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# Metabolic Effects of Inhibitors of Two Enzymes of the Branched-Chain Amino Acid Pathway in *Salmonella typhimurium*

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**The metabolic effects of inhibitors of two enzymes in the pathway for biosynthesis of branched-chain amino acids were examined in *Salmonella typhimurium* mutant strain TV105, expressing a single isozyme of acetohydroxy acid synthase (AHAS), AHAS isozyme II. One inhibitor was the sulfonylurea herbicide sulfometuron methyl (SMM), which inhibits this isozyme and AHAS of other organisms, and the other was *N*-isopropyl oxalylhydroxamate (IpOHA), which inhibits ketol-acid reductoisomerase (KARI). The effects of the inhibitors on growth, levels of several enzymes of the pathway, and levels of intermediates of the pathway were measured. The intracellular concentration of the AHAS substrate 2-ketobutyrate increased on addition of SMM, but a lack of correlation between increased ketobutyrate and growth inhibition suggests that the former is not the immediate cause of the latter. The levels of the keto acid precursor of valine, but not of the precursor of isoleucine, were drastically decreased by SMM, and valine, but not isoleucine, partially overcame SMM inhibition. This apparent stronger effect of SMM on the flux into the valine arm, as opposed to the isoleucine arm, of the branched-chain amino acid pathway is explained by the kinetics of the AHAS reaction, as well as by the different roles of pyruvate, ketobutyrate, and the valine precursor in metabolism. The organization of the pathway thus potentiates the inhibitory effect of SMM. IpOHA has strong initial effects at lower concentrations than does SMM and leads to increases both in the acetohydroxy acid substrates of KARI and, surprisingly, in ketobutyrate. Valine completely protected strain TV105 from IpOHA at the MIC. A number of explanations for this effect can be ruled out, so that some unknown arrangement of the enzymes involved must be suggested. IpOHA led to initial cessation of growth, with partial recovery after a time whose duration increased with the inhibitor concentration. The recovery is apparently due to induction of new KARI synthesis, as well as disappearance of IpOHA from the medium.**

Enzymes that participate in biosynthetic pathways of essential amino acids have been recognized as targets for a number of safe and effective herbicides (18). The biosynthetic pathway to the branched-chain amino acids valine, leucine, and isoleucine is of special importance in this respect. At least three classes of very potent and extensively used herbicides, the sulfonylureas, the imidazolinones, and the sulfonanilides (26, 30, 39), are known to inhibit the first common enzyme in this pathway (Fig. 1), acetohydroxy acid synthase (AHAS; EC 4.1.3.18). The second common enzyme in the pathway (Fig. 1), ketol-acid reductoisomerase (KARI; EC 1.1.1.86), is the target for two additional inhibitors with potential herbicidal activity, 2-dimethylphosphinoyl-2-hydroxyacetate (42) and *N*-isopropyl oxalylhydroxamate (IpOHA) (3).

Sulfometuron methyl (SMM), which belongs to the sulfonylurea class of herbicides and inhibits AHAS activity, is an extremely potent herbicide and bacteriostatic agent (23). The potency of SMM has been ascribed in part to the toxicity of one of the substrates of AHAS, 2-ketobutyrate, which accumulates upon inhibition of the enzyme. It has been proposed that high 2-ketobutyrate levels interfere with a number of metabolic pathways, including synthesis of coenzyme A, by several mechanisms (6–8, 24, 25, 44, 46).

IpOHA also causes the accumulation in bacterial growth media of a substrate of the enzyme which it inhibits, acetolactate (41). However, there are no reports in the literature on the intracellular concentrations of the KARI substrate acetolactate or acetohydroxybutyrate. In the enterobacteria, transcription of *ilvC*, the gene which encodes KARI, is induced by the enzyme's substrates (38). Thus, an increase in substrate concentrations due to partial inhibition of the enzyme should lead to a compensating enhancement of the rate of the reaction by increasing enzyme synthesis (as well as by mass action, if KARI normally works well below its $K_m$). It is also not clear whether IpOHA has any effect on the intracellular levels of ketobutyrate (the toxic metabolite).

In the present study, we examined the effects of the inhibitors SMM and IpOHA at the level of intact cells to learn how inhibition of two different target enzymes in the same pathway affects cellular metabolism and growth. As a model system, we chose *Salmonella typhimurium* TV105, which expresses only SMM-sensitive AHAS isozyme II. This isozyme is similar in substrate specificity to the enzyme found in plants and is the isozyme responsible for most of the acetohydroxy acid production in *S. typhimurium* growing with glucose as the carbon source. The effects of SMM and IpOHA on growth, enzyme activities, and levels of intermediates in the branched-chain amino acid pathway were analyzed in this system.

## MATERIALS AND METHODS

**Materials.** The materials used included the following: amino acids; 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride; antibiotics; creatine; thiamine $PP_i$; flavin adenine dinucleotide; dithiothreitol; reduced forms of the sodium salts of β-NADH and β-NADPH; methyl acetoxyacetoacetate; isopropyl-β-D-thiogalactopyranoside (IPTG); *o*-nitrophenyl-β-D-galactoside; and the sodium salts of pyruvic acid, 2-ketobutyric acid, 2-ketovaleric acid, 2-ketoisovaleric acid, 2-ketoisocaproic acid, D,L,-2-keto-3-methylvaleric acid, and phosphoenolpyruvate (Sigma Chem-

