

## Toxic Accumulation of $\alpha$ -Ketobutyrate Caused by Inhibition of the Branched-Chain Amino Acid Biosynthetic Enzyme Acetolactate Synthase in *Salmonella typhimurium*

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**Biochemical and genetic analyses of the bacterium *Salmonella typhimurium* suggest that accumulation of  $\alpha$ -ketobutyrate partially mediates the herbicidal activity of acetolactate synthase inhibitors. Growth inhibition of wild-type bacteria by the herbicide sulfometuron methyl was prevented by supplementing the medium with isoleucine, an allosteric inhibitor of threonine deaminase-catalyzed synthesis of  $\alpha$ -ketobutyrate. In contrast, isoleucine did not rescue the growth of a mutant containing a threonine deaminase unresponsive to isoleucine. Moreover, the hypersensitivity of seven *Tn10* insertion mutants to growth inhibition by sulfometuron methyl and  $\alpha$ -ketobutyrate correlated with their inability to convert  $\alpha$ -ketobutyrate to less noxious metabolites. We propose that  $\alpha$ -ketobutyrate accumulation is an important component of sulfonylurea and imidazolinone herbicide action.**

Blockage of a metabolic pathway usually causes two primary biochemical alterations: end product depletion and accumulation of abnormally high levels of pathway intermediates. Either biochemical change may contribute to cessation of the growth of an organism. Supplementation of the culture medium with the end product of the obstructed pathway normally allows growth to resume. Since this addition usually lowers the levels of pathway intermediates by feedback inhibition of the first pathway enzyme and also satisfies the nutritional requirement, the cause of growth inhibition is often undefined.

Pathways may be blocked either by mutation or by the addition of enzyme inhibitors to cells. Alterations leading to the accumulation of  $\alpha$ -ketobutyrate (AKB) are of current interest for several reasons. This compound, a by-product of the transsulfuration pathway of L-methionine catabolism in animals, accumulates in individuals having a newly described inborn error of human metabolism (29). In plants, a major AKB-consuming enzyme, acetolactate synthase (ALS; EC 4.1.3.18), is inhibited by two new and potent herbicides (14, 15, 20) of vastly different chemical structures.

In microbes and plants, ALS catalyzes  $\alpha$ -acetolactate formation from two molecules of pyruvate and  $\alpha$ -aceto- $\alpha$ -hydroxybutyrate synthesis from pyruvate and AKB (24). These reactions are components of the biosynthetic pathways for the formation of the branched-chain amino acids and pantothenate (Fig. 1). Two ALS isozymes are present in the bacterium *Salmonella typhimurium* (11). ALS II is catalytically competent in both reactions, while ALS I utilizes AKB inefficiently (18, 21). Sulfometuron methyl {SM; N-[(4,6-dimethylpyrimidin-2-yl)-aminocarbonyl]-2-methoxycarbonylbenzenesulfonamide}, a sulfonylurea herbicide, inhibits most ALS species of microbial and plant origin (7, 12, 15, 19), including *S. typhimurium* isozyme II (15); *S. typhimurium* isozyme I, however, is SM insensitive (16). L-Valine displays a complementary inhibition spec-

trum; it interferes with the activity of ALS I without affecting that of ALS II (11).

In this report, we demonstrate that AKB accumulation is triggered by exposing *S. typhimurium* to the sulfonylurea herbicide SM. Analyses of SM-hypersensitive *S. typhimurium* insertion and point mutants indicate the AKB accumulation is toxic. It is thus likely that AKB toxicity is an important contributor to the detrimental effects of those inborn errors of human metabolism leading to AKB accumulation and to the herbicidal action of the two new agrichemicals that specifically inhibit ALS.

### MATERIALS AND METHODS

**Materials.** All compounds were of reagent grade. SM was obtained from the Agricultural Products Department of E. I. du Pont de Nemours & Co., Inc., Aquasol-2 and L-[U-<sup>14</sup>C]threonine were purchased from the New England Nuclear Division of E. I. du Pont de Nemours. Agar was obtained from Difco Laboratories. 2,4-Dinitrophenylhydrazine, cellulose (6065) sheets, and silica gel (6061) sheets were products of Eastman Kodak Co. L-Valine and L-isoleucine were obtained from Fisher Scientific Co. L-Threonine, pyruvate, AKB, and  $\alpha$ -keto- $\beta$ -methylvalerate were purchased from Sigma Chemical Co.  $\alpha$ -Acetohydroxybutyrate, produced enzymatically with *S. typhimurium* ALS II purified to homogeneity, was the gift of D. E. Van Dyk and J. V. Schloss.

