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Acetolactate Synthase Is the Site of Action of Two Sulfonylurea Herbicides in Higher Plants

Abstract. *Biochemical and genetic studies of a tobacco mutant resistant to the herbicides chlorsulfuron and sulfometuron methyl have demonstrated that these sulfonylurea herbicides inhibit acetolactate synthase, the first enzyme specific to the branched chain amino acid biosynthetic pathway. Resistance of this mutant is accomplished by production of a form of the enzyme that is insensitive to inhibition by the two herbicides.*

Chlorsulfuron and sulfometuron methyl are the active ingredients in the two herbicides, Glean and Oust (DuPont), respectively. *Nicotiana tabacum* mutants resistant to these compounds have been isolated from cell cultures. One such mutation, *S4*, increased resistance of plants to chlorsulfuron at least 100-fold when present in the homozygous state (*1*). Biochemical characterization of mutant plants has now revealed both the site of action of these herbicides and the mechanism by which resistance is effected.

The discovery that sulfometuron methyl inhibits growth of *Salmonella typhimurium* in the presence of valine and that this inhibition is reversed specifically by isoleucine first suggested that this herbicide acts by interfering with an early step in the biosynthesis of the branched-chain amino acids (*2*). Similar patterns of reversal of growth inhibition were obtained with pea (*Pisum sativum*) and the yeast *Saccharomyces cerevisiae*. Growth inhibition by chlorsulfuron of pea seedlings and root cultures was prevented by supplementation of the medium with isoleucine and valine (*3*). Growth of yeast in the presence of sulfometuron methyl was restored by addition of isoleucine, leucine, and valine (*4*). Direct assay of the first enzyme specific to the isoleucine-leucine-valine biosynthetic pathway, acetolactate synthase (ALS) (E.C. 4.1.3.18), demonstrated the sensitivity of the ALS activities of these three organisms (*2–4*) and of tobacco (Fig. 1) to extremely low concentrations of chlorsulfuron and sulfometuron methyl.

However, demonstration of the sensitivity of an enzyme to a herbicide is necessary, but not sufficient, evidence to identify the site of action of that herbicide. A possibility that is too often overlooked in such investigations is that the herbicide inhibits other enzymes in addition to the one identified (perhaps within the same metabolic pathway) and that it is primarily the action of the herbicide on one of these other activities that determines the response of the whole organism.

A particular enzyme can be established as the primary (or sole) site of action of a herbicide by a combination of genetic and biochemical lines of evidence that include demonstration that mutants resistant to a herbicide possess an altered form of an enzyme that is insensitive to the herbicide and that the resistant form of the enzyme cosegregates with the resistance phenotype in genetic crosses. We now report the results of such experiments, which provide strong evidence that ALS is the site of action of the sulfonylurea herbicides chlorsulfuron and sulfometuron methyl in higher plants.

A plant heterozygous for the *S4* mutation was constructed by a series of backcrosses of a plant regenerated from a herbicide-resistant tobacco cell line with plants of the parental variety (*N. tabacum* cv. Xanthi). Self-fertilization of this heterozygous individual yielded homozygous mutant, heterozygous, and ho-

