imazethapyr	1.78	1.56	1.55	1.28	1.82	1.04	0.44	**1.15**	0.53	0.74	0.30	0.50	**0.37**	**0.58**	**0.49**	**0.53**	**0.61**	0.27	15.53
chlorimuron-ethyl	1.79	1.51	1.58	1.28	1.89	1.01	0.46	1.07	0.49	0.79	0.31	**0.44**	**0.34**	**0.38**	**0.31**	**0.42**	**0.59**	**0.30**	14.98
LSD at 5%	0.19	0.17	0.17	0.15	0.18	0.13	0.08	0.13	0.09	0.10	0.06	0.06	0.08	0.10	0.10	0.07	0.09	0.05	1.62

[a] The boldface values are significantly different from their respective controls at $p < 5\%$.

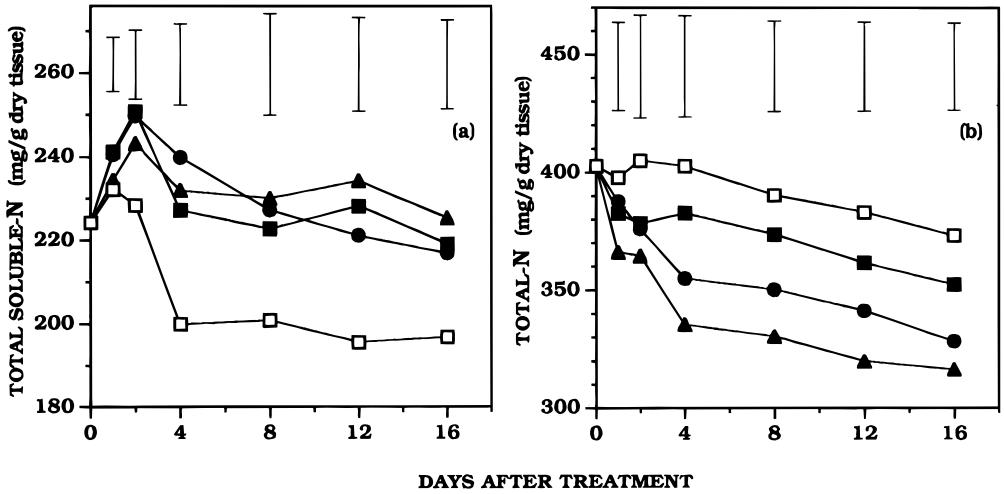

Figure 3. Contents of total soluble N (a) and total N (b) in *V. faba* L. seedlings: (□) control; (▲) chlorimuron-ethyl; (●) imazethapyr; (■) propachlor. Each value is the mean of three determinations. Vertical bars represent LSD at $p < 0.05$.

the eighth day after treatment, after which time an almost constant reduction of 31% was maintained. The connections between AHAS inhibition and valine, leucine, and isoleucine depletions can be clearly seen in Figure 2: chlorimuron-ethyl and imazethapyr determined abrupt simultaneous decreases in valine, leucine, and isoleucine contents, as they did in the extractable AHAS activity. Compared to the untreated control, the percent decreases in valine, leucine, and isoleucine contents in the chlorimuron-ethyl-treated samples reached levels of 58.7, 62.9, 56.1, respectively, and in the imazethapyr-treated samples 49.6, 46.7, 33.9, respectively. The decreases were maintained at an almost steady level during the experimental period, with the exception of a partial recovery in the valine content. No significant changes were observed in the content of the three amino acids in the propachlor-treated samples. These results confirm that the loss of protein content and the alteration in the profile of free amino acids in samples treated with chlorimuron-ethyl and imazethapyr are consequences of inhibition of AHAS activity. This assumption does not appear to be applicable for propachlor treatment; in fact, the protein loss preceded the inhibition of AHAS activity, and consequently the