**Strains.** All strains were derivatives of *S. typhimurium* LT2. The SM-sensitive insertion mutants, created by insertion of the transposon *Tn10*, were described previously (27). Strains TV087 (*ilvA*<sup>+</sup>) and TV088 (*ilvA219*), an isogenic pair differing only at the *ilvA* locus encoding the biosynthetic threonine deaminase (EC 4.2.1.16), were constructed in two steps. First, a null allele (*ilvA::Tn10*) (3) of the structural gene for threonine deaminase was introduced into strain LT2 by generalized transduction (10), with selection for tetracycline resistance and screening for isoleucine auxotrophy, by using a P22HT  $\Delta$ int-4 (10) phage stock grown on strain TT58 (*ilvA::Tn10*) (3). The resultant strain, TV086 (*ilvA::Tn10*),

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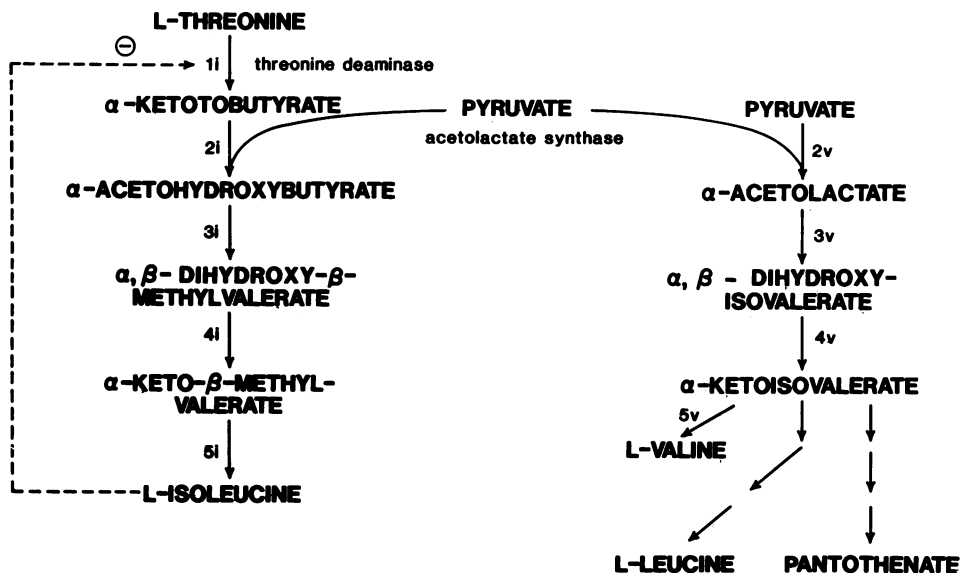


FIG. 1. Branched-chain amino acid biosynthetic pathways. Reaction 1i is catalyzed by threonine deaminase. The isozymes of ALS catalyze reactions 2v and 2i in *S. typhimurium*. L-Valine is an inhibitor of ALS I, but not of ALS II, while SM inhibits only ALS II (11, 15, 16). As indicated by the dashed line, L-isoleucine inhibits threonine deaminase-mediated formation of AKB (24).

was used as the recipient in the subsequent transductional crosses to introduce other *ilvA* alleles by selection for prototrophy. The *ilvA219* allele encoding an isoleucine-insensitive form of threonine deaminase was introduced with strain DU24 (6) as the donor. The *ilvA*<sup>+</sup> allele was introduced with wild-type strain LT2 as the donor. Transductants containing the isoleucine-insensitive *ilvA219* form of threonine deaminase were distinguished from recombinants containing an *ilvA*<sup>+</sup> isoleucine-inhibited form of this enzyme by the ability of the former to utilize L-threonine as a sole nitrogen source in the presence of L-isoleucine (6).

**Media.** The rich medium L broth and the minimal medium E supplemented with 0.33% glucose were used (10).

**SM sensitivity.** SM sensitivity was determined by monitoring growth on derivatives of medium E containing 0.33% glucose and solidified with 2% agar. One medium was unsupplemented, while the second medium contained 0.1 mM L-valine and 0.137 mM SM. Added to the third medium were 0.1 mM L-valine, 0.1 mM L-isoleucine, and 0.137 mM SM. Single-colony isolations were performed on these media. Growth, resulting from incubation at 37°C for 17 h, was recorded.

