Chapter 6

IMIDAZOLINONE-ACETOHYDROXYACID SYNTHASE INTERACTIONS

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I. INTRODUCTION

Research on the mode of action of the imidazolinone herbicides led to the conclusion that the sole mechanism was the inhibition of the enzyme acetohydroxyacid synthase (AHAS, acetolactate synthase, ALS, E.C. 4.1.3.18). Five key experiments led to this conclusion. First, the treatment of susceptible maize cell cultures with imidazolinones resulted in reducing the levels of only three amino acids: valine, leucine, and isoleucine.[1] These amino acids share four enzymes in their biosynthetic pathway; AHAS is the first in the sequence (Figure 1). Second, supplementation of herbicide-treated maize with the three amino acids resulted in the alleviation of herbicidal symptoms.[1,2] Third, AHAS extracted from susceptible maize was inhibited by the imidazolinones *in vitro*.[3] Fourth, imidazolinone-treated plants were found to have reduced AHAS activity.[4] Fifth, AHAS from imidazolinone-tolerant maize derived from cell culture selection was not inhibited by the imidazolinones, and tolerance cosegregated with insensitive AHAS.[5,6] Many other observations support this hypothesis of the mode of action of the imidazolinone herbicides. Thus, a discussion of AHAS and the interactions of the imidazolinones with this enzyme is essential for a complete description of the imidazolinone herbicides.

II. AHAS BACKGROUND

AHAS is required for the biosynthesis of two acetohydroxyacids: acetolactate and acetohydroxybutyrate (Figure 2). AHAS catalyzes the condensation of pyruvate either with a second pyruvate to yield acetolactate or with 2-oxobutyrate to yield acetohydroxybutyrate. Figure 3 shows the structures of the cofactors of AHAS and the amino acid end products of the pathway. Both thiamine pyrophosphate and divalent magnesium activate the enzyme, while flavin adenine dinucleotide (FAD) acts as a stabilizing cofactor with no role in catalysis.[4,7] AHAS is the first enzyme common to valine, leucine, and isoleucine biosynthesis and is feedback inhibited by the pathway end products.[8-10] AHAS has been found in bacterial extracts,[11,13-15] yeast and other fungi,[12,16-20] archaebacteria,[21] algae,[22,23] and plants.[3,5,6,8-10,24-33] The majority of studies on AHAS have been performed on the enzyme extracted from microbial sources.

A. MICROBIAL AHAS

In enterobacteria, as many as six AHAS isozymes are encoded by different genes. Isozyme I of *E. coli*[13] and isozyme II of *Salmonella typhimurium* have been purified to homogeneity.[14] AHAS II was carefully studied by DuPont researchers after discovering that AHAS is the site of action of the sulfonylurea herbicides.[34] Although the sulfonylureas are structurally unrelated to the imidazolinones, the studies on AHAS inhibition by sulfonylureas reveal much about the mechanism of AHAS. Inhibition of AHAS II by sulfonylureas is characterized as slow, tight binding. Stopped-flow kinetic studies showed that the first step in catalysis, the binding and decarboxylation of pyruvate, occurs unimpeded in the presence of sulfometuron methyl (SM). It is the second step, the binding and condensation with the second pyruvate, that is inhibited by SM.[35] AHAS II activity was not influenced by the redox state or the reduction potential of the FAD bound to the enzyme;[7] however, the absorption spectrum of the FAD bound to the enzyme changes during catalysis, and these changes were diminished by SM.[36] Also, substitution of reduced FAD ($FADH_2$) for FAD resulted in an increase in the binding constant for SM. Thus, the binding of SM, which results in the inhibition of AHAS, must occur proximal to the FAD binding site and near the binding site of the second pyruvate (or the binding site of the 2-oxobutyrate). The binding of SM first occurs with low affinity followed by formation of the final high-affinity complex.[34]

Certain conclusions about the structure of the catalytic site of AHAS can be inferred from these studies. First, FAD must serve a structural role for the enzyme while being

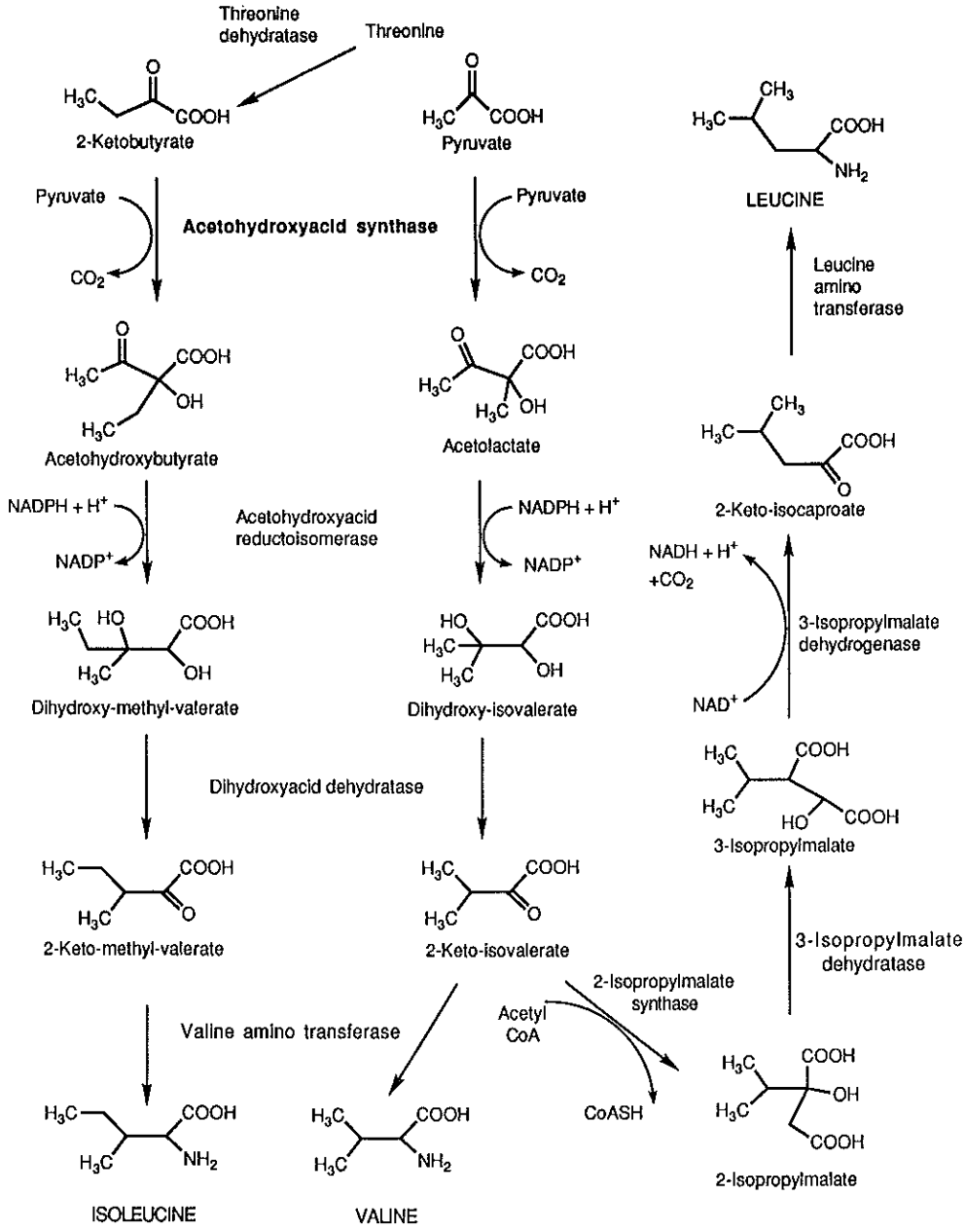


FIGURE 1. Biosynthetic pathway of the branched-chain amino acids.

associated with conformational changes in the enzyme occurring during catalysis. Second, the FAD binding site must be proximal to the second pyruvate binding site. Third, the sulfonylurea binding site must be proximal to both the FAD and the second pyruvate binding site. Fourth, all of these sites must be somewhat removed from the first pyruvate binding site. The implications of these studies on the mechanism of plant AHAS inhibition by the imidazolinones remain to be investigated.