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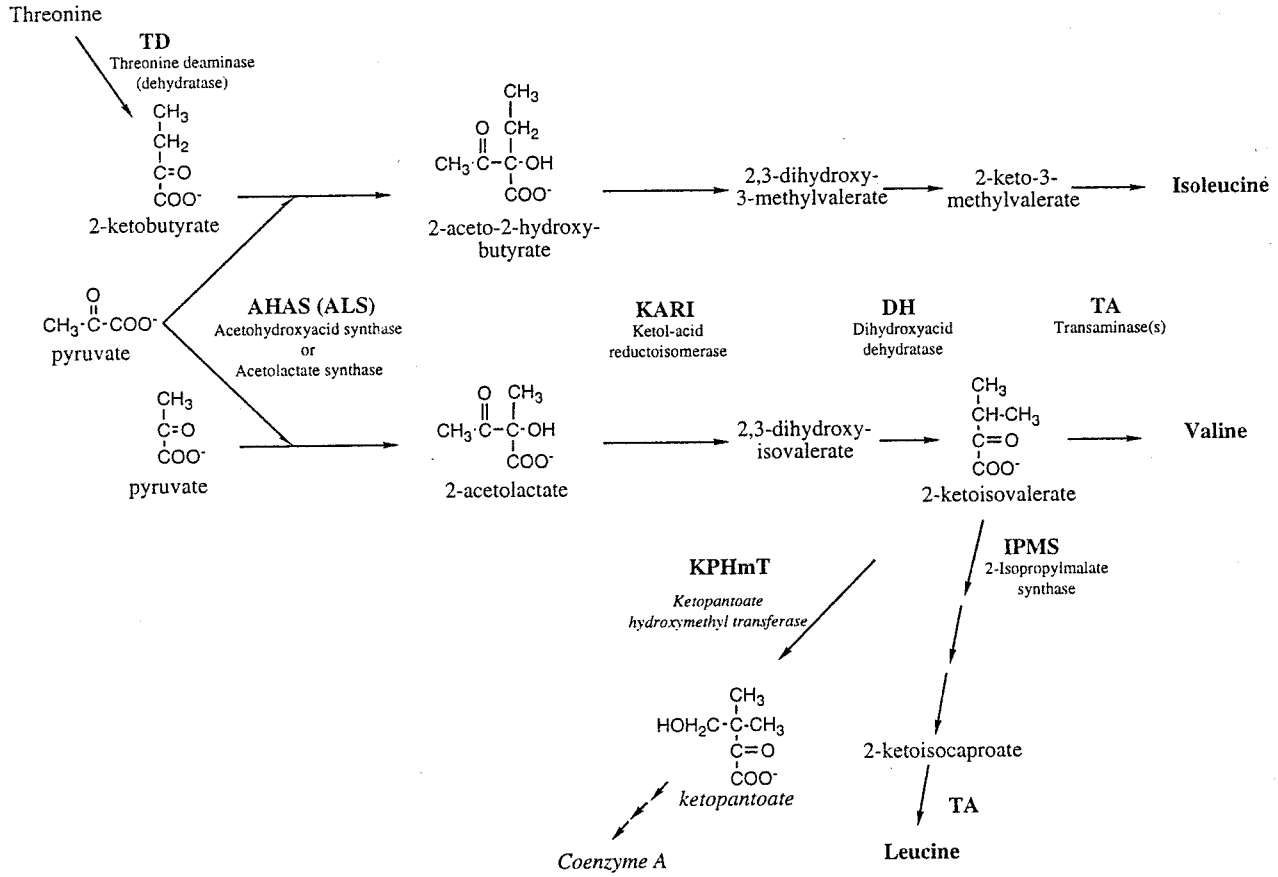



FIG. 1. Pathway for the biosynthesis of branched-chain amino acids.

ical Co., St. Louis, Mo.). Calcium pantothenate was purchased from Calbiochem, La Jolla, Calif. Methanol and acetonitrile (high-pressure liquid chromatography [HPLC] grade) were purchased from Bio-Rad Laboratories. 2,3-Pentanedione and 2,3-butanedione were purchased from Aldrich Chemical Co., Milwaukee, Wis.

Sulfometuron methyl was a gift of E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. Solutions of SMM in dimethyl sulfoxide were prepared freshly for every experiment. Control experiments showed no effect of dimethyl sulfoxide on AHAS activity at the volumes used in our experiments.

Racemic acetolactate was prepared chemically (21) from 1 eq of methyl acetoxyacetoacetate mixed with 2 eq of NaOH and incubated at room temperature for 2 h. Analysis of acetolactate content was performed as described below for determination of AHAS activity. Aliquots of the product were also analyzed for the presence of acetoin by the Westerfeld assay (49) without addition of $H_2SO_4$. The acetolactate solutions were found to contain about 5% acetoin.

IpOHA, a specific KARI inhibitor, was prepared essentially as described by Aulabaugh and Schloss (3).

All of the other chemicals used were of analytical grade.

**Bacterial strains.** The bacterial strain used in most of this work was the *S. typhimurium* LT2 derivative F′lac/TV105 (F′*pro-lac zzf1836*::Tn*10* Cm$^r$ *ilvBN*::Mud*J*), which expresses only SMM-sensitive AHAS isozyme II. The KARI$^-$ *S. typhimurium* LT2 strain TV191 (*ilvC19 zid-64*::Tn*10*) was also used in one experiment. These strains were obtained from R. A. LaRossa (Central R&D Department, E. I. du Pont de Nemours & Co., Inc.). The F′lac episome, encoding IPTG-inducible β-galactosidase, made it possible to monitor the polypeptide chain growth rate (see below).

**Growth of bacteria.** The growth media used were LB rich medium (29) and morpholinepropanesulfonic acid (MOPS) minimal medium (32). Glucose was added to the minimal media to a final concentration of 0.4%. Bacterial cultures were grown with shaking (200 rpm) in a gyratory water bath at 37°C. Growth was monitored with a Klett-Summerson colorimeter equipped with a no. 66 filter. For analysis of enzyme activities, keto acids, and acetohydroxy acids in cells, 250 ml of prewarmed medium in a 1-liter Erlenmeyer flask was inoculated with a 1% bacterial culture from a starter culture in the exponential phase of growth and grown to 40 Klett units (KU). To study the effects of supplemental nutrients on inhibition, cells were allowed to double twice in minimal medium before introduction of SMM or IpOHA (1 μM). After inhibition was confirmed (15 min), supplements were added at the following concentrations: Ile, Val, Leu, Asp, and Met, 0.38 mM; pantothenate, 0.34 mM; acetolactate, 6 mM; 2-ketoisovalerate, 0.4 mM.

**Determination of enzyme activities.** Bacterial cultures (250 ml) in the exponential growth phase (40 KU; about $4 \times 10^8$ cells ml$^{-1}$) were centrifuged (5,000 × *g* for 10 min at 0 to 4°C). Cells were washed twice and resuspended in the relevant sonication buffer at pH 7.6 to allow determination of enzyme activities under nearly physiological conditions. Buffers were otherwise as described for each enzyme. The washed cell suspensions were sonicated at 0°C with an XL2015 ultrasonic liquid processor (Heat Systems Inc., New York, N.Y.) by using the flat-tip probe at a power of 4 (output, 120 kW at 20 kHz) for 1 min (30 s of sonication, 1 min of cooling, 30 s of sonication). The broken cells were centrifuged for 20 min at 10,000 × *g* at 0 to 4°C, and the resulting supernatant was used for analysis of enzyme activities and protein content.

Threonine deaminase activity was determined essentially as described by Burns (5). The reaction mixture contained, in a total volume of 1 ml, 100 μmol of Tris-HCl (pH 7.6), 100 μmol of $NH_4Cl$, 80 μmol of threonine, and 50 to 200 μl of bacterial extracts. The reaction was initiated by addition of the extract, terminated after 20 min at 37°C, and analyzed as previously described (5).