**Accumulation of isoleucine biosynthetic intermediates.** L-[U-<sup>14</sup>C]threonine was added at a specific activity of 10 mCi/mmol and a final concentration of 100  $\mu$ M to cultures (at  $2.5 \times 10^8$  to  $6 \times 10^8$  cells per ml) of *S. typhimurium* growing in minimal medium E (27). Accumulation of L-threonine-derived aldehydes and ketones in the culture was monitored as described elsewhere (27). The following inhibitors of ALS and threonine deaminase were added simultaneously: L-valine (0.85 mM), SM (100  $\mu$ M), and L-isoleucine (0.76 mM). At various times, 0.2-ml culture samples were mixed with 0.5 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl. After at least 5 min at room temperature, during which dinitrophenylhydrazones formed, the acidified culture was blended for 30 s with 0.5 ml of toluene. Phases were separated by centrifugation. Radioactivity in 0.15 ml of the toluene (upper) phase containing dinitrophenylhydrazones was determined by liquid scintillation counting with 10 ml of Aquasol-

2 as the scintillant. In many instances, the identity of the radiolabeled material in the toluene extract was analyzed by thin-layer chromatography (TLC). The chromatograms were visualized under UV light and subjected to autoradiography.

**AKB utilization.** Production of radiolabeled AKB from L-threonine was accomplished by incubating cultures (at  $3 \times 10^8$  cells per ml) for 20 min at 37°C in the presence of L-[U-<sup>14</sup>C]threonine, SM, and L-valine as specified in the preceding section. This treatment resulted in the accumulation of approximately 25  $\mu$ M AKB without a diminution in CFU. Further AKB synthesis was then prevented by supplementing the cultures with L-isoleucine (0.76 mM). AKB remaining in the culture medium was analyzed as described in the preceding paragraph. Residual AKB was expressed as the percentage of AKB remaining relative to that present at the time of isoleucine addition. The disappearance of AKB observed in the presence of valine and SM was attributed to activities other than that of ALS.

**TLC.** Dinitrophenylhydrazone derivatives of  $\alpha$ -ketoacids were prepared by mixing 1  $\mu$ mol of 2,4-dinitrophenylhydrazine with 0.5  $\mu$ mol of  $\alpha$ -ketoacid in 0.5 ml of 0.8 N HCl and incubating the mixture for 10 min at room temperature. The resulting products were extracted with an equal volume of toluene. Samples (10  $\mu$ l) of the toluene phases containing dinitrophenylhydrazone derivatives were spotted on TLC plates. Three chromatographic systems were used: system I, silica gel plates developed with dioxane; system II, cellulose plates developed with tertiary amyl alcohol-ethanol-water (5:1:4); and system III, cellulose plates developed with *n*-butanol-ethanol-water (4:1:5) (30).

**Threonine deaminase.** Threonine deaminase was measured by a modification of the method of Uzan and Danchin (26). Cultures of strains TV087 and TV088 were grown at 37°C in medium E supplemented with 0.4% glucose to a density of approximately  $8 \times 10^8$  cells per ml. After being collected by centrifugation at 4°C, chilled cells were suspended in 20 ml of cold (0°C) medium E. The cells were pelleted before being frozen in an ethanol-dry-ice bath. The frozen material was stored at -20°C. The cells (approximately 0.8 g) were