Inhibition of bacterial AHAS by the imidazolinones has been documented in only one report. Schloss et al.[7] determined the binding constants of imazaquin with the three bacterial isozymes (3.6 mM, 0.32 mM, and 3.2 mM for isozymes I, II, and III, respectively).

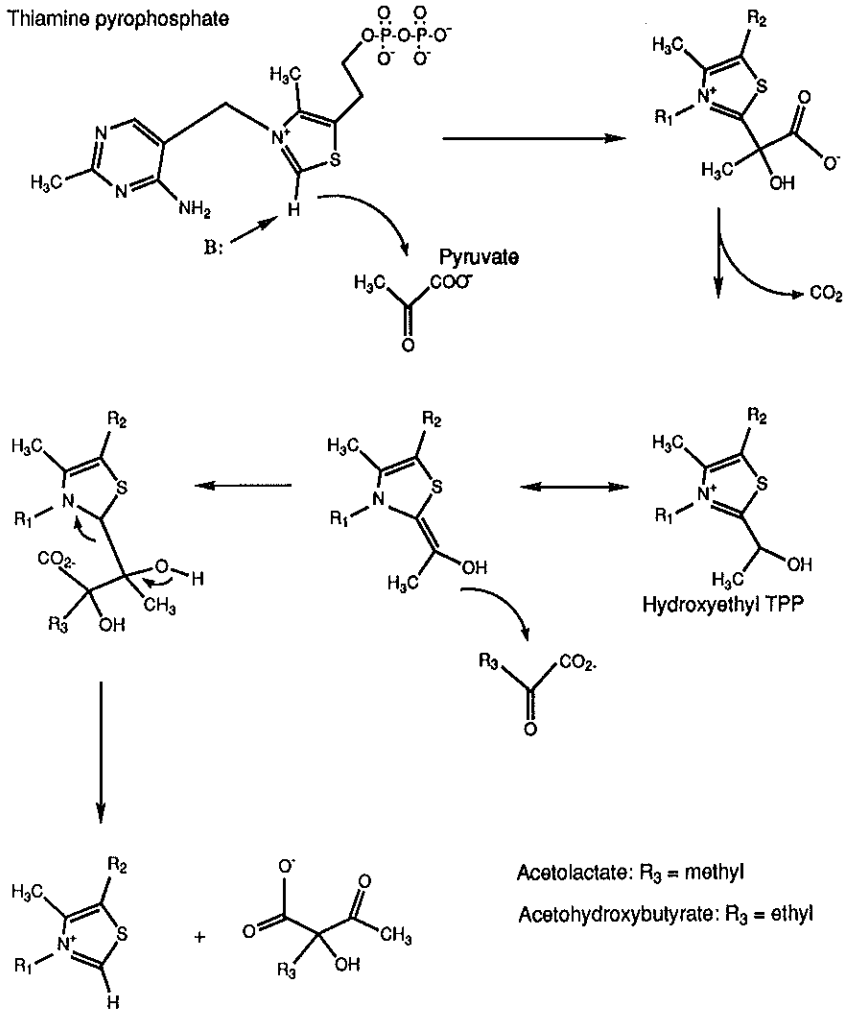


FIGURE 2. Proposed reaction mechanism for AHAS.

Equilibrium binding studies using AHAS II and radiolabeled SM proved that SM could be displaced by imazaquin or ubiquinone-0. The amino acid sequences of all of the AHAS isozymes are similar to the sequence of pyruvate oxidase,[37,38] a mechanistically related enzyme, which catalyzes the oxidative decarboxylation of pyruvate. FAD undergoes cyclical oxidation and reduction during the cycle, and the reoxidant for $FADH_2$ is ubiquinone. Schloss speculated that the herbicide binding sites of AHAS are derived from the vestigial ubiquinone binding site of the ancestral pyruvate oxidase.[7]

B. GENERAL PROPERTIES OF PLANT AHAS

AHAS occupies the same central role in valine, leucine, and isoleucine biosynthesis in plants as it does in microbes. Feedback of all three amino acids inhibits the enzyme, but a cooperative inhibition is observed when both valine and leucine are present.[9] AHAS occurs in small quantities in plant tissues and, like the bacterial isozymes, the plant enzyme requires FAD for stability.[4,6,32]

Like a majority of the amino acid biosynthetic enzymes, AHAS is nuclear encoded and plastid localized.[8,28] AHAS gene sequences from *Arabidopsis* and tobacco have been published[39] and show a high degree of homology at the amino acid level both between plant

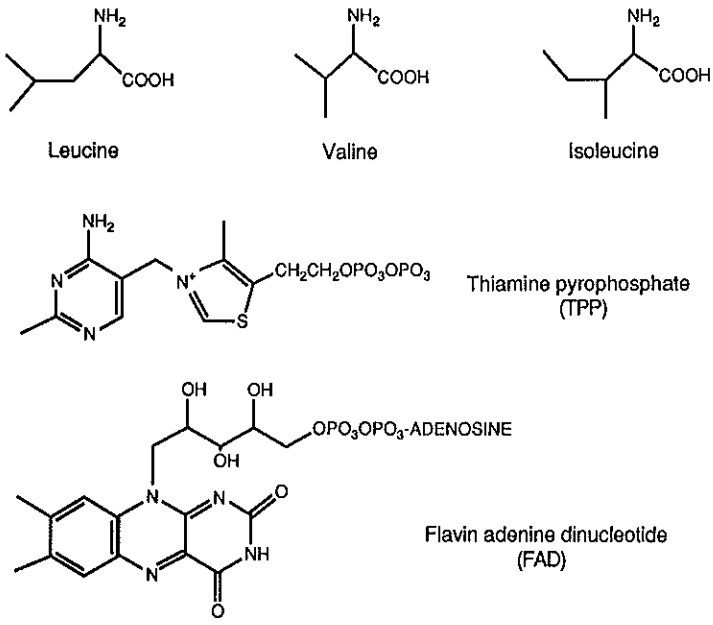


FIGURE 3. Structures of end products of AHAS pathway and cofactors of AHAS.

species and with microbial enzymes. A European patent application on the AHAS gene has been published, showing sites of mutation in the gene that confer resistance to sulfonylurea and imidazolinone herbicides.[40]

III. AHAS INHIBITION BY THE IMIDAZOLINONES

A. *IN VITRO* EXPERIMENTS WITH AHAS

Initial experiments on the inhibition of AHAS by the imidazolinones were conducted on AHAS from corn roots. Substrate-inhibitor studies suggest that the inhibition of AHAS by imazapyr is uncompetitive with respect to pyruvate (Figure 4). Uncompetitive inhibition implies that imazapyr binds to AHAS only after the formation of the ternary enzyme-pyruvate-TPP complex.

Muhitch et al.[4] reported on a number of experiments that demonstrated a more complex interaction of imazapyr with AHAS than was assumed in the earlier studies. When AHAS activity was measured over an extended assay period (4 h) in the presence of various imazapyr concentrations, inhibition was found to increase with time (Figure 5). These results suggest that the equilibrium between imazapyr and the enzyme is reached slowly, a feature typical of many tight-binding inhibitors. The initial and final K_i values for the inhibition of AHAS from black Mexican sweet corn suspension cell cultures by imazapyr were 15 and 0.9 μM, respectively.

The structural requirements for inhibition of AHAS by the imidazolinones correlate, to a certain degree, with the structural requirements for herbicidal activity. Figure 6 shows a summary of some of these structural requirements. Individually, neither the nicotinic acid ring nor the imidazolinone ring is inhibitory. Neither the des-carboxy imidazolinone nor the esters of the nicotinic acid are inhibitory. In contrast, among those imidazolinones containing nitrogen in the aromatic ring, only the nicotinic imidazolinone is an effective herbicide.