AHAS activity was determined as previously described (10) in a reaction mixture containing 100 mM phosphate buffer (pH 7.6), 0.1 mM thiamine $PP_i$, 40 mM pyruvate, 10 mM $MgCl_2$, 0.025 mM flavin adenine dinucleotide, and bacterial extract.

KARI activity was determined in a 1-ml reaction mixture containing 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6), 10 mM $MgCl_2$, 2.5 mM chemically synthesized racemic acetolactate, 0.2 mM NADPH, and bacterial extract. The reaction was monitored at 37°C by recording the disappearance of NADPH $A_{340}$ (2).

The protein content in all extracts was determined by the procedure of Bradford (4). Bovine serum albumin served as the standard.

**Identification and determination of intracellular keto acids.** Extracts for analysis of keto acid content were prepared (generally, in duplicate) as previously described (34), with some modifications. Exponentially growing bacteria ($10^{11}$ cells) were filtered quickly (<1 min) through a polycarbonate filter (132-mm diameter, 0.2-μm pore size). To stop metabolism, the filtered cells were imme-

diately frozen in liquid nitrogen. The frozen filter was disrupted mechanically with forceps, and 4 ml of $HClO_4$ (0.3 M) containing 1 mM EDTA was added. The tube was vortexed for 1 min and then centrifuged at 10,000 × *g* for 15 min. The supernatant was kept at −70°C until further analysis. Ketovalerate (25 μl of a 4 mM solution) was usually added to one extract as an internal standard after addition of perchloric acid.

Determination of intracellular keto acids was performed essentially as described by Wang et al. (48), with some modifications. Bacterial extracts (50 to 200 μl) were derivatized with 1,2-diamino-4,5-methylenedioxybenzene. The derivatization mixture (total volume, 0.5 ml) contained 2.5 mM 1,2-diamino-4,5-methylenedioxybenzene, 0.18 M $HClO_4$, and ketovalerate as an internal standard (20 pmol per injection volume) when not added to cell extracts. The tubes were capped and incubated for 50 min at 100°C. The samples were cooled on ice, diluted fivefold in the starting elution buffer, and passed through a 0.2-μm-pore-size Prep-Disc membrane filter (Bio-Rad) before injection for HPLC.

HPLC was done with a TSK gel ODS-80TM column (250 by 4.6 mm [inside diameter]; particle size, 5 μm; Tosoh, Tokyo, Japan) with a Waters M-45 pump equipped with a Rheodyne 7125 syringe-loading sample injector valve (50-μl loop), a Jasco 821-FP dual monochromator fluorescence detector (Japan Spectroscopic, Tokyo, Japan), and a Waters workstation (Waters-Millipore, Milford, Mass.). Separation of the keto acid derivatives was performed at ambient temperature by using a linear gradient of 40 mM phosphate buffer (pH 7.0)–double-distilled water–acetonitrile (12:8:5, vol/vol/vol) as the starting elution buffer and 40 mM phosphate buffer–methanol–acetonitrile (12:8:5, vol/vol/vol) as the final elution buffer over 100 min, followed by 20 min more with the final eluant. The keto acids were eluted at a flow rate of 0.8 ml/min, and the eluate was monitored at an excitation wavelength of 365 nm and an emission wavelength of 445 nm.

We have found that phosphoenolpyruvate, a major cellular metabolite under some conditions, is labile and detected as pyruvate in the assay described above. Analysis of a phosphoenolpyruvate standard after 1,2-diamino-4,5-methylenedioxybenzene derivatization showed that the phosphoenolpyruvate gave a molar response equivalent to 0.7 mol of pyruvate. Therefore, we determined pyruvate independently by using the spectrophotometric lactate dehydrogenase assay (20). Bacterial extracts were prepared as described above. The resulting supernatant was neutralized with $K_2CO_3$ to pH 7.0 to 7.2. After separation of the resulting precipitate from the neutralized supernatant, 0.5 to 1.5 ml of this solution was added to the lactate dehydrogenase reaction mixture (20), enzyme was added to start the reaction, and the decrease in $A_{340}$ was monitored.

**Analysis of acetohydroxy acid contents in cells and medium.** To measure the intracellular levels of acetohydroxy acids, we had to find a way to stop metabolic activity and extract these compounds without causing their destruction; the acetohydroxy acids are converted to acetoin at low pH. We were able to extract the acetohydroxy acids from cells by using a buffer at pH 4.0. Cultures were grown and treated as described above, except that cells were broken by addition of 2 ml of sodium citrate buffer (1 M, pH 4.0).

Analysis of acetohydroxy acids was performed as described by Gollop et al. (11, 12). Samples consisted of 1.5 ml of bacterial extract, obtained as described above, or 5 ml of medium, separated from cells by filtration, to which 1.5 ml of citrate buffer (1 M, pH 4.0) was added. The reaction volume was brought to 10 ml with distilled water, and $FeCl_3$ and $FeSO_4$ were added to a final concentration of 0.15 mM each. Separation and identification of acetolactate and acetohydroxybutyrate were performed by gas chromatography after their conversion to their respective diketones, butanedione and pentanedione. Commercially available butanedione and pentanedione served as standards. Addition of acetolactate to duplicate extracts led to 95% recovery.

**Calculation of intracellular concentrations.** Intracellular metabolite concentrations were calculated from the cellular contents measured by assuming that $10^{11}$ cells contain a cytoplasmic volume (excluding the periplasmic volume) of 0.06 ml (14, 31). Measurements with a Coulter Counter and no. 66 filter verified that 1 KU is equal to $10^7$ cells $ml^{-1}$. Enzyme activities are reported in the tables as specific activities; they can also be calculated in units of flux (millimolar per minute) by dividing measured activities by the internal cell volume represented by the sample measured.

**Determination of average cell size.** Samples (0.5 ml) of bacterial cultures were fixed in formaldehyde (0.25% final concentration), and aliquots (10 to 30 μl) were mixed with 5 ml of an electrolyte solution (Isoton; Coulter Euro Diagnostics GmbH). Average cell size and cell numbers were measured in a Coulter Counter (model ZM).

**Polypeptide chain growth rate.** The polypeptide chain growth rate was determined as described by Palmer et al. (33) by monitoring the appearance of β-galactosidase activity after induction (37). IPTG (1 mM) was added to 10-ml cultures grown at 37°C to 40 KU. Samples (0.5 ml) were taken before and after addition of IPTG at the time intervals indicated in Results and added to 0.5 ml of cold sodium phosphate buffer (0.1 M, pH 7.0) containing 600 μg of chloramphenicol per ml. The mixture was vortexed and kept on ice. The samples were analyzed for β-galactosidase activity as previously described (28).