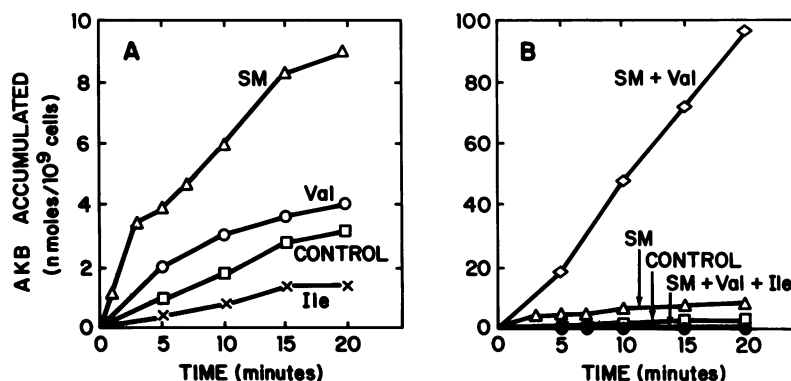


FIG. 2. Accumulation of isoleucine biosynthetic intermediates in *S. typhimurium*. To strain LT2 (wild type; at a density of  $2.9 \times 10^8$  cells per ml) with threonine were added 100  $\mu$ M SM ( $\Delta$ ); 0.85 mM valine ( $\circ$ ); 0.76 mM isoleucine ( $\times$ ); 100  $\mu$ M SM and 0.85 mM valine ( $\diamond$ ); 100  $\mu$ M SM, 0.85 mM valine, and 0.76 mM isoleucine ( $\bullet$ ); or nothing ( $\square$ ). Note that the scale of the y axis differs in panels A and B. TLC demonstrated that the material accumulating upon treatment with SM and valine was AKB.

thawed in 4 ml of 50 mM potassium phosphate (pH 7.2)–0.2 mM dithiothreitol. Sonic extracts were prepared and clarified by centrifugation as described elsewhere (16).

The protein content of the resulting supernatant was determined by the method of Bradford (5) with reagents supplied by Bio-Rad Laboratories.

The 1-ml reaction mixtures used to measure threonine deaminase activity contained 100 mM Tris hydrochloride (pH 8), 0.1 mM pyridoxal phosphate, 20 mM  $\text{NH}_4\text{Cl}$ , 0.5 mM dithiothreitol, 10 mM L-[ $^{14}\text{C}$ (U)]threonine at a specific activity of 50  $\mu\text{Ci}/\text{mmol}$ , L-isoleucine varying from 0 to 44.5 mM, and the clarified sonic extract containing between 400 and 800  $\mu\text{g}$  of protein. The components of the reaction mixture were preincubated for 5 min at 37°C before initiation by the addition of L-threonine. Reactions were terminated after 15 min at 37°C by the addition of 0.5 ml of 0.1% dinitrophenylhydrazine in 2 N HCl. The acidified solution was added to 2 ml of toluene before it was mixed on a vortex mixer for 30 s. The organic phase containing dinitrophenylhydrazones and the aqueous phase containing L-threonine were separated by centrifugation. Formation of AKB was calculated after the radioactivity present in the toluene phase was determined by liquid scintillation counting. One unit of threonine deaminase activity was the amount of enzyme needed to catalyze the formation of 1 nmol of AKB in 1 min.

## RESULTS

**AKB accumulation.** Growth of wild-type *S. typhimurium* occurred in the presence of SM. At least one ketone accumulated in the culture in response to SM (Fig. 2A). Growth inhibition was observed upon supplementation of the culture with both L-valine and SM. Under the latter condition, both ALS isozymes were inhibited. In this case, the ketone accumulation rate increased approximately 10-fold over that observed in cells treated only with SM (Fig. 2B).

The L-threonine-derived accumulating ketone was suspected to be AKB, as judged by the established modes of SM (15) and L-valine (11) action. The three known routes by which L-threonine is metabolized in aerobically grown enteric bacteria are initiated by the *ilvA*-encoded threonine deaminase, thronyl-tRNA synthetase (EC 6.1.1.3), and L-threonine 3-dehydrogenase (EC 1.1.1.103) (17, 24, 25). The dinitrophenylhydrazine-toluene extraction assay could detect metabolic intermediates formed in both the threonine deaminase- and L-threonine dehydrogenase-containing path-

ways. These two enzymes differ in their responses to L-isoleucine. Threonine deaminase is end product inhibited by this compound (24), while the threonine dehydrogenase activity of *E. coli* is refractile to this amino acid in vitro (17). Supplementation of the SM- and L-valine-containing labeling medium with L-isoleucine suppressed ketone accumulation by at least 50-fold (Fig. 2B), suggesting that the accumulating ketone was a biosynthetic intermediate in the pathway from L-threonine to L-isoleucine.