Among the herbicidal imidazolinones, the benzene imidazolinone is the best inhibitor, followed by the quinoline and the pyridine (Figure 7). In the pyridine series, all of the

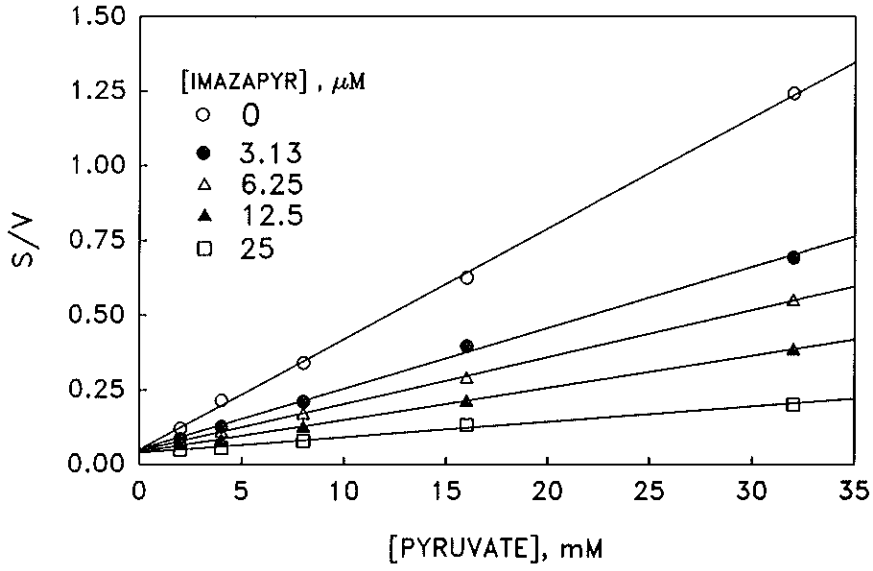


FIGURE 4. Effects of imazapyr on AHAS dependence on pyruvate (Hanes-Woolf plot).

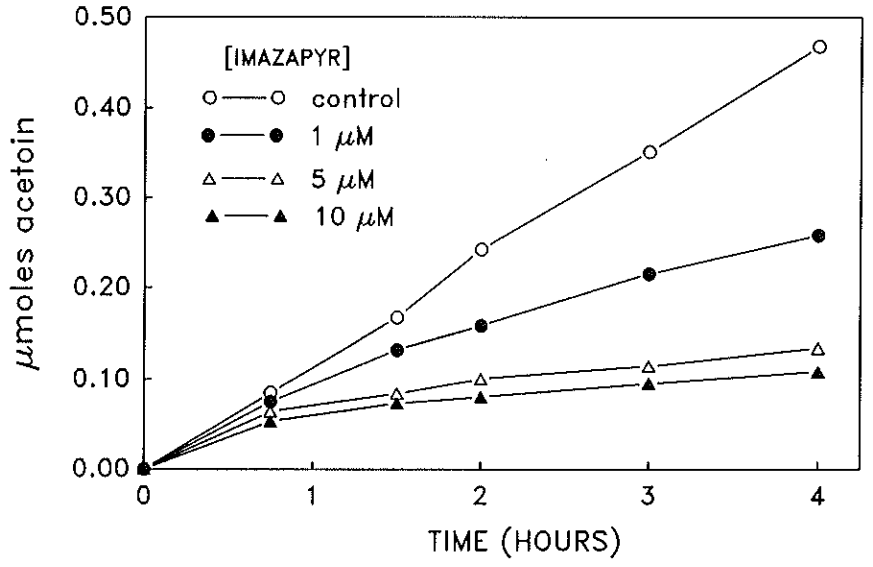


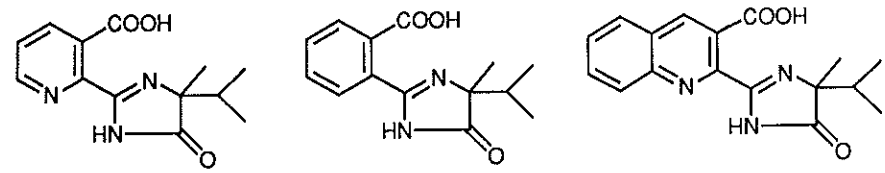
FIGURE 5. Time course for inhibition of AHAS by imazapyr. (Adapted from Muhitch, M. J., Shaner, D. L., and Stidham, M. A., *Plant Physiol.*, 83, 451, 1987. With permission.)

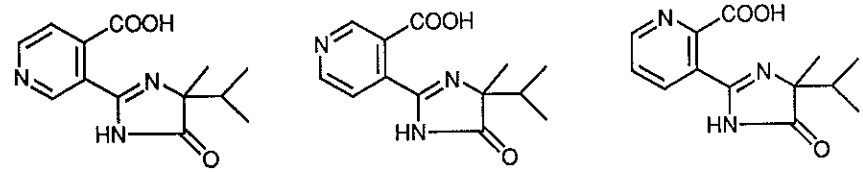
positional isomers that have adjacent imidazolinone and carboxylic acid substitutions on the ring are active inhibitors of AHAS. In the imidazolinone ring, the chiral carbon at the attachment of the isopropylmethyl group is important in determining enzyme inhibition: the *R* isomer is about 10 times more inhibitory than the *S* isomer.

B. EFFECTS OF IMIDAZOLINONE TREATMENT ON THE LEVEL OF EXTRACTABLE AHAS

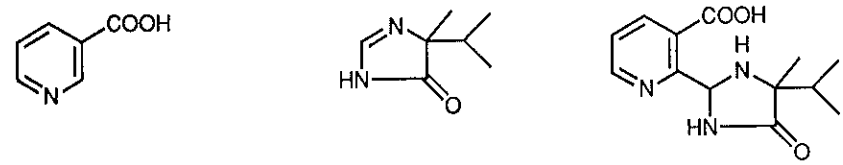
When AHAS is extracted from corn tissue treated with imazapyr, the amount of extractable AHAS is dractically reduced compared to that in untreated tissue (Table 1). This

Active





Inactive



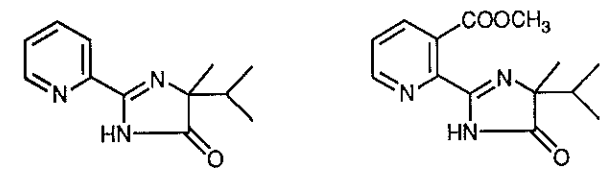
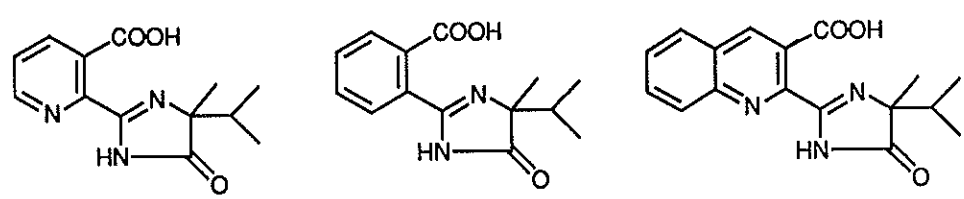


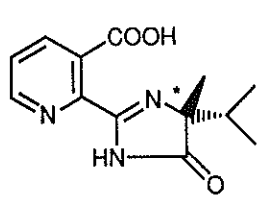
FIGURE 6. Structural requirements for AHAS inhibition.



I_{50} = 4 μM

I_{50} = 0.1 μM

I_{50} = 1 μM



*
R, S : I_{50} = 4 μM
R : I_{50} = 2.4 μM
S : I_{50} = 18 μM

FIGURE 7. Relative inhibition of AHAS by imidazolinones.