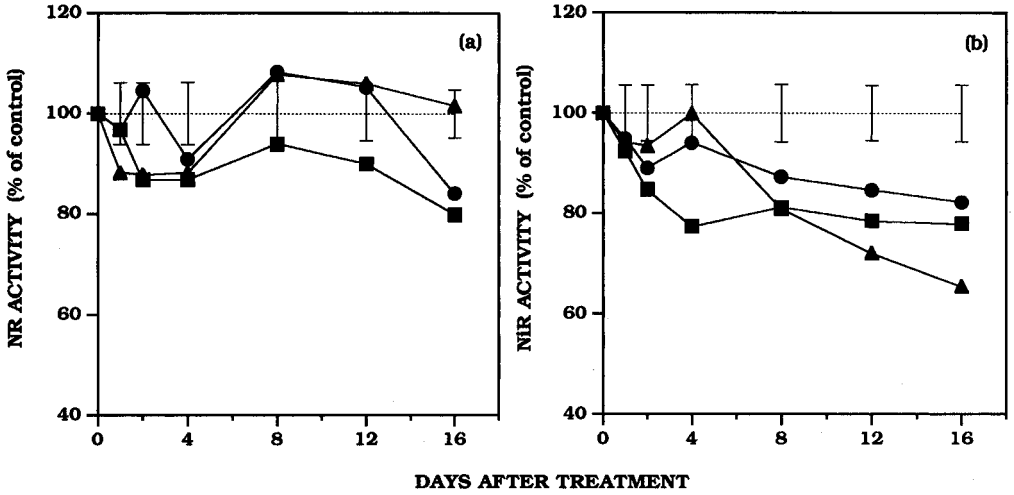

Figure 4. Activity of NADH-dependent nitrate reductase (a) and nitrite reductase (b) in *V. faba* L. seedlings as percent of control, (▲) chlorimuron-ethyl, (●) imazethapyr, and (■) propachlor. Each value is the mean of three determinations. Vertical bars represent LSD at $p < 0.05$. [Minimum and maximum values of NR and NiR activity in the controls during the experimental period were 11.5 and 41.1 mg of nitrite formed h^{-1} (g of dry weight)$^{-1}$ and 12.4 and 33.7 μg of nitrite disappeared h^{-1} (g of dry weight)$^{-1}$, respectively.]

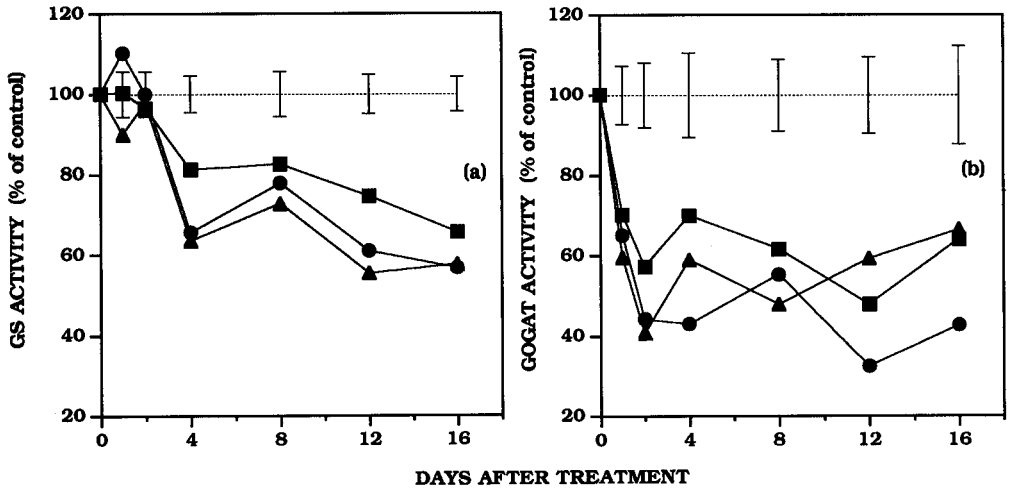

Figure 5. Activity of glutamine synthetase (a) and glutamate synthase (b) in *V. faba* L. seedlings as percent of control, (▲) chlorimuron-ethyl, (●) imazethapyr, and (■) propachlor. Each value is the mean of three determinations. Vertical bars represent LSD at $p < 0.05$. [Minimum and maximum values of GS and GOGAT activity in the controls during the experimental period were 25.7 and 30.7 μg of γ-glutamylhydroxamate formed h^{-1} (g of dry weight)$^{-1}$ and 6.2 and 16.7 absorbance decrease min^{-1} (g of dry weight)$^{-1}$, respectively.]

reduced AHAS activity in propachlor-treated samples seems to be a consequence, rather than a cause, of protein depression.