The radiolabeled dinitrophenylhydrazone formed by reaction of the culture with 2,4-dinitrophenylhydrazine was analyzed by TLC. More than 99% of the radioactivity in this sample comigrated with the dinitrophenylhydrazone of commercial AKB in three different systems (Fig. 3, lane A of each panel). L-Isoleucine prevents growth inhibition mediated by the combination of SM and L-valine (15). As well as satisfying the nutritional requirement imposed by inhibition of both ALS isozymes, L-isoleucine prevented AKB accumulation, presumably by restriction of threonine deaminase activity (Fig. 2A and B) (24). L-Isoleucine thus corrected two

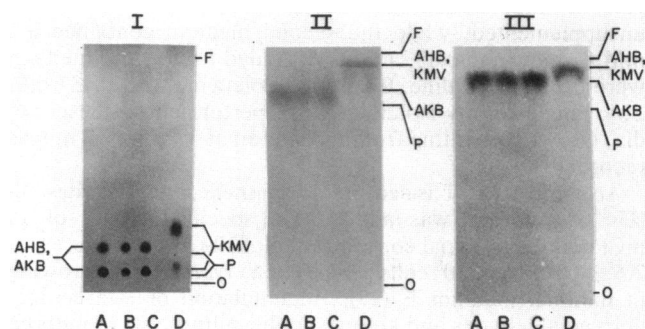


FIG. 3. TLC analyses of dinitrophenylhydrazone derivatives. In each panel is an autoradiogram of a TLC plate showing the results with system I, II, or III, as described in Materials and Methods. The positions of the standards, dinitrophenylhydrazones of pyruvate (P), AKB,  $\alpha$ -aceto- $\alpha$ -hydroxybutyrate (AHB), or  $\alpha$ -keto- $\beta$ -methylvalerate (KMV), are indicated. O and F indicate the origins and fronts, respectively. Cultures of strains LT2 (wild type), TV087 (*ilvA*<sup>+</sup>), and TV088 (*ilvA219*) treated with radioactive L-threonine for 20 min in the presence of SM and L-valine were the sources of samples applied to lanes A, B, and C, respectively. A culture of strain TV088, labeled for 20 min in the absence of the ALS inhibitors, was used to prepare the sample applied to lanes D.

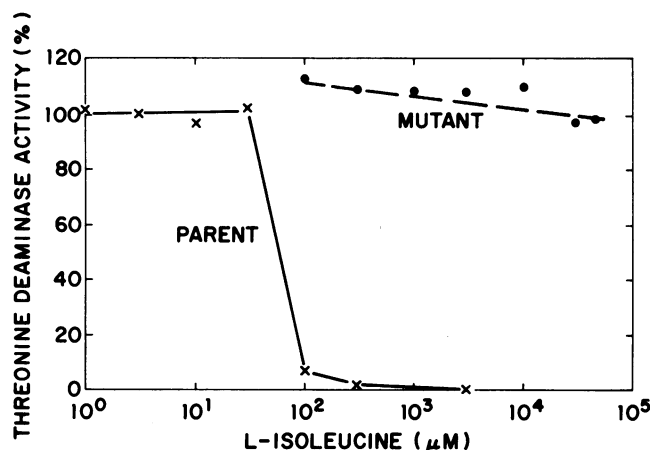


FIG. 4. Inhibition of threonine deaminase activities by L-isoleucine in vitro. Clarified sonic extracts were assayed in reaction mixtures containing the indicated amounts of L-isoleucine. The extract from parent strain TV087 (*ilvA*<sup>+</sup>) had, in the absence of L-isoleucine, a specific activity of 13.1 U/mg of protein, while that of mutant strain TV088 (*ilvA219*) had a specific activity of 11.6 U/mg of protein. The activities measured in the presence of L-isoleucine were normalized to these values.

abnormal conditions, i.e., AKB overabundance and amino acid starvation.

**Unimpeded AKB synthesis.** Wild-type threonine deaminase, the product of the *ilvA* gene, is end product inhibited by L-isoleucine (24). The activity extracted from the *ilvA219*-harboring strain, TV088, was catalytically active and refractile to end product inhibition by 40 mM L-isoleucine (Fig. 4). In contrast, the threonine deaminase activity present in extracts of the isogenic *ilvA*<sup>+</sup> strain TV087 was severely restricted by 100  $\mu$ M L-isoleucine.