TABLE 1
Effect of Imazapyr on Extractable AHAS in Excised Maize Shoots

Treatment	Extractable AHAS activity (4 h after treatment)	
	µg Acetoin/mg protein/h	% of control
Control	3.73	—
Control + Imazapyr spike[a]	3.80	101
Imazapyr fed[b]	0.37	10

[a] 100 n*M* imazapyr/ml in extraction buffer
[b] 57 n*M* imazapyr/ml internal concentration of maize leaves

From Stidham, M. A. and Shaner, D. L., *Pestic. Sci.*, 29, 335, 1990. With permission.

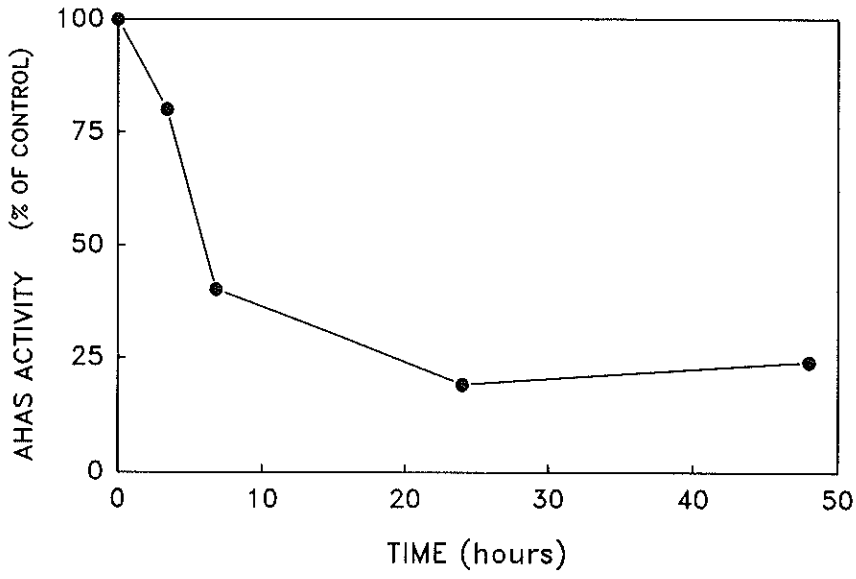


FIGURE 8. *In vivo* inhibition of AHAS by imazaquin. (From Shaner, D., Singh, B., and Stidham, M., *J. Agric. Food Chem.*, 38, 1279, 1990. With permission.)

decrease is unexpected because preparation of AHAS from the plant tissue involves dilution steps that should result in a dissociation of reversibly bound imazapyr. Decreases in extractable AHAS are not observed when untreated tissue is extracted in a buffer containing high concentrations of imazapyr. Thus, it appears that the imidazolinone effect on extractable AHAS is not due to the mixing of AHAS with imazapyr during extraction. Radiolabeled imazapyr was used in an excised shoot experiment to quantify the amount of imazapyr carried over in the AHAS extraction procedure. The imazapyr associated with the protein was 0.05 nm/mg protein; this level translated to an imazapyr concentration of 50 n*M* in the assay. Since this concentration of imazapyr is roughly 100 times less than the concentration required for 50% inhibition of the enzyme, the imazapyr carried over could not account for the *in vitro* inhibition of the extracted AHAS.

The reduction in extractable AHAS caused by imazapyr occurs when either excised leaves or intact plants are used; this effect is observed with other imidazolinones as well. Figure 8 shows the amount of AHAS activity extracted from corn treated with imazaquin in a soil drench. Eight hours after application, the extractable AHAS in plants treated with

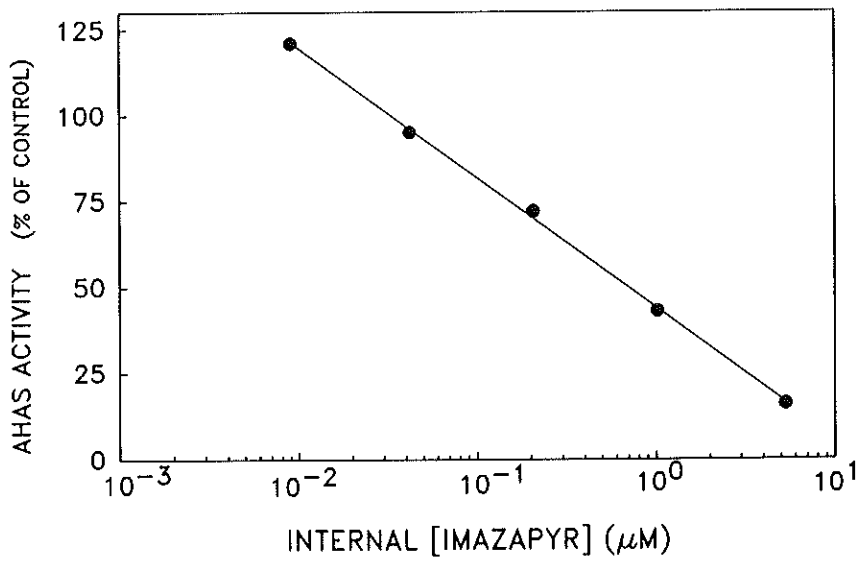


FIGURE 9. Effect of imazapyr on extractable AHAS from excised corn. (From Shaner, D., Singh, B., and Stidham, M., *J. Agric. Food Chem.*, 38, 1279, 1990. With permission.)

imazaquin is reduced by 60% relative to that in the untreated plants, and one day after treatment, the quantity is 20% of that in the untreated plant. The effect is specific for AHAS, since the levels of other enzymes are unaffected after imidazolinone treatment.[4]

The dependence of AHAS inactivation on imidazolinone concentration was determined using an excised leaf system, wherein corn leaves were placed in solutions containing different levels of imazapyr and then extracted for AHAS activity 4 h after complete uptake of the solution. The extractable AHAS activity decreased as the semilogarithm of the calculated internal imazapyr concentration (Figure 9). The I_{50} value calculated from the plot was one tenth the I_{50} value calculated from *in vitro* experiments on the enzyme. Since the enzyme was extracted 4 h after exposure to the herbicide, the drop in the I_{50} value could reflect the slow tight-binding inhibition observed *in vitro*. Alternatively, the herbicide could concentrate in the chloroplast via weak-acid trapping.[41] Since the calculation of the internal imazapyr concentration is averaged over the entire cell volume, accumulation in the chloroplast would give higher concentrations at the site of action.

A number of interpretations could explain the effect of the imidazolinones on the amount of extractable AHAS. One interpretation could be that upon prolonged exposure *in vivo* the imidazolinones form an irreversible complex with AHAS. Another interpretation is that AHAS-binding of imidazolinones results in the destabilization of AHAS, which may or may not be reflected in degradation of the enzyme *in vivo*. Antibodies raised against AHAS will be valuable in determining which of the above interpretations is correct.