## RESULTS

**Growth inhibition.** As shown in Fig. 2, both SMM and IpOHA caused immediate inhibition of growth of strain TV105. However, the patterns of inhibition by the two inhibitors were different. Inhibition by SMM led to decreased growth rates with apparently constant doubling times. IpOHA led to initial cessation of growth, with partial recovery of growth after lag periods whose length increased with increasing IpOHA concentrations. The MIC of SMM that led to complete inhibition of growth was about 2 μM, whereas that of IpOHA was about 1 μM in this experiment.

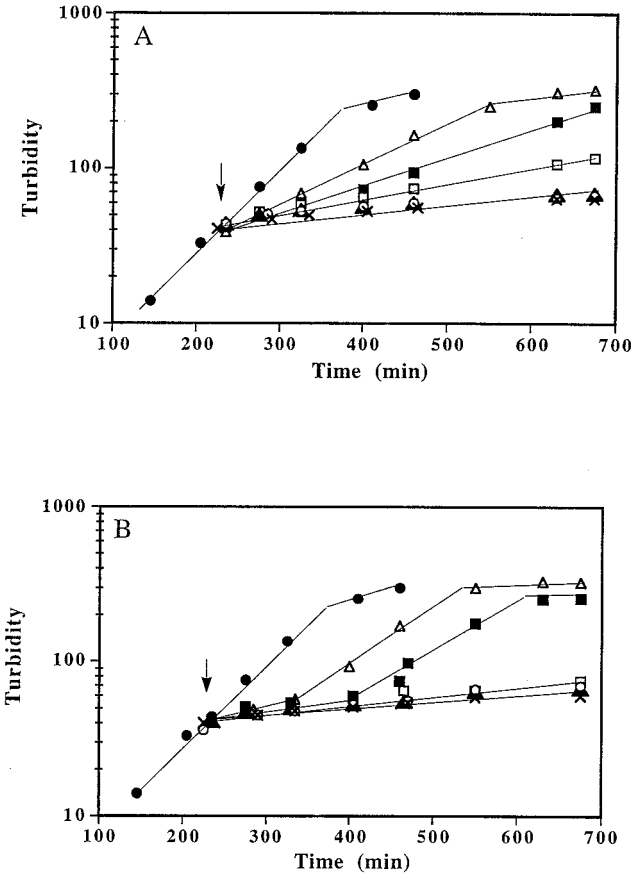



FIG. 2. Growth behavior of strain TV105 in the presence of the inhibitor SMM (A) or IpOHA (B). Strain TV105 was grown in minimal glucose medium. SMM or IpOHA was added at the indicated time (arrow). The inhibitor concentrations were 0 (●), 0.2 (△), 0.5 (■), 1 (□), 2 (△), 5 (○), and 10 (×) μM. Growth was monitored with a Klett-Summerson colorimeter equipped with a no. 66 filter.

We examined the ability of supplements added to the medium to overcome the growth inhibition caused by either SMM or IpOHA at a 1 μM concentration. Bacterial cells were allowed to double twice before introduction of the inhibitors, and supplements were added only after inhibition of growth had been confirmed. The supplements included end products and intermediates of the branched-chain amino acid pathway (Val, Ile, Leu, ketoisovalerate, and acetolactate), as well as compounds whose synthesis has been suggested to be affected by high ketobutyrate levels (pantothenate, Met, and Asp) (8, 25, 46). Inhibition of growth by SMM was partially overcome (doubling time, 2.6 h as opposed to 1.3 h in the absence of inhibitors) by addition of valine. Complete recovery from growth inhibition required the addition of valine and isoleucine together, as expected. Ile, Met, or pantothenate alone had no effect. Interestingly, in the case of cells inhibited by IpOHA, inhibition was overcome completely by addition of valine or its

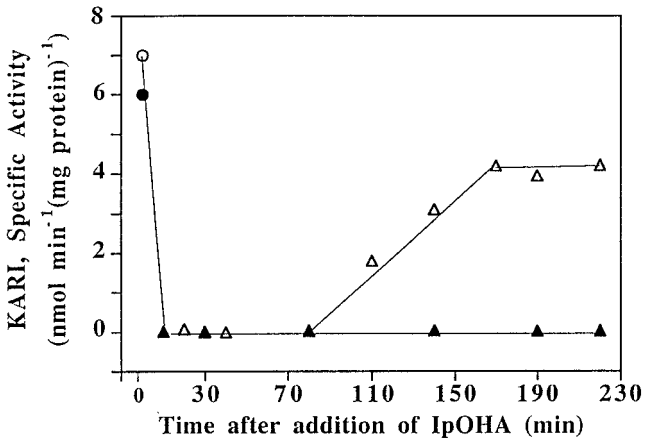



FIG. 3. KARI activity extracted from IpOHA-inhibited cells. *S. typhimurium* TV105 was grown in minimal glucose medium. At time zero, which corresponded to a cell density of $4 \times 10^8$/ml, IpOHA was added to the different cultures (△, 0.2 μM; ▲, 100 μM). Each culture (250 ml) was harvested at the time indicated, and KARI activity was extracted and determined as described in Materials and Methods. Control cultures (○, ●) were harvested without addition of inhibitors.

TABLE 1. Effect of IpOHA on acetohydroxy acid levels in strain TV105[a]

| Time (min) after addition of 1 μM IpOHA | Intracellular acetohydroxy acid level (mM) | | Acetohydroxy acid level in medium (nmol/ml of filtrate)[b] | |
|---|---|---|---|---|
| | Acetolactate | AHB[c] | Acetolactate | AHB |
| Control | 0.39 | 0.05 | ND | ND |
| 10.0 | 3.81 | 0.28 | 9.8 | ND |
| 20.0 | 4.29 | 0.43 | 10.9 | ND |
| 40.0 | 3.79 | 0.28 | 10.9 | ND |
| 80.0 | 4.17 | 0.30 | 41.7 | ND |
| 120.0 | 5.72 | 0.30 | 50.9 | ND |
| 150.0 | 4.71 | 0.32 | 59.0 | ND |

[a] Cells were grown to 40 KU, and 1 μM IpOHA was added to each flask except the control. Separation of cells from medium and preparation of extracts for determination of intracellular acetohydroxy acid levels were performed as described in Materials and Methods. Filtrate samples were also taken and analyzed for acetohydroxy acid content. Analysis of acetohydroxy acid contents in cells and filtrate was performed as described in Materials and Methods. In duplicate experiments, the variation in the measured concentrations was about 10 to 15%.
[b] ND, not detected (≤9 nmol/ml for acetolactate; ≤3 nmol/ml for AHB).
[c] AHB, acetohydroxybutyrate.

precursor ketoisovalerate alone, whereas acetolactate partially relieved growth inhibition (doubling time, 2.2 h). No combination of compounds which did not include valine or ketoisovalerate relieved inhibition by IpOHA.