Since nitrogen availability is considered to be a prerequisite for amino acid and, hence, protein formation, the contents of total soluble N and total N as well as the activities of the NR−NiR system, as parameters of ammonia production, were determined and compared among the controls and the variously herbicide-treated plants.

The data depicted in Figure 3a show that soluble N content was unchanged during the first day interval following all herbicide treatments and at the second day following chlorimuron-ethyl treatment. Afterward, and up to the end of the experiment, soluble N content was significantly increased with no great differences among the effects of the three herbicides. Total N content (Figure 3b) was unaffected by propachlor but was significantly depressed by chlorimuron-ethyl and imazethapyr starting from the fourth day after treatment. The NR activity (Figure 4a) was generally unaffected with the exception of an inhibiting effect at the 16th day after propachlor and imazethapyr treatments, while NiR activity (Figure 4b) was significantly inhibited starting from the 2nd day by propachlor treatment and from 8th and 12th days by chlorimuron-ethyl and imazethapyr treatments, respectively. Therefore, NiR inhibition preceded AHAS inhibition and was concomitant with protein depression in the propachlor-treated plants, while it was subsequent to both AHAS inhibition and protein depression in the chlorimuron-ethyl- and imazethapyr-treated plants. As a whole, the results reported in the Figures 3 and 4 suggest that in samples treated with the two AHAS inhibitors the increase of soluble N, concomitant with the decreases of total N and of NiR activity, is consequent to protein depression. Otherwise, in propachlor-treated samples, the increase in soluble N, without a corresponding decrease in total N, does not appear to be derived from protein depression. It does seem attributable to the inhibition of NiR activity, which can result in a hindrance to ammonia availability.

As parameters of ammonia assimilation, the activities of GS and NADH-GOGAT enzymes were checked. The

data reported in Figure 5a show that none of the employed herbicides significantly affected GS activity during the first 2 days from treatment, but a significant inhibition of the enzyme activity was apparent thereafter. On the other hand, the activity of NADH-GOGAT (Figure 5b) was significantly inhibited by all herbicides throughout the experimental period, without great difference among them. Singh et al. (1986) and Yamaya et al. (1995) observed a rapid response of NADH-GOGAT to a supply of ammonium ion in *Vigna mungo* and *Oryza sativa*, respectively. A similarly rapid, but inverse, NADH-GOGAT response to the lack of ammonium ion could be hypothesized. This could have occurred in our experiment following NiR inhibition. In addition, the consequent nitrate accumulation may have reduced the activity of GS and GOGAT. In agreement with this hypothesis, it has been reported that high nitrate levels reduced the activity of GS and GOGAT in nodules of *V. faba*, the second being more markedly inhibited than was the first (Cordovilla et al., 1996). The responses of NR, NiR, GS, and NADH-GOGAT to treatments confirm the disturbance induced by all of the herbicides on nitrogen assimilation into the organic form. However, it is important to note that in chlorimuron-ethyl- and imazethapyr-treated samples, the inhibition of GS activity preceded that of NiR and followed that of AHAS, while in propachlor-treated samples an opposite sequence of changes in the activities of these enzymes was observed. Therefore, the reduction in protein formation and the disturbance in free amino acid profile in *V. faba* appear to be due to inhibition of AHAS following treatment with chlorimuron-ethyl or imazethapyr and to ammonia starvation deriving from the inhibition of NiR following treatment with propachlor.

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