IV. AHAS FROM VARIOUS SPECIES

AHAS activity in the intact plants ranged widely among different species (Table 2). The greatest AHAS activity was found in pea. However, compared to AHAS activity from intact plants, the BMS (Black Mexican Sweet) corn cells grown in suspension culture had even higher AHAS activity. On a fresh weight basis, BMS cells had 2.8-fold higher AHAS activity than the next highest source (pea). Similarly, on a protein basis, BMS cells had over 10-fold higher activity than any intact plant species used in this survey. It is important to note that actively dividing BMS cells were used in this study. Therefore, the higher

TABLE 2
Acetohydroxyacid Synthase from Different Species

Plant	Protein (mg)	Total AHAS (μmol/h)	Specific activity of AHAS (μmol/mg protein/h)	Specific activity of AHAS (μmol/g fresh wt/h)
Matricaria	50	2.0	0.04	0.2
Flax	59	3.3	0.06	0.3
Sunflower	81	5.7	0.07	0.6
Arabidopsis	56	6.5	0.12	0.7
Ambrosia	90	6.7	0.07	0.7
Barley	97	8.9	0.09	0.9
Wheat	120	9.4	0.08	0.9
Rape	59	12.4	0.21	1.2
Amaranthus	68	12.8	0.19	1.3
Spinach	53	16.6	0.31	1.7
Tobacco	51	17.4	0.34	1.7
Sorghum	66	18.1	0.28	1.8
Soybean	97	21.0	0.22	2.1
Mustard	51	21.4	0.42	2.1
Corn	76	28.4	0.37	2.8
Lima bean	110	28.9	0.26	2.9
Pea	117	58.6	0.50	5.9
BMS cells	61	164.1	5.36	16.4

From Singh, B. K., Stidham, M. A., and Shaner, D. L., *J. Chromatogr.*, 444, 251, 1988. With permission.

specific activities of exponentially growing cell cultures are consistent with studies on lima bean in which the younger leaves (undergoing rapid cell division) have higher specific activities of AHAS than do the older leaves. This aspect is discussed later in this chapter.

AHAS activity from all species showed feedback inhibition by valine, leucine, and isoleucine (Table 3) as was reported earlier in other studies.[8-10] A combination of valine and leucine was most inhibitory. There were significant differences in the kinetics of inhibition between species (Figure 10); however, maximum inhibition was similar to the values presented in Table 3.

AHAS from all species was highly sensitive to inhibition by imazapyr and imazethapyr, but in general, imazethapyr was a stronger inhibitor than imazapyr (Table 4). There were large differences in the I_{50} for herbicides among species. These differences may be attributed to the inherent differences in the AHAS protein itself and/or differences in the stability of the enzyme. Interestingly, AHAS activity from pea, lima bean, and soybean appeared to be the most tolerant to the herbicides. These same species are also tolerant of both herbicides at the whole-plant level because of their ability to metabolize these compounds.

Besides their morphological and physiological characteristics, plant species used in this survey were chosen based on their sensitivity to imidazolinones at the whole-plant level. For example, *Amaranthus* is one of the most sensitive species, whereas *Ambrosia* is one of the less sensitive ones. Previous work has demonstrated that detoxification of an imidazolinone is the basis for tolerance of some of these species (e.g., soybean). However, tolerance to an imidazolinone herbicide can also result from a higher level of AHAS activity or from the presence of a form of AHAS that is insensitive to inhibition by imidazolinone. Results from this survey showed large differences between species in the specific activity of AHAS (Table 2) and in the sensitivities of these activities to herbicides (Table 4). However, no correlation was found between glasshouse control rates (the minimum amount of herbicide required for complete or nearly complete kill of plants) of imazapyr and imazethapyr and the I_{50} of AHAS activities for the respective herbicides (Figure 11; $r^2 = 0.03$ for imazapyr and 0.0001 for imazethapyr). Similarly, no correlation was found between these control

TABLE 3
Effects of Valine, Leucine, and Isoleucine on AHAS Activity

Plant	Inhibition caused by various amino acids at 1 m*M* (%)						
	L[a]	V[b]	I[c]	L+I	V+I	L+V	L+V+I
Flax	26	15	32	29	37	42	37
Amaranthus	39	35	10	45	25	40	45
Soybean	33	44	33	51	36	50	46
Barley	55	26	20	45	31	49	48
Pea	41	9	21	46	27	41	49
Lima bean	24	17	6	33	30	55	53
BMS cells	26	5	12	44	19	57	59
Wheat	60	49	33	62	45	61	59
Rape	54	30	28	61	34	62	62
Mustard	46	29	19	61	26	65	62
Sunflower	52	40	34	60	44	63	63
Arabidopsis	52	47	33	64	50	67	67
Spinach	48	43	32	64	47	71	71
Tobacco	50	43	31	65	45	70	71
Corn	49	34	16	57	43	76	76
Sorghum	69	61	41	69	66	72	77
Ambrosia	66	54	40	70	58	74	77
Matricaria	72	54	50	73	54	75	79

[a] L = Leucine.
[b] V = Valine.
[c] I = Isoleucine.

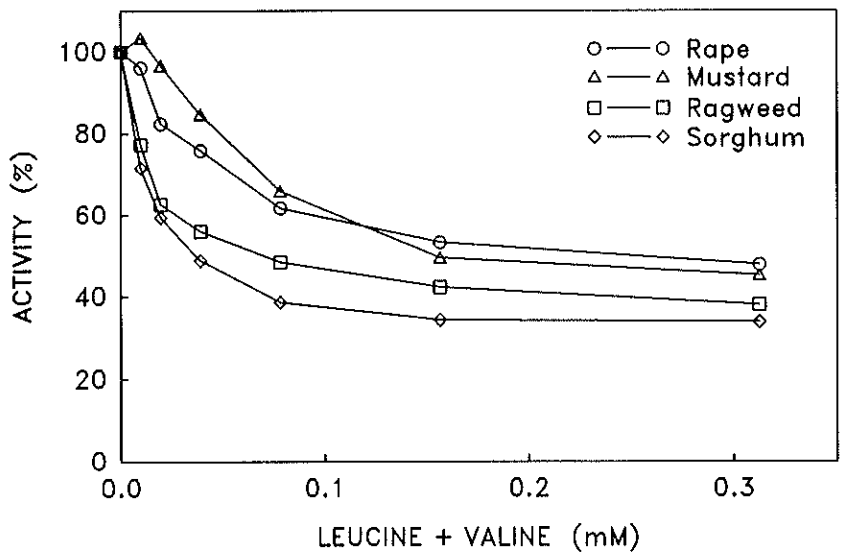


FIGURE 10. Inhibition of AHAS with leucine + valine.

rates and the specific activities of AHAS in different species (Figure 12; $r^2 = 0.08$ for imazapyr and 0.15 for imazethapyr). The absence of any relationship between the control rates and other characteristics of AHAS in these species (Figures 11 and 12) implies that AHAS does not significantly contribute to the natural tolerance of different species to imidazolinones at the whole-plant level.

TABLE 4
Inhibition of AHAS from Various Species by Different Herbicides

Plant	I_{50} Imazapyr (μM)	Imazethapyr (μM)	Sulfometuron methyl (nM)
Matricaria	9.5	7.9	74.1
Flax	2.8	1.7	50.1
Sunflower	4.7	2.9	31.3
Arabidopsis	5.1	1.8	7.3
Ambrosia	14.1	5.4	21.9
Barley	7.6	4.0	38.0
Wheat	17.8	5.7	32.4
Rape	2.9	1.3	20.5
Amaranthus	3.5	2.0	21.0
Spinach	1.4	0.4	4.0
Tobacco	3.0	0.8	7.9
Sorghum	5.9	2.1	27.6
Soybean	19.4	51.3	95.5
Mustard	2.6	1.5	17.8
Corn	4.0	1.2	23.4
Lima bean	7.5	18.4	160.0
Pea	31.7	83.2	53.7

From Singh, B. K., Newhouse, K. E., Stidham, M. A., and Shaner, D. L., in *Biosynthesis of Branched-Chain Amino Acids*, Barak, A., Schloss, J. V., and Chipman, D. M., Eds., VCH Publishers, New York, 1990, 357. With permission.