**KARI activity and growth recovery in IpOHA-inhibited cultures.** Given the low rate of release of IpOHA from KARI (half-time for release, 6 days [9, 41]), the level of inhibition of KARI achieved in vivo after addition of IpOHA to cells in culture was readily determined. The extractable KARI activity declined almost to zero within the first 10 min after application of IpOHA (Fig. 3). At a high IpOHA concentration, this inhibition persisted, while upon addition of 0.2 μM IpOHA, the extractable activity recovered. Recovery from growth inhibition in the presence of the low IpOHA concentration (Fig. 2B) paralleled the gradual increase in enzyme activity. However, the maximal recovery of enzyme activity observed was only approximately 65% of the original activity (Fig. 3).

The pattern of inhibition seen with IpOHA is suggestive of inactivation of the inhibitor by the bacteria. In accord with this, the inhibition period on introduction of cells into a medium containing IpOHA appeared to decrease with increased inoculum concentrations (data not shown). To examine the effect of growing cells with IpOHA in the medium, strain TV105 was grown as described in the legend to Fig. 2 and 0.2 μM IpOHA was added. The cells recovered partially from inhibition (Fig. 2), and after the turbidity doubled from 20 to 40 KU, the culture was centrifuged to separate cells and medium. A fresh starter inoculum was added to this conditioned medium, and the cells grew without a lag with a doubling time of 1.3 h (data not shown), as they did in fresh medium without IpOHA. The cells which had partially recovered from IpOHA inhibition grew with a normal doubling time in fresh medium but were inhibited when transferred to medium containing fresh IpOHA, although they showed a shorter lag time until subsequent recovery (2 h, compared with 6 h for control cells; data not shown). IpOHA itself is stable in the medium for several hours in the absence of bacteria, and cells added to medium containing fresh or aged IpOHA were inhibited as expected.

**Substrate accumulation in the presence of IpOHA.** The levels of the KARI substrates acetolactate and acetohydroxybutyrate were determined before and at various times after addition of IpOHA (at the MIC). As can be seen from Table 1, after addition of 1 μM IpOHA, intracellular acetolactate and acetohydroxybutyrate levels increased approximately 10- and 6-fold, respectively, so that the acetolactate/acetohydroxybutyrate ratio increased in inhibited cells almost twofold compared with that in control cells. Similar changes were observed after 15 min of incubation with 0.2 μM IpOHA (data not shown). In the medium, we detected only acetolactate, probably because its intracellular concentrations were much higher than those of acetohydroxybutyrate (Table 1).

**AHAS activity extracted from SMM-inhibited cultures.** AHAS activity was extracted from cultures of strain TV105 growing on glucose for various times after addition of 1 or 10 μM SMM. As SMM inhibition is a reversible process with isolated AHAS isozyme II (41) and the contents of the harvested cells were diluted 2,000 to 3,000-fold during extraction of enzyme activities, the enzyme activity extracted from inhibited cells reflected not the actual situation in vivo but rather the total amount of the enzyme. At 1 μM SMM, a threefold increase in activity after 3 h compared with the level found in the control was observed (data not shown). This suggests that new AHAS was synthesized because of derepression of *ilvGM*. Despite this specific enhancement, the flow through the newly synthesized AHAS isozyme II must have been severely inhibited, since growth was still retarded at this concentration of SMM (Fig. 2A).

Addition of SMM at 10 μM, a concentration well above the MIC, led to a four- to fivefold decrease in the AHAS activity extracted from the cultures over 3 h (data not shown). This decrease might be due to irreversible formation of a tight complex or irreversible breakdown of the inactivated enzyme.

**Intracellular keto acid levels after brief incubation with SMM or IpOHA.** We measured intracellular keto acid levels in strain TV105 before and after inhibition with each of the inhibitors at concentrations well above their MICs (Fig. 4 and Table 2). After addition of 10 μM SMM, ketobutyrate levels were still low, although growth was inhibited completely (Fig. 2A). Only at higher SMM concentrations were ketobutyrate levels elevated. Thus, the ketobutyrate concentration per se does not seem to be the cause of the persistent growth inhibition. However, it has been suggested (25, 47) that one of the mechanisms of ketobutyrate toxicity is competition with ke-

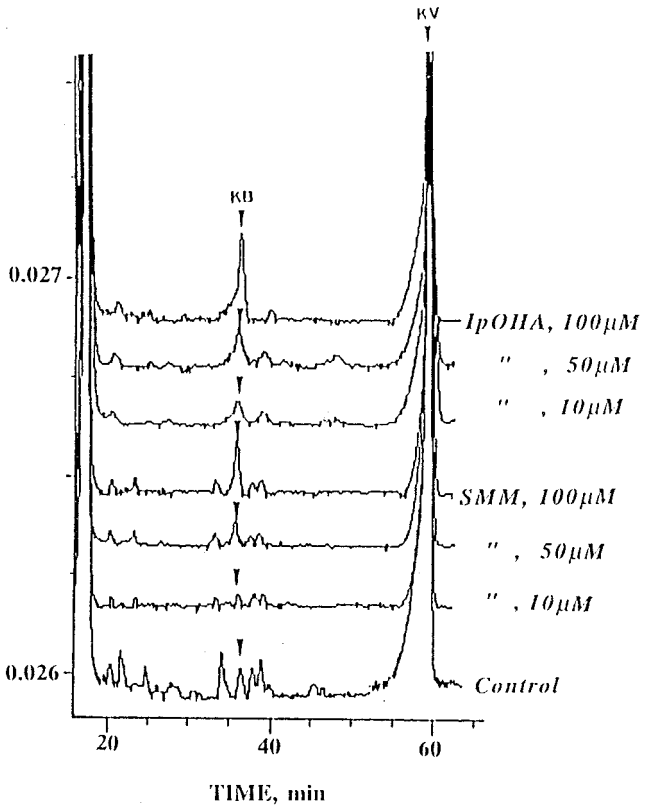



FIG. 4. 2-Ketobutyrate levels after addition of different concentrations of SMM and IpOHA. At 40 KU, SMM or IpOHA was added. After 20 min, cultures were filtered, keto acids were extracted and derivatized, and the fluorescent derivatives were separated by HPLC as described in Materials and Methods. The lowest trace shows the chromatogram of 200 μl of 4 ml of extracts from cells grown without additions, and the upper traces show chromatograms of 100 μl of 4 ml of extracts from cells grown with the indicated inhibitor at the concentration shown. Only the first portion of the trace is shown. On the ordinate is the detector voltage in volts.

toisovalerate for the first enzyme in the coenzyme A biosynthesis pathway. Indeed, ketoisovalerate levels decreased after addition of the inhibitors, thereby decreasing the ketoisovalerate/ketobutyrate ratio, even in the absence of high ketobutyrate levels. The intracellular level of the keto acid precursor of leucine, ketoisocaproate, also was decreased after addition of SMM, while that of the isoleucine precursor ketomethylvalerate was, if anything, increased. The effect of SMM on keto acid levels when cells were grown to a steady state with the inhibitor (Table 2) are discussed below.