The kinetics of ketone accumulation were examined in this isogenic pair (Fig. 5). The *ilvA219* mutant in minimal medium consumed a great deal of L-threonine and transformed the radiolabeled amino acid into compounds which reacted with 2,4-dinitrophenylhydrazine (Fig. 5B, control curve). TLC analyses of this toluene-extractable radiolabeled material were performed (Fig. 3). The predominant species comigrated with the  $\alpha$ -keto- $\beta$ -methylvalerate derivative in each separation system (Fig. 3, lane D of each panel). The utilization of L-threonine by strain TV087, the *ilvA*<sup>+</sup> strain, was much smaller (Fig. 5A, control curve). The combined

administration of SM and L-valine increased the accumulation of pathway intermediates by both strains (Fig. 5). Under this condition, the radioactive dinitrophenylhydrazone extracted from each strain cochromatographed with the AKB derivative (Fig. 3, lanes B and C of each panel). Moreover, the SM- and L-valine-mediated accumulation of radiolabeled ketones, prevented by L-isoleucine in *ilvA*<sup>+</sup> strains (Fig. 2 and 5A), was not curtailed by this compound in the *ilvA219* strain (Fig. 5B).

Despite the regulatory defect displayed by the *ilvA219* mutant, the growth rates of strains TV087 and TV088 were similar in minimal and rich liquid media (data not shown). Nonetheless, the capacity of threonine deaminase to be end product inhibited by L-isoleucine appeared to be important for growth under conditions of diminished ALS function. Both strain TV087 (*ilvA*<sup>+</sup>) and strain TV088 (*ilvA219*) were inhibited by media containing SM and L-valine (Fig. 6, central petri dish). More importantly, inhibition of growth of the feedback-insensitive mutant by SM and L-valine was not prevented by inclusion of L-isoleucine in the medium (Fig. 6, righthand sector of righthand petri dish). Similarly, L-isoleucine did not prevent SM inhibition of the feedback-insensitive mutant in the absence of L-valine (data not shown). Thus, AKB accumulation, rather than L-isoleucine starvation, correlated with the SM sensitivity of the feedback-insensitive mutant.

**Defects in AKB turnover.** The kinetics of AKB metabolism have been studied by pulse-chase experiments with radiolabeled L-threonine as a tracer. The chase was initiated by adding L-isoleucine to a culture of wild-type strain LT2 preincubated for 20 min at 37°C with L-[U-<sup>14</sup>C]threonine in the presence of the two ALS inhibitors SM and L-valine. The residual AKB concentration was monitored at various times after L-isoleucine supplementation. AKB consumption proceeded in the presence of the two ALS inhibitors (Fig. 7).

If AKB accumulation contributed to SM toxicity, then SM-sensitive mutants might be defective in AKB consumption. AKB turnover of 15 SM-sensitive mutants (27) was therefore examined. These Tn10 insertion mutants of *S. typhimurium*, containing an active ALS I, are inhibited by SM in the absence of L-valine (27). As in the wild-type strain, the collection of SM-sensitive insertion mutants accumulated AKB upon blocking both ALS isozymes. Representative results of the turnover experiments are shown in Fig. 7. Eight of the SM-sensitive insertion mutants, exemplified by *aspC* strain SMS409 (27), utilized AKB, with kinetics indistinguishable from those of wild-type strain. Seven others

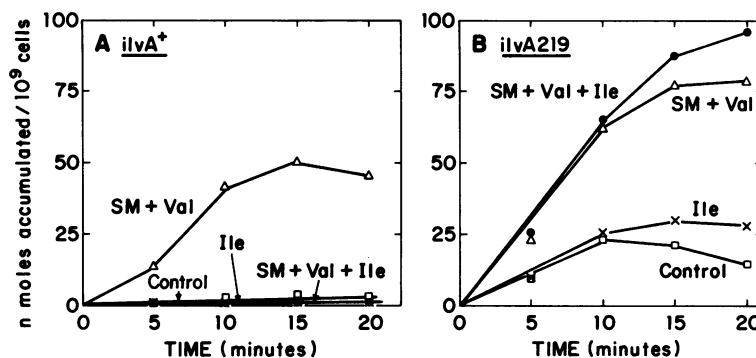


FIG. 5. Accumulation of isoleucine biosynthetic intermediates by strains harboring either a wild-type (*ilvA*<sup>+</sup>, strain TV087) (A) or a feedback-insensitive (*ilvA219*, strain TV088) (B) form of threonine deaminase. Simultaneously added to each culture (at a density of  $6 \times 10^8$  cells per ml) with L-threonine were 100  $\mu$ M SM and 0.85 mM L-valine ( $\Delta$ ); 0.76 mM L-isoleucine ( $\times$ ); 100  $\mu$ M SM, 0.85 mM valine, and 0.76 mM isoleucine ( $\bullet$ ); or nothing ( $\square$ ).