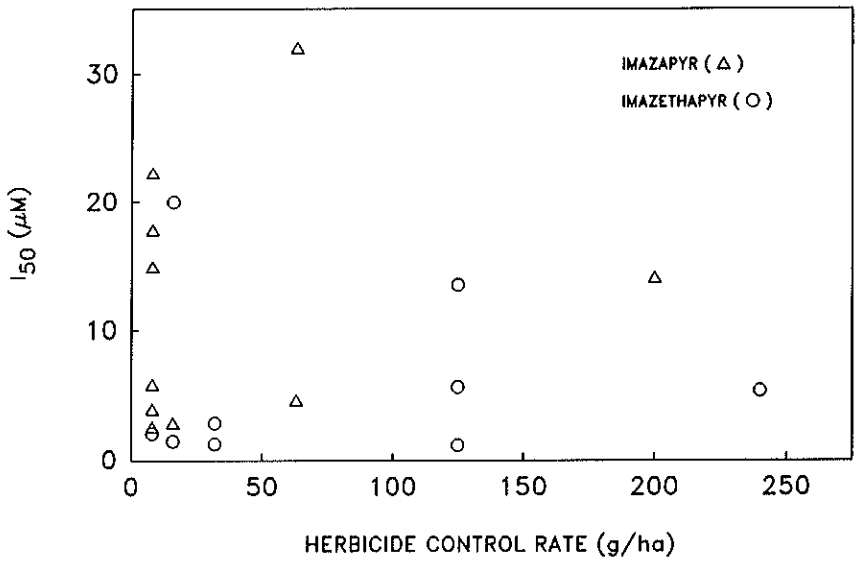


FIGURE 11. Correlation between glasshouse control rates and I_{50} for AHAS from different species. (From Singh, B. K., Newhouse, K. E., Stidham, M. A., and Shaner, D. L., in *Biosynthesis of Branched-Chain Amino Acids*, Barak, A., Schloss, J. V., and Chipman, D. M., Eds., VCH Publishers, New York, 1990, 357. With permission.)

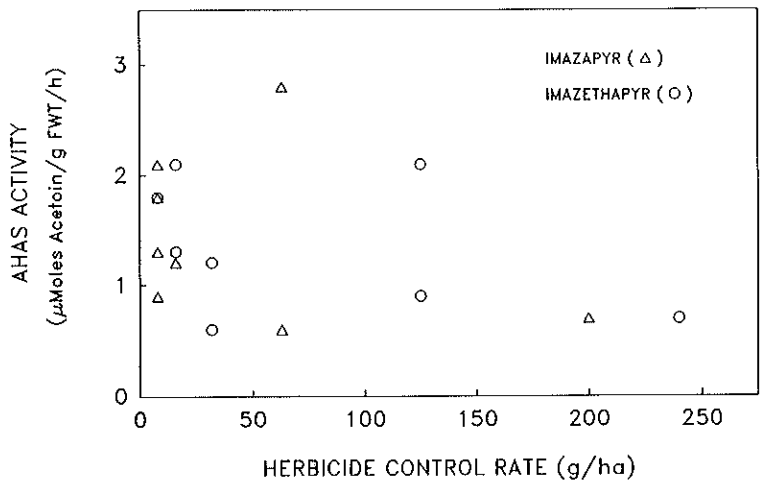


FIGURE 12. Correlation between glasshouse control rates and total AHAS activity from different species. (From Singh, B. K., Newhouse, K. E., Stidham, M. A., and Shaner, D. L., in *Biosynthesis of Branched-Chain Amino Acids*, Barak, A., Schloss, J. V., and Chipman, D. M., Eds., VCH Publishers, New York, 1990, 357. With permission.)

V. DEVELOPMENTAL REGULATION OF AHAS ACTIVITY

There are two principal mechanisms of metabolic regulation in microbes: variation of enzyme concentration and alteration of enzyme activity.[42,43] In plants, the contribution of these mechanisms to the coordination of metabolism is not yet clear. The only known mechanism of regulation of AHAS in plants is feedback inhibition of the enzyme by valine, leucine, and isoleucine, which are the end products of the pathway. In order to understand growth-dependent regulation of AHAS in plants, AHAS activity was examined in lima beans at different stages of growth.

The first pair of leaves from lima bean plants at different ages were used for this study. On the first sampling day, 5 d after planting, these unifoliate leaves had emerged from the cotyledons. The fresh weight of leaves increased with advancing leaf age and was maximal on the last sampling day, 12 d after planting, when this leaf was fully expanded. The leaf growth measured in this way showed a typical sigmoidal pattern. Soluble protein content per leaf showed a similar increase in response to leaf growth (Figure 13).

AHAS activity per leaf increased about threefold from days 5 to 7 after planting, then remained relatively stable throughout the sampling period. However, the specific activity of AHAS declined sharply from days 5 to 10 after planting, then remained steady (Figure 13). The lack of further increase in AHAS activity per leaf after several days of growth suggests that no apparent net increase in AHAS activity occurs after about 2 d of leaf growth. Since soluble protein content per leaf continues to increase with advancing leaf age (Figure 13), lack of further increase in AHAS activity after day 7 is not due to a cessation of protein synthesis. The first few days of leaf growth involve rapid cell division, whereas most of the subsequent growth can be attributed to cell expansion. Therefore, it appears that net synthesis of AHAS occurs only during cell division. Consistent with this theory were the results of experiments using a tissue culture system as discussed earlier. The results of these experiments, however, do not rule out the possibility that increases in the enzyme activity observed here were due to enzyme activation. Quantification of AHAS protein using an antibody is required to resolve this issue.

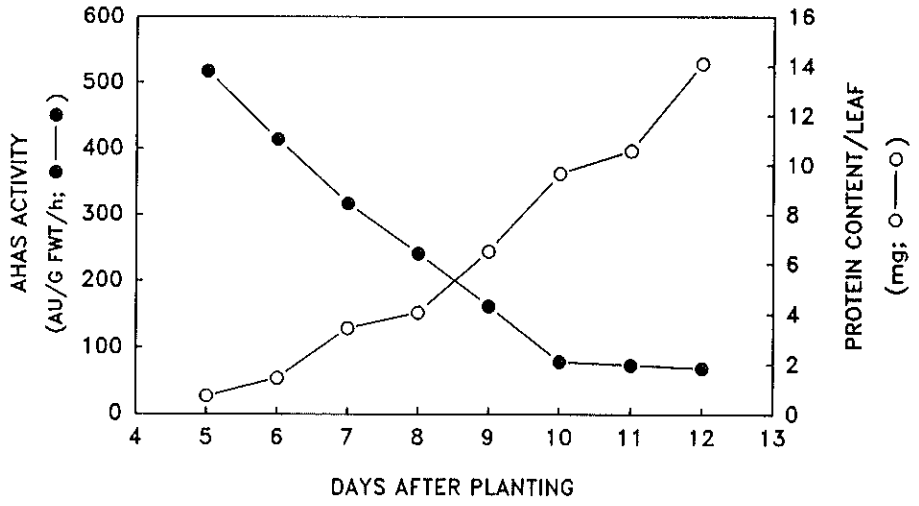


FIGURE 13. Total soluble protein and AHAS activity in lima bean leaves.

In a previous study, the properties of AHAS isolated from shoots of peas appeared to differ from the properties of the enzyme isolated from seeds: AHAS activity from seeds was less sensitive to inhibition by the pathway end products, either alone or in combination.[10] This observation may reflect a type of growth-dependent regulation similar to that observed with homoserine dehydrogenase: its sensitivity to inhibition by threonine may vary significantly during plant growth.[44] The present study did not reveal differences in the sensitivity of AHAS to inhibition by the leucine-valine combination at any stage of growth. This observation could suggest that there is no growth-dependent difference in the regulatory properties of AHAS in lima bean plants. However, it is possible that the observation in the previous case is tissue specific[10] and not a growth-dependent response. Further studies are needed to evaluate the possibility that the differences in the sensitivity of AHAS to feedback inhibition by amino acids appear at a later stage of leaf growth.

VI. DIFFERENT FORMS OF AHAS

Microorganisms may possess as many as six isozymes of AHAS with different sensitivities to feedback inhibition by amino acids.[43] Similarly, bacterial AHAS isozymes differ in their sensitivities to sulfonylureas.[7] Even though genetic evidence for the presence of AHAS isozymes in tobacco was present,[45] these isozymes could not be resolved by chromatography. Using BMS corn cells from suspension cultures as the enzyme source, we were successful in separating different forms of AHAS with different physical and kinetic characteristics.