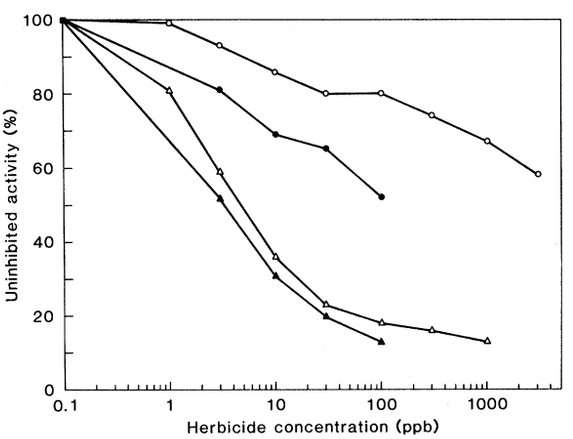

Fig. 1. Responses to chlorsulfuron and sulfometuron methyl of ALS activities in extracts of normal (H1) and homozygous mutant (Isolate 16) cell lines. Cell suspension cultures were washed in liquid medium (*6*) and extracted in a 100 m*M* potassium phosphate buffer (*p*H 7.5) containing 0.5 m*M* $MgCl_2$, 1 m*M* sodium pyruvate, 0.5 m*M* thiamine pyrophosphate, 10 μ*M* flavine-adenine dinucleotide (FAD), and 10 percent (by volume) glycerol. Polyclar AT (BDH Chemicals Ltd.) was added to a final concentration of 250 mg per gram fresh weight of tissue. After centrifugation at 27,000*g* for 15 minutes at 4°C, an equal volume of a saturated solution of ammonium sulfate was added to the supernatant fraction. The resultant precipitate (representing a 0 to 50 percent ammonium sulfate fraction) was redissolved in 100 m*M* potassium phosphate buffer (*p*H 7.5) containing 0.5 m*M* $MgCl_2$ and 1 m*M* sodium pyruvate, and salts were removed by passage through a Sephadex G-25 (Pharmacia PD-10) column. ALS activity was assayed in a total volume of 500 μl containing 100 μl of the column eluate, 65 m*M* potassium phosphate buffer (*p*H 7.5), 40 m*M* sodium pyruvate, 10 m*M* $MgCl_2$, 250 μ*M* thiamine pyrophosphate, 23 μ*M* FAD, and the indicated concentration of chlorsulfuron or sulfometuron methyl. After incubation at 30°C for 90 minutes, 250 μl of 6*N* H_2SO_4 was added to each assay tube, and the tubes were incubated at 55°C for 10 minutes to terminate the enzymatic reaction and achieve complete conversion of acetolactate to acetoin. Acetoin content was then measured by a modification of the procedure described (*14*). To each tube were added sequentially 100 μl of 50 percent NaOH, 150 μl of 0.5 percent creatine, and 150 μl of 5 percent α-naphthol (in 2.5*N* NaOH). Color development was accelerated by incubation at 55°C for 10 minutes. Tubes were centrifuged, and the optical density was read at 530 nm. Background optical densities, which were subtracted from the optical density values of the reactions, were determined for each extract from parallel assays to which the enzyme extract was added after the addition of 6*N* H_2SO_4. Protein concentrations were measured with Bio-Rad Protein Assay reagent with bovine serum albumin as a standard. Data points and specific activities (see text) are the average values of at least three independent determinations. ALS activities are presented as percent activity in the absence of herbicide. (▲) H1 activity in the presence of sulfometuron methyl, (△) H1 activity in the presence of chlorsulfuron, (●) *S4*/*S4* mutant activity in the presence of sulfometuron methyl, and (○) *S4*/*S4* mutant activity in the presence of chlorsulfuron.

Table 1. Sensitivity to chlorsulfuron of ALS activities in progeny of a *S4*/+ heterozygote. Data are presented as percent of ALS activity in the absence of chlorsulfuron. Each number represents the average of at least three independent experiments. Callus cultures initiated from the individual isolates were tested for growth on medium supplemented with chlorsulfuron at 5 ppb (14 n*M*). Callus cultures capable of growth on this medium are designated *R* (resistant). *S* denotes sensitive callus cultures that did not grow in the presence of the herbicide.

Isolate	Genotype	Callus phenotype	Uninhibited activity (percent) in the presence of chlorsulfuron		
			10 ppb	30 ppb	100 ppb
H1	+	*S*	40	23	18
1	+/+	*S*	42	29	23
7	+/+	*S*	36	22	16
9	*S4*/+	*R*	59	50	42
10	*S4*/+	*R*	61	52	45
16	*S4*/*S4*	*R*	86	80	80
23	*S4*/+	*R*	64	53	46
24	*S4*/+	*R*	60	47	41
27	*S4*/*S4*	*R*	88	82	79
30	*S4*/*S4*	*R*	89	83	81

mozygous normal progeny in the ratio of 1 to 2 to 1, which is expected for segregation of a single semidominant nuclear allele. The genotypes of these progeny plants were confirmed by analysis of a subsequent generation (*1*). Callus cultures were established (*5*) from several of the genetically defined isolates produced by self-fertilization of the *S4*/+ heterozygote. Unlike normal *N. tabacum* callus cultures and callus cultures initiated from segregants that produced only herbicide-sensitive progeny, callus cultures derived from all plants that yielded resistant progeny and, therefore, that carried the *S4* mutation were capable of growth on medium supplemented with either chlorsulfuron or sulfometuron methyl at 5 parts per billion (ppb) (14 n*M*). Cell suspension cultures were initiated from these callus cultures and from the parental haploid cell line (H1) by transferring callus to a liquid medium (*6*). ALS activity was assayed in extracts prepared from these cell suspension cultures.