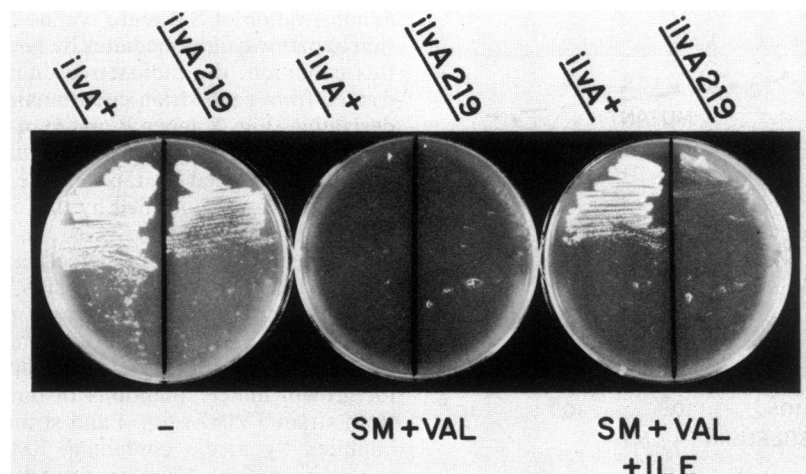


FIG. 6. Enhanced SM sensitivity of a feedback-insensitive threonine deaminase (*ilvA219*) mutant. Isogenic strains TV087 (*ilvA*<sup>+</sup>) and TV088 (*ilvA219*) were streaked on the left and right sectors, respectively, of three plates. These plates contained minimal glucose medium with no addition (left); 0.1 mM L-valine and 0.137 mM SM (center); and 0.1 mM L-valine, 0.137 mM SM, and 0.1 mM L-isoleucine (right).

(represented in Fig. 7 by strains SMS401, SMS408, and SMS429) consumed AKB at greatly reduced rates in comparison with that of the wild-type strain. Strains SMS103 and SMS429, the two mutants most sensitive to SM in the collection (Table 1), displayed an almost complete inability to utilize AKB in pathways unrelated to branched-chain amino acid biosynthesis. Five other strains had intermediate AKB turnover phenotypes. Table 1 summarizes the properties of these insertion mutants. Thus, herbicide sensitivity can be correlated with deficiencies in AKB utilization by enzymes other than ALS.

### DISCUSSION

Potential targets for rational herbicide design include the 49 enzymes involved in producing the 10 amino acids required in the mammalian diet (14). Such inhibitors are not expected to display toxicity towards animals because the primary targets of the inhibitors are absent. In fact, four discrete classes of herbicides, identified by empirical screening, interfere with essential amino acid synthesis (14). Surprisingly, two distinct groups, sulfonyleurea and imidazolinone compounds, inhibit a single enzymic target, the branched-chain amino acid biosynthetic enzyme ALS (14). This fact suggests that the 49 enzymes of essential

amino acid biosynthesis may not be equivalent targets. Metabolic consequences of the biosynthetic blockades, such as energy drain or intermediate accumulation, may confer greater utility upon inhibitors of a particular enzyme.

Of the 15 *Tn10* insertion mutants of *S. typhimurium* that we studied which were hypersensitive to SM, 7 displayed defects in catabolism of AKB, a normal cellular constituent. These seven mutations represent at least four distinct genetic loci (T. K. Van Dyk and R. A. LaRossa, unpublished data). Since the preponderance of AKB catabolic defects in the set of SM-sensitive mutants is not caused by a genetic hot spot for *Tn10* insertion, other explanations are necessary. We have suggested that elevated intracellular levels of AKB, triggered by SM inhibition of ALS II, are toxic (14, 27). Two findings presented in this report strongly support this proposal. First, in the presence of branched-chain amino acids, SM inhibited growth of a mutant harboring an isoleucine-insensitive form of threonine deaminase. In contrast, growth of an isogenic *ilvA*<sup>+</sup> strain was not impeded by this environment. Furthermore, one-half of the SM-

TABLE 1. Properties of herbicide-sensitive insertion mutants

Strain	MIC of <sup>a</sup> :		AKB degradation rate <sup>b</sup> (nmol/min per 10 <sup>9</sup> cells)
	SM ( $\mu$ M)	AKB (mM)	
Wild type (LT2)	>2,800	2.5	2.6
SMS001	1,400	5.0	3.0
SMS424	1,400	0.4	3.6
SMS404	820	10.0	2.8
SMS307	410	10.0	2.9
SMS301	410	10.0	2.7
SMS421	550	0.4	2.8
SMS211	410	1.0	2.6
SMS409	210	0.4	2.2
SMS419	210	1.0	0.95
SMS408	210	1.0	0.84
SMS406	210	1.0	1.4
SMS209	210	1.0	1.3
SMS401	140	0.4	0.87
SMS103	100	0.4	0.27
SMS429	60	0.4	0.13

<sup>a</sup> Data from reference 27.