Two peaks of AHAS activity were seen after anion exchange chromatography on a Mono Q* column (Figure 14). The major peak, designated AHAS I, was eluted during gradient elution and contained about 90% of the total recovered enzyme activity. A minor peak of AHAS activity, designated AHAS II, which contained nearly 10% of the total AHAS activity, was recovered in the unbound fraction. The proportions and recoveries of enzyme activity of the two peaks of AHAS activity were similar to those observed during chromatofocusing.[33] Rechromatography of each of these two peaks separately on the Mono Q column yielded single peaks of AHAS activity that eluted at their original retention times.

* Trademark of Pharmacia, Piscataway, NJ.

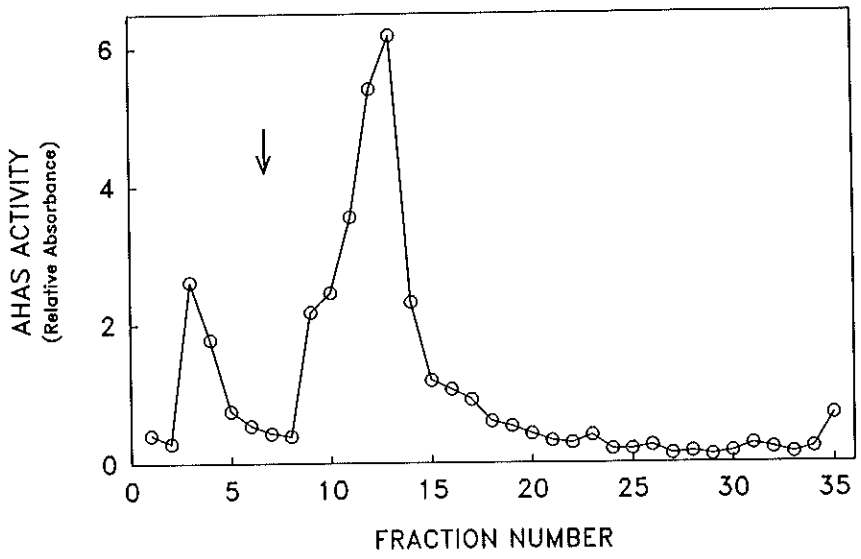


FIGURE 14. Anion exchange chromatography of AHAS from BMS cells on a Mono Q column. The arrow indicates the beginning of salt gradient. (From Singh, B. K., Stidham, M. A., and Shaner, D. L., *J. Chromatogr.*, 444, 251, 1988. With permission.)

TABLE 5
Physical and Kinetic Properties of AHAS I and AHAS II

Property	AHAS I	AHAS II
Molecular weight	193,000	55,000
pH optimum	6—7	7
K_m for pyruvate (m*M*)	5	8
I_{50}		
Leucine + valine (m*M*)	0.1	[a]
Imazapyr (μ*M*)	2.0	1.5
Sulfometuron methyl (n*M*)	10.0	10.0

[a] Less than 10% inhibition.

From Singh, B. K., Stidham, M. A., and Shaner, D. L., *J. Chromatogr.*, 444, 251, 1988. With permission.

The properties of the two peaks of AHAS activity were significantly different. AHAS I was sensitive to inhibition by both leucine and valine as well as to inhibition by imazapyr. In contrast, AHAS II was insensitive to inhibition by leucine and valine but was more sensitive to inhibition by imazapyr than was AHAS I. Further characterization revealed differences in the molecular weights and in other kinetic properties of the two activities.

The native molecular weights of AHAS I and AHAS II were estimated to be 193,000 and 55,000, respectively, by chromatography on an HPLC gel filtration column (Table 5). AHAS I had a broad pH optimum between pH 6 and 7. The pH response was not affected by the type of buffer system used. In contrast, AHAS II had a very distinct pH optimum at pH 7 in phosphate buffer (Table 5) but showed very little activity in MES (2-[*N*-morpholino]ethanesulfonic acid) or Tris (*tris*[hydroxymethyl]aminomethane). The pyruvate saturation curves of AHAS I and AHAS II are hyperbolic, and both forms of AHAS were

TABLE 6
Effects of Valine, Leucine, and Isoleucine on the Activity of AHAS I and AHAS II

Form of AHAS	Inhibition caused by various amino acids at 1 m*M* (%)						
	L[a]	V[b]	I[c]	L+I	V+I	L+V	L+V+I
AHAS I	34	34	16	59	50	66	63
AHAS II	4	0	0	0	10	8	0

[a] L = Leucine.
[b] V = Valine.
[c] I = Isoleucine.

From Singh, B. K., Stidham, M. A., and Shaner, D. L., *J. Chromatogr.*, 444, 251, 1988. With permission.

saturated at about 100 m*M* pyruvate.[33] The Hanes-Woolf plot of the data gave a K_m value of 5 m*M* pyruvate for AHAS I and 8 m*M* for AHAS II (Table 5).

Feedback inhibitors — valine, leucine, and isoleucine — were used to test the sensitivity of the two forms of AHAS to these inhibitors. The degree of inhibition of the enzyme activity was compared at 1 m*M* for each amino acid. AHAS I was inhibited by all of the amino acids, singly and in combination (Table 6), and to the greatest degree (66%) by a combination of leucine and valine. In contrast, AHAS II is relatively unaffected by these amino acids (Tables 5 and 6).

The kinetics of inhibition of AHAS I and AHAS II by imazapyr differed significantly.[33] AHAS II was much more sensitive to the herbicide than was AHAS I and was almost completely inhibited (>90%) by imazapyr. In contrast, the maximum inhibition of AHAS I caused by imazapyr (100 μM) was approximately 70%. Despite these differences in the degree of inhibition, imazapyr concentrations required for 50% inhibition of the enzyme (I_{50}) were the same for both isozymes (Table 5).

The origin and relationship of the two enzyme forms are unclear at this time. Recent results suggest that the two types of AHAS observed represent different aggregation states of the same enzyme with AHAS I being the multimeric form and AHAS II the monomeric form. Additional experiments are in progress to determine the origin and relationship of different forms of AHAS in plants.

VII. PROPERTIES OF AHAS FROM IMIDAZOLINONE-TOLERANT CORN

The development of imidazolinone-tolerant corn is extensively reviewed in Chapter 10. AHAS activity from tolerant corn lines is detailed in this section.

Tissue culture selection methods were used to obtain imidazolinone tolerance in corn,[5] and a number of mutant lines were selected. Three lines, XA17, XI12, and QJ22, were regenerated into plants, and conventional breeding techniques were used to obtain the homozygous tolerant plants that were used in the study. An inbred line, B73, was used as the sensitive control. Two imidazolinones — imazaquin and imazethapyr — and one sulfonylurea — sulfometuron methyl — were used to examine the sensitivity at the whole-plant level as well as at the enzyme level.

The inbred corn line B73 was killed at low rates of each of the herbicides tested (Figure 15; only data for imazaquin presented). Similar to this whole-plant response, all of these herbicides inhibited AHAS activity from B73 (Figure 16; only data for imazaquin presented). Sulfometuron methyl was the most active herbicide as well as the most potent enzyme inhibitor.