The specific activities of ALS in H1 extracts and in extracts of a homozygous mutant (Isolate 16) cell line were 161 and 168 nmole of acetoin per milligram of protein per hour, respectively. ALS activity of the H1 cell line was inhibited 50 percent by chlorsulfuron at approximately 5 ppb (14 n*M*). In contrast, this degree of inhibition of ALS activity in extracts of the mutant was not achieved by chlorsulfuron at 3000 ppb (8 μ*M*), the highest concentration tested. ALS activity of the mutant cell line was also more resistant than the parental activity to inhibition by sulfometuron methyl. However, the mutant activity displayed a higher degree of resistance to the former than to the latter compound (Fig. 1). The relative resistances of ALS activity in the *S4*/*S4* extract to chlorsulfuron and sulfometuron methyl corresponded to the growth responses of this cell line to these two compounds. Growth of this same homozygous mutant cell line was inhibited 64 percent by sulfometuron methyl at 10 ppb (28 n*M*) and less than 5 percent by chlorsulfuron at 10 ppb (*1*). This correspondence between the relative responses of mutant cell cultures and ALS activity in cell-free extracts to the two herbicides provided the first indication that the altered enzyme was the basis for the resistance phenotype.

The responses to chlorsulfuron were determined for ALS activities in extracts of cell suspension cultures derived from nine individuals that differed with respect to the *S4* mutation. As described above, these individuals were produced by self-fertilization of an *S4*/+ heterozygote, and their genotypes were established by means of progeny analysis. Callus and plant phenotypes were determined by growth responses of cell cultures and seedlings, respectively, on herbicide-supplemented medium (*1*). All normal, sensitive segregants had a chlorsulfuron-sensitive form of ALS that was indistinguishable from that of the parental H1 cell line. In contrast, all individuals homozygous for the *S4* mutation contained a highly resistant form of the enzyme. The ALS activities in extracts of heterozygotes displayed an intermediate degree of resistance to chlorsulfuron (Table 1). The correlation between both callus and plant phenotype and the response of ALS activity in vitro to the herbicide is both absolute and quantitative. The maintenance through genetic crosses of an association between the responses of the plant, cell culture, and ALS activity to chlorsulfuron provides a convincing argument that this enzyme is the site of action of the herbicide in plants and that the production of an insensitive form of the enzyme is the basis for resistance in the mutant.

Although the *S4* mutation effects production of a chlorsulfuron- and sulfometuron methyl–insensitive form of ALS, this mutation cannot be assumed to define the structural gene for this enzyme. It is possible that the *S4* mutation alters a regulatory apparatus that normally prevents expression of a more resistant ALS isozyme encoded in the tobacco genome.

Tobacco mutants resistant to chlorsulfuron and sulfometuron methyl have been employed to define the site of action of these herbicides in a higher plant. These mutants are among a small but ever-increasing number of plant mutants to be isolated from cell cultures (*7*). Few such mutants, however, have been characterized biochemically, and in only two other cases has a correlation been established between an altered phenotype and an altered biochemical function (*8, 9*). Demonstration of such a correlation is especially important in assigning the basis for mutant phenotypes selected in vitro because of the enormous genetic variability accumulated by plant cells during maintenance in culture (*10*). Hence, detection of an altered enzyme in a variant cell line or in a regenerated plant does not by itself indicate that alteration of that particular activity is the basis of the new phenotype. Rather, the biochemical alteration detected and the phenotypic difference observed could result from unrelated genetic events.

Herbicides that inhibit synthesis of essential amino acids have been identified earlier: amitrole apparently interferes with histidine biosynthesis (*11*), and glyphosate inhibits the synthesis of aromatic amino acids (*12, 13*). The low application rates of chlorsulfuron and sulfometuron methyl set these sulfonylurea compounds apart from conventional herbicides. The observed biological potency of chlorsulfuron and sulfometuron methyl is consistent with the low concentrations of these compounds that we have shown to inhibit the biosynthetic enzyme ALS.

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Human Colon Cells: Culture and in Vitro Transformation

Abstract. *Normal human colon mucosal epithelial cells were cultured in vitro and treated with the oncogenic simian DNA virus (SV40) and the chemical carcinogen azoxymethane. Both SV40 and azoxymethane altered a number of phenotypic characteristics of the normal human colon cells, including their morphology, culture longevity, growth in soft agar, substrate adherence, and peanut agglutinin binding. The SV40 transformants synthesized intranuclear T antigen. These data indicate that normal human colon mucosal cells were transformed toward the malignant phenotype.*

Mechanisms of carcinogenesis are poorly defined not only because of the complexity of the process but also because of the model systems chosen for study. Approximately 80 percent of human neoplasms are carcinomas and originate from epithelial cells. However, most experimental models of neoplastic transformation in vitro are based on the use of fibroblastic cells, because these cells predominate in the outgrowths from most tissue explants and survive subculture under the usual conditions for growing cells in vitro. Consequently, the features of fibroblast transformation and growth are better defined than those of epithelial cells (*1, 2*). Thus there is a need to develop successful models for the initiation and characterization of epithelial cells transformed in vitro.