<sup>b</sup> Data compiled from experiments similar to that for which the results are depicted in Fig. 7.

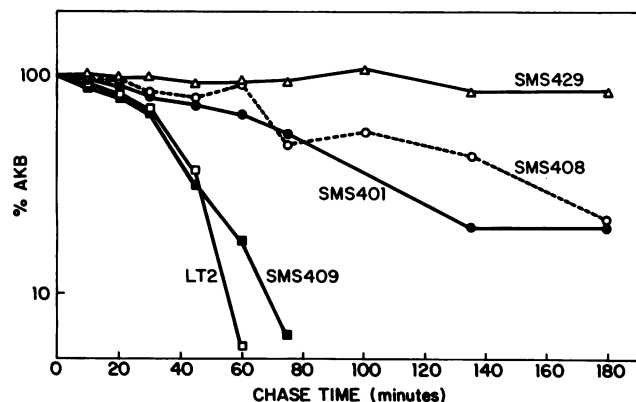


FIG. 7. AKB utilization by enzymes other than ALS in herbicide-sensitive mutants of *S. typhimurium*. LT2 was the wild-type strain.

hypersensitive insertion mutants were defective in alternative pathways of AKB metabolism (Fig. 7; Table 1).

Toxicity associated with the accumulation of metabolic intermediates is not uncommon, having been observed in several anabolic and catabolic pathways (4, 13, 23). Not all intermediates of a single pathway are toxic at high concentrations. For example, the accumulation of the pyrimidine intermediate carbamyl aspartate is deleterious to *S. typhimurium*, although that of its precursor, carbamyl phosphate, is not (23). Similarly, the clinical manifestations of human inborn errors affecting steps 1 and 4 in the phenylalanine catabolic pathway are markedly different. Phenylketonuria, a genetic disease in which the initial, phenylalanine oxidase-catalyzed step is obstructed, results in the severe mental retardation of afflicted infants. In contrast, the symptoms of alcaptonuria, the inborn error in which the homogenistic acid oxidase-catalyzed step 4 is blocked, are benign (2).

In most instances, the biochemical events inhibited by accumulated intermediates are undefined. Proposals for AKB targets range from stimulation of isoleucine catabolism (18) to inhibition of valine (21) and aspartate (9, 27) synthesis, sugar transport (8), pantothenate production (18), aminoacylation (22) of tRNA<sup>Leu</sup> and tRNA<sup>Val</sup>, and acetyl coenzyme A formation (8, 18). Inhibition of AKB condensation with coenzyme A is the cause of a newly identified inborn error of human metabolism (29). Furthermore, AKB is metabolized to propionyl coenzyme A (9), whose accumulation is associated with another severe inborn error (28). Thus, AKB accumulation may elicit a highly pleiotropic response.

The modes by which various herbicides inhibit cellular growth also suggest the importance of AKB accumulation. Four chemically distinct inhibitors of essential amino acid biosynthesis are useful herbicides (14). Two interfere with ALS, an enzyme of branched-chain amino acid biosynthesis (14). Obviously, the branched-chain amino acid pools eventually diminish after addition of these two herbicides to plants (1). Each herbicide will also rapidly increase the intracellular AKB concentration causing metabolic imbalances. We propose that these AKB-mediated imbalances contribute to the potency of herbicides interacting with ALS. The toxicity of AKB towards plants, therefore, merits study.

We further suggest that rational design of drugs and agrichemicals may require not only the synthesis of potent enzyme inhibitors, but also the judicious choice of enzymic target based on knowledge of toxicity associated with accumulation of the substrate for the target. Normally, end product inhibition precludes accumulation of such potentially toxic intermediates in auxotrophic mutants. Nonetheless, metabolic poisons may be discovered by the introduction of a feedback-resistant mutation into a series of auxotrophs blocked in subsequent pathway steps. A systematic application of this approach may reveal the most potent metabolic poisons.

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