Surprisingly, addition of IpOHA also caused ketobutyrate levels to rise; ketobutyrate levels exceeded control levels by twofold at 1 μM IpOHA and by eightfold at 100 μM IpOHA (Fig. 4 and Table 2). One should keep in mind that IpOHA is presumably specific for the reaction catalyzed by KARI (3, 40). We further confirmed by in vitro enzyme assay that IpOHA does not inhibit any AHAS catalytic activity expressed in strain TV105 (data not shown).

**Inhibition and recovery of the polypeptide chain elongation rate.** The effect of SMM on the availability of amino acids was tested by using the protein chain elongation assay. In this assay, the rate of polypeptide chain elongation ($cgr_p$) is determined by measurement of the appearance of β-galactosidase activity above the basal level at brief intervals after addition of IPTG to induce expression from the F′lac episome (33). Results of a typical experiment are shown in Fig. 5. At 20 min after exposure to 0.5 μM SMM, the $cgr_p$ was decreased more than twofold, from 14 to 7 amino acids $s^{-1}$ (induction lags of 74 and 150 s, respectively). This suggests starvation for charged tRNAs. After 2 h, the $cgr_p$ had recovered to 14 amino acids $s^{-1}$, while the slope of the β-galactosidase production rate remained low, as would be expected if the number of active ribosomes in each cell were decreased. This is also consistent with a lower rate of total protein synthesis and limited production of branched-chain amino acids. Note that the growth rate of strain F′lac/TV105 decreased by about fourfold in the presence of 0.5 μM SMM (Fig. 2A). A decrease in $cgr_p$, followed by a decrease in the number of active ribosomes and recovery of the $cgr_p$, has been observed on metabolic shift down in *Escherichia coli* (16).

TABLE 2. Effects of inhibitors on keto acid concentrations in strain TV105[a]

| Inhibitor | Concn (μM) | Intracellular concn (μM)[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Pyr + PEP | Pyr | KB | KIVA | KICAP | KMV | KIVA/KB |
| None | | 1,188 | 952 | 13 | 31 | 5.1 | 15 | 2.4 |
| SMM[c] | 0.2 | 2,184 | 2,229 | 71 | ND | ND | 13 | ≤0.02 |
| SMM | 1 | NM | | 11 | 4.8 | NM | NM | 0.4 |
| SMM | 10 | 593 | | 12 | ND | ND | 23 | ≤0.08 |
| SMM | 50 | 526 | | 27 | ND | ND | 18 | ≤0.04 |
| SMM | 100 | 683 | | 77 | ND | ND | 46 | ≤0.01 |
| IpOHA | 1 | 1,266 | | 28 | ND | ND | NM | ≤0.035 |
| IpOHA | 10 | 784 | | 34 | ND | ND | 18 | ≤0.03 |
| IpOHA | 50 | 806 | | 74 | ND | ND | 17 | ≤0.01 |
| IpOHA | 100 | 784 | | 103 | ND | ND | 35 | ≤0.009 |

[a] Unless otherwise stated, a 250-ml volume of cells was grown to 40 KU, either SMM or IpOHA was added at the indicated concentrations, and keto acids were extracted after 20 min and determined as described in Materials and Methods.

[b] Pyruvate (Pyr) + phosphoenolpyruvate (PEP) was determined by the HPLC method, and pyruvate was determined by the lactate dehydrogenase assay (see Materials and Methods). Intracellular keto acid concentrations were calculated by assuming that $10^{11}$ cells contain 0.06 ml of intracellular water (see Materials and Methods). Duplicate experiments gave measured values within 10 to 15% for (Pyr + PEP) and within 25% for the other acids. ND, not detected (<1 μM). NM, not measured. KB, 2-ketobutyrate; KIVA, 2-ketoisovalerate; KICAP, 2-ketoisocaproate; KMV, 2-keto-3-methylvalerate.

[c] Cells were grown for at least 10 generations in the presence of 0.2 μM SMM, and keto acids were extracted at 40 KU and determined as described in Materials and Methods.

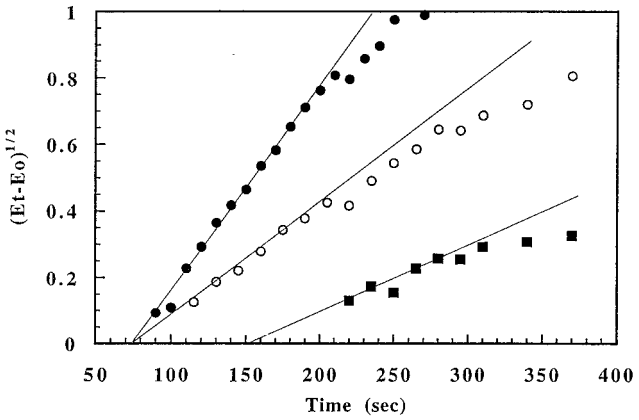



FIG. 5. Induction of polypeptide synthesis in cultures inhibited with SMM. Cultures were induced with IPTG (1 mM) before (●) or 20 min (■) or 2 h (○) after addition of SMM (0.5 μM). Samples (0.5 ml) were taken at the given times after induction and analyzed for β-galactosidase activity as described in Materials and Methods. The square root of the activity ($A_{420}$, $E_t$, corrected for basal activity, $E_0$) is plotted against time. The intercept on the abscissa is the lag for completion of the first polypeptide chains (33).

**Cell size during inhibition by SMM.** The average cell size of cultures before and after addition of SMM (0.5 μM) was measured in a Coulter Counter. In a typical experiment (Fig. 6), the average cell size decreased by some 15% over the first hour after addition of SMM and then returned almost to normal within an additional 2 h. This time course appears to be parallel to the changes we measured in the polypeptide chain growth rate under the same conditions. The phenomenon of a decrease in average cell size and recovery has been observed for metabolic shift down in *E. coli* (51).