The incidence of large bowel cancer currently is one of the highest among all human neoplastic diseases in the United States. Although cultured cells have been used to study colon cancer, studies performed in vitro have been limited. Normal human colon mucosal (NCM) epithelial cells have not been used for transformation studies in vitro because only short-term cultures of such gastrointestinal cells have been achieved (*3*). However, we have developed methods that permit longer term culture of human colon and other gastrointestinal epithelial cells (*4, 5*). We now report evidence that NCM cells can be transformed in vitro with the oncogenic simian virus 40 (SV40) or with the chemical carcinogen azoxymethane (Azm). SV40 was chosen because it has been shown to transform a variety of human cells, and many aspects of SV40 genetics and biology are well defined (*6*). Azoxymethane is a direct-acting, carcinogenic derivative of dimethylhydrazine, a potent chemical carcinogen with demonstrated colon specificity in animal model systems (*7*).

The NCM cultures were initiated in vitro (*4*); the culture medium was an enriched L15:S-MEM base medium supplemented with 2 percent fetal bovine serum (MA Bioproducts) (*8*), L broth (*9*), pituitary extract (*10*), and other factors (*11*). The NCM cultures were characterized as epithelial on the basis of several criteria, including morphology, presence of keratin, little or no fibronectin, and synthesis of colon-specific mucins and carcinoembryonic antigen (*4, 12*). The cells were maintained and subcultured in suspension without the use of standard dissociating agents (*4*). At the second subculture, NCM cells were plated (2×10^6 to 3×10^6 cells per flask) into plastic flasks (25 cm^2). Four separate experiments were performed, with three experimental groups in each (controls, SV40-infected, and Azm-treated). Control cultures received 0.1 ml of phosphate-buffered saline. The SV40-treated cells received approximately five virus plaque-forming units (*13*) per cell. Azoxymethane(10 μg/ml in 0.1 ml of phosphate-buffered saline) was added to each appropriate culture for 3 weeks at weekly intervals. Each group had three or four replicate cultures. The suspension cultures were observed daily to assess differences in morphology and growth and were subcultured at weekly intervals (*4*). At various subcultures they were analyzed for altered phenotypic characteristics (Tables 1, 2, and 3).

Significant differences were noted between the viral or chemical carcinogen–treated cells and the control cells. These included changes in cell size, decreased proportions of morphologically differentiated epithelial cells in the population, and altered growth, as evidenced by enhanced adherence to the culture substrate, a reduced requirement for NCM-conditioned medium, the ability to grow in soft agar, and increased culture longevity (Tables 1 and 2). Viability after chemical dissociation and binding of peanut agglutinin lectin (PNA; Sigma) were evident only in the transformants (Table 1). In addition, only SV40-infected cells expressed SV40-specific intranuclear T

Table 1. Phenotypic characteristics displayed by NCM control and transformed cells.

Group	Phenotypic characteristics									
	Altered morphology	Adherence to culture substrate* (%)	Viability (%)		Total number of subcultures in experiment				Binding of PNA§ (%)	
			After dissociation†	Without NCM-conditioned medium‡	I	II	III	IV	III	IV
Control		5 to 10	0 to 1	0 to 5	5	3	2	4	0 to 10	0 to 10
SV40	±‖	10 to 25	5 to 20	10 to 20	8	8	5	10	60 to 75	75 to 90
Azm	±‖	10 to 20	5 to 20	10 to 20	7	6	5	8	50 to 80	80 to 90

*Under standard culture conditions, substrate uncoated. †Chemical dissociation was accomplished by centrifuging cells (800*g*), resuspending the pellet for 3 to 5 minutes at 37°C in 0.25 percent trypsin–0.02*M* EDTA, then replating cells in complete culture medium. ‡Analyzed by centrifuging cells and replating in complete culture medium without NCM-conditioned medium; viability determined by trypan blue dye exclusion of cells 24 to 48 hours after plating. §Performed by direct assay with fluorescein isothiocyanate (FITC)–labeled PNA (E-Y Laboratories); cells (5 to 8 × 10^4 per well) were rinsed with phosphate-buffered saline then incubated for 45 minutes at 4°C with a 1:30 dilution of FITC-PNA stock in phosphate-buffered saline. Not done for experiments I and II. ‖±, Similar to controls but cells commonly somewhat larger.