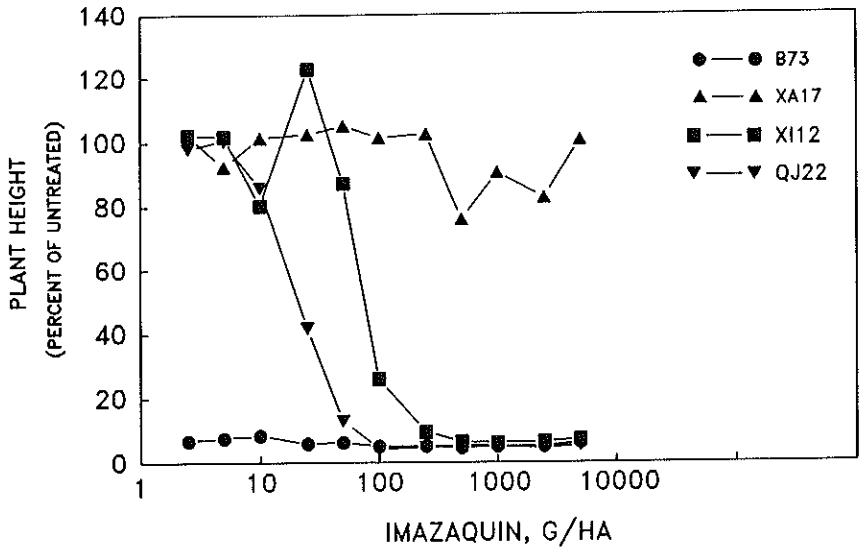


FIGURE 15. Effects of imazaquin on plant growth. (From Singh, B. K., Newhouse, K. E., Stidham, M. A., and Shaner, D. L., in *Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry*, Monogr. No. 42, Copping, L. G., Dalziel, J., and Dodge, A. D., Eds., British Crop Protection Council, Farnham, England, 1989, 87. With permission.)

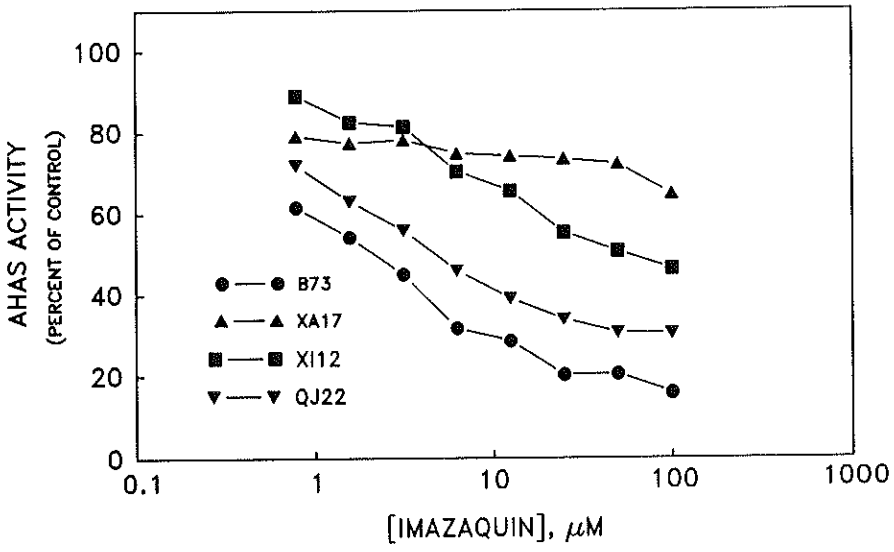


FIGURE 16. Effects of imazaquin on the AHAS activity from wild-type corn (B73) and various herbicide-tolerant mutants. (From Singh, B. K., Newhouse, K. E., Stidham, M. A., and Shaner, D. L., in *Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry*, Monogr. No. 42, Copping, L. G., Dalziel, J., and Dodge, A. D., Eds., British Crop Protection Council, Farnham, England, 1989, 87. With permission.)

Homozygous XA17 was the most tolerant line and could not be killed even at the highest rate of herbicides used (Figure 15; only data for imazaquin presented). AHAS extracted from XA17 plants was insensitive to inhibition by these herbicides (Figure 16; only data for imazaquin presented). In contrast to XA17, the XI12 and QJ22 genotypes were selectively tolerant to the imidazolinone herbicides. Compared to XA17 and XI12, QJ22 has the lowest

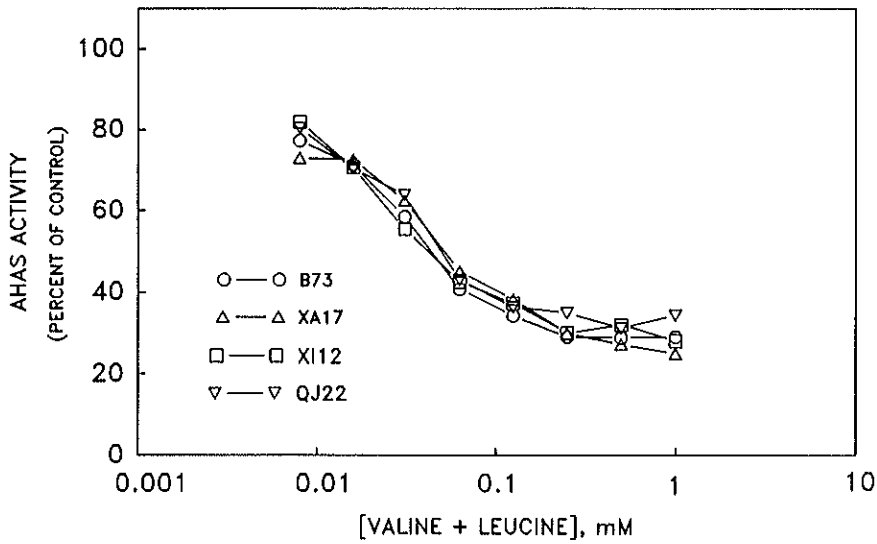


FIGURE 17. Effects of valine + leucine on the AHAS activity from the wild-type corn (B73) and various herbicide-tolerant mutants. (From Singh, B. K., Newhouse, K. E., Stidham, M. A., and Shaner, D. L., in *Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry*, Monogr. No. 42, Copping, L. G., Dalziel, J., and Dodge, A. D., Eds., British Crop Protection Council, Farnham, England, 1989, 87. With permission.)

degree of tolerance to imidazolinones (see Figures 15 and 16; only data for imazaquin presented). The herbicide tolerance of XI12 and QJ22 shown at the whole-plant level was also displayed by AHAS from these lines.

The herbicide tolerance in the three corn mutants described above differs in both the spectrum of herbicide tolerance and the level of tolerance expressed at the whole-plant level. Herbicide tolerance at the whole-plant level is explained by the presence of a form of AHAS that shows a similar degree of tolerance by the respective herbicides. Insensitivity of AHAS from XA17 to the imidazolinones and sulfonylureas and the sensitivity of AHAS from XI12 and QJ22 to sulfonylureas suggests that the binding sites of the imidazolinones and sulfonylureas are not the same, but that they may share a common binding domain. However, the possibility that AHAS extracted from XA17 contains two separate mutations, one in each herbicide-binding domain, cannot be ruled out. Characterization of these mutants at the gene level will explain the differential response of these genotypes to various herbicides.

An interesting property of AHAS from each of these genotypes is the identical response to inhibition by the combination of valine and leucine, feedback inhibitors of the enzyme (Figure 17). It has been speculated that the imidazolinones are analogs of the feedback inhibitors and therefore they bind at the feedback inhibitor bind site.[46,47] Experimental results presented here clearly demonstrate that the feedback inhibitor-binding site is separate from the herbicide-binding site.

VIII. CONCLUSIONS

A complete understanding of the mechanism of action of the imidazolinone herbicides begins at the level of AHAS. Sensitivity of a plant to the imidazolinones is a result of the sensitivity of AHAS from that plant to the imidazolinones. Although natural tolerance to these herbicides cannot be attributed to AHAS, it is clear that plants obtained through tissue culture selection methods are tolerant to imidazolinones because of the presence of an imidazolinone-insensitive form of AHAS.

Many questions remain concerning AHAS, the biosynthesis of leucine, isoleucine, and valine, and the inhibition of AHAS by the imidazolinones. These questions include the molecular interactions between the imidazolinones and AHAS, the rate of turnover of AHAS, tissue localization of AHAS, and AHAS isozymes. An increased understanding of the role of AHAS in amino acid biosynthesis will help answer the important remaining question of why plants die upon inhibition of AHAS.

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