**Keto acid and enzyme levels at steady-state growth with SMM.** The above observations on the effect of SMM on the $cgr_p$ (Fig. 5) and on cell size (Fig. 6) indicated that despite the apparent immediate switch to a new constant and lower growth rate, cell cultures do not grow at a steady state for quite some time after SMM inhibition. We were able to perform steady-state experiments with a low concentration of SMM, such that doubling times were only slightly longer than in controls. In the presence of 0.2 μM SMM, strain TV105 adapted slowly after several dilutions, to a constant doubling time of 90 min (compared with 80 min for the parallel control), and maintained this generation time for 10 doublings. Similar experiments could not be performed with IpOHA, since growth inhibition at low concentrations of the inhibitor was overcome (Fig. 2).

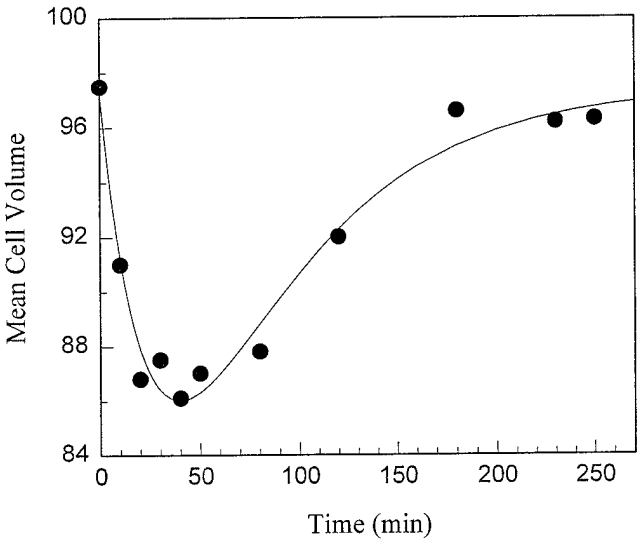



FIG. 6. Change in relative cell volume of strain TV105 after addition of 0.5 μM SMM. Cultures (25 ml) were grown in minimal glucose medium. When steady-state growth was achieved, SMM was added to $2 \times 10^8$ cells per ml and relative cell volume was measured in a Coulter Counter as described in Materials and Methods.

TABLE 3. Enzyme specific activities of strain TV105 under steady-state growth conditions[a]

| Addition(s) | Sp act (nmol min$^{-1}$ mg$^{-1}$) | | |
|---|---|---|---|
| | TD[b] | AHAS isozyme II | KARI |
| None | 528 | 30.4 | 11.0 |
| Ile, Val | 560 | 31.0 | 10.1 |
| Ile, Val, Leu | 35 | 3.7 | 4.5 |
| SMM | 2,693 | 60.7 | 8.5 |

[a] Strain TV105 was grown on glucose minimal medium for at least seven generations without additions; with isoleucine and valine at 0.38 mM each; with isoleucine, valine, and leucine at 0.38 mM each; or with 0.2 μM SMM. Enzyme activities were determined in extracts of strain TV105 as described in Materials and Methods.

[b] TD, threonine deaminase.

In the steady-state cultures partially inhibited by SMM, changes in several of the keto acid levels were observed (Table 2). Ketobutyrate and pyruvate levels increased 5- and 2.5-fold, respectively, compared with the control level. Ketoisovalerate and ketoisocaproate levels decreased below the level of detection, while the 2-keto-3-methylvalerate concentration remained almost unchanged. In addition, accumulation of a new, unidentified keto acid eluting later than ketomethylvalerate (data not shown) was observed. Further experiments are required to determine its structure and the possible relationship between its appearance and SMM inhibition.

Enzyme activities were extracted from cells grown under the same conditions (Table 3). Threonine deaminase and AHAS isozyme II activities were elevated because of derepression of the *ilvGMEDA* operon. On the other hand, KARI activity was slightly lower than in the control, implying that the levels of acetolactate and acetohydroxybutyrate were lower under these conditions. Flux through the AHAS was thus slightly limited, despite the very high levels of measured enzyme activities.

## DISCUSSION

The aim of this study was to gain insight into the metabolic effects of the AHAS inhibitor SMM (23) and the KARI inhibitor IpOHA (3, 40) by determining the changes in intracellular enzyme and metabolite levels in *S. typhimurium* during inhibition. Inhibition of growth by these inhibitors could, in principle, be caused by starvation for the vital end products of the branched-chain amino acid pathway, by accumulation of intermediates which have toxic effects, or by a combination of these effects.

**Effect of SMM.** The particular efficacy of AHAS inhibitors has been ascribed to the buildup of ketobutyrate to toxic levels (23–25). Pioneering experiments used labeled threonine to study the effect of SMM on the buildup and fate of ketobutyrate in *S. typhimurium* (25). Later experiments with plant cells showed that the AHAS inhibitor imazaquin significantly raised intracellular ketobutyrate concentrations, but it was

concluded that this increase was not the cause of the herbicidal effect, since similar levels could be induced by external 2-aminobutyrate without consequent growth inhibition (43). We observed the immediate intracellular accumulation of ketobutyrate upon inhibition with SMM in the alga *Chlorella emersonii* (22). However, once again, intracellular ketobutyrate was not clearly correlated with growth inhibition, as its concentration subsequently declined almost to control levels even though growth inhibition persisted.

The present study also does not demonstrate a good correlation between the effects of SMM on growth rates and on measured ketobutyrate levels in *S. typhimurium*. The elevation of the intracellular ketobutyrate concentration above control levels in the first 20 min of incubation with SMM, a period in which growth inhibition is clear, was observed only at concentrations above 10 μM, well above the MIC of SMM (Table 2). The rate of protein synthesis, as measured by polypeptide chain elongation rates (Fig. 5), was also decreased significantly under conditions in which no elevation of cellular ketobutyrate was observed. The intracellular ketobutyrate level by itself, therefore, does not seem to be the proximate cause of growth inhibition by SMM.

When SMM was added to a culture of strain TV105, the growth of the culture immediately slowed to a new, apparently constant, exponential rate (Fig. 2A). However, examination of the effects of SMM on protein synthesis (Fig. 5) and cell volume (Fig. 6) showed that cells were not at a physiological steady state for about 2 h after SMM addition. Analysis of the effects of SMM after longer incubation with bacterial cultures was thus desirable. Experiments were carried out with a low concentration of SMM (0.2 μM), so that doubling times were only slightly longer than in controls (1.5 h) and extracts could be analyzed at steady state (10 doubling times after SMM addition). Ketobutyrate levels were 5-fold higher and ketoisovalerate levels were at least 30-fold lower in the SMM-inhibited culture than in the control culture (Table 2).

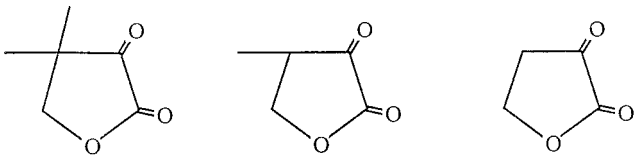
In both the short-term and steady-state inhibition experiments, we found that SMM led to a marked decrease in the intracellular levels of the keto acid precursors of valine and leucine (ketoisovalerate and ketoisocaproate), while the levels of ketomethylvalerate, the precursor of isoleucine, were almost unaffected. These results are in accord with the observation that valine alone was able to partially overcome inhibition by SMM, whereas isoleucine alone could not. We believe that this inequality in the effects of SMM on the valine and isoleucine arms of the branched-chain amino acid pathway can be understood if we consider the organization of the pathway and the intracellular concentrations of its substrates.

The rates of the two possible reactions catalyzed by a single AHAS isozyme, leading to the formation of acetolactate ($v_{AL}$) and acetohydroxybutyrate ($v_{AHB}$), are given by equations 1 and 2 (13), where $K_m$ is the Michaelis constant for pyruvate and $R$ is the specificity constant for the competition between ketobutyrate and pyruvate.

$$v_{AL} = \frac{V_{max}\cdot[Pyr]}{K_m + [Pyr]} \cdot \frac{[Pyr]}{[KB]R + [Pyr]} \quad (1)$$

$$v_{AHB} = \frac{V_{max}\cdot[Pyr]}{K_m + [Pyr]} \cdot \frac{[KB]R}{[KB]R + [Pyr]} \quad (2)$$

Studies of purified AHAS isozyme II (data not shown) show that these equations hold at physiological concentrations, as they do for AHAS isozyme III (13). From the concentrations of ketobutyrate and pyruvate in strain TV105 under uninhibited conditions (Table 2) and the $R$ value of 65 for AHAS isozyme II (13), one can calculate that $v_{AL}$ would be nearly equal to $v_{AHB}$. (The fluxes calculated on the basis of the measured $V_{max}$ would each be 1.6 mM $min^{-1}$, in approximate agreement with the minimal required fluxes of 3.6 and 1.0 mM $min^{-1}$ to [valine + leucine] and isoleucine, respectively, calculated from the amino acid contents of the bacteria and their doubling time.) Inhibition by SMM decreases $V_{max}$ and thus decreases the flux of ketobutyrate and pyruvate to acetohydroxybutyrate and that of pyruvate to acetolactate. Since pyruvate is a major cellular metabolite, present at a concentration of nearly 1 mM, and the AHAS reactions are only a minor fraction of the total metabolic flux through pyruvate (15), this inhibition should not lead directly to significant changes in the pyruvate concentration. On the other hand, the concentration of ketobutyrate is low and its transformation to acetohydroxybutyrate represents the major route for its metabolism (25), so that the decrease in $v_{AHB}$ should lead to a much more significant relative increase in the intracellular ketobutyrate concentration. An increase in ketobutyrate relative to pyruvate can partially compensate for the effect on $v_{AHB}$ of inhibition of AHAS by SMM (equation 2), but it will have the opposite effect on $v_{AL}$ (equation 1). If there were no other metabolic changes, inhibition of AHAS isozyme II in strain TV105 by up to about 50% could lead to a new, higher steady-state ketobutyrate concentration and a much more serious decrease in the flux in the valine-leucine arm of the pathway than in the isoleucine arm. A greater extent of inhibition of AHAS isozyme II would be expected to lead to a runaway increase in intracellular ketobutyrate. There are, of course, further effects: the rate of the threonine deaminase reaction is under feedback control by the level of isoleucine in the cell, so that inhibition of AHAS is expected to increase the rate of synthesis of ketobutyrate, further decreasing $v_{AL}$. The organization of the pathway thus amplifies the effect of AHAS inhibition on the flux to valine and leucine.




(1) (2) (3)

FIG. 7. Structural similarities between ketopantoyl lactone (structure 1), its desmethyl analog (structure 2), and the known inhibitor of ketopantoate reductase, 2-keto-4-hydroxybutyrolactone (structure 3).

There are a number of additional possible effects of an increase in intracellular ketobutyrate. Several enzymes which utilize ketoisovalerate as their physiological substrate are susceptible to competition between ketoisovalerate and ketobutyrate (35, 47). A decrease in the ratio of the two keto acids might be expected to lead to formation of analogs of the normal products and perhaps have deleterious metabolic effects (35, 47). For example, the competition of ketobutyrate with ketoisovalerate in the reaction catalyzed by ketopantoate hydroxymethyltransferase (35) would result in the formation of the desmethyl analog (Fig. 7, compound 2) of ketopantoyllactone (compound 1). As the analog 2-keto-4-hydroxybutyrolactone (compound 3) is a very potent inhibitor of the next enzyme on the coenzyme A pathway, ketopantoate reductase (17), compound 2 might well also be an inhibitor. There have been suggestions that other metabolic fates of ketobutyrate further affect the coenzyme A pool and other biosynthetic activities (24, 46).

We tentatively conclude that growth inhibition by SMM is largely due to the limited flux in the valine arm of the branched-chain amino acid pathway. The potency of the inhibitor might be further increased under some conditions by the imbalance induced by SMM between the intracellular levels of the keto acids ketobutyrate and ketoisovalerate, which might lead to the formation of an antimetabolite(s). What is not clear, given these problems, is how cells continue to grow at a steady state in the presence of 0.2 μM SMM, despite the observed decrease of 2 orders of magnitude in the ketoisovalerate/ketobutyrate ratio (Table 2), and how they maintain a sufficient supply of valine when their intracellular pool of ketoisovalerate is so drastically lowered.

**Effect of IpOHA.** We were somewhat surprised when analysis of keto acids in strain TV105 after brief incubations with IpOHA showed elevated levels of ketobutyrate (Table 2); the elevation of intracellular ketobutyrate levels upon inhibition of KARI had not previously been observed or proposed. However, release of threonine deaminase from feedback inhibition by isoleucine, as well as derepression of *ilvA*, is expected to lead to an increased rate of synthesis of ketobutyrate in the presence of IpOHA. As we have pointed out above, the rate of utilization of ketobutyrate by AHAS (equation 2) is not far from saturation under control conditions in strain TV105. AHAS can also catalyze an oxygenase reaction which converts an acetohydroxy acid to acetate and the keto acid (1, 45), and it is conceivable that under conditions under which KARI is inhibited, this could contribute to ketobutyrate accumulation. Whichever effect is more important, IpOHA seems to be even more efficient than SMM in increasing the intracellular ketobutyrate level, on the one hand, and in decreasing the intracellular ketoisovalerate level, on the other hand (Table 2).

Another unexpected property of IpOHA is its apparent specificity for inhibition of the valine branch of the pathway. Valine alone completely reversed the growth inhibition by IpOHA at its MIC, 1 μM, although concentrations higher than 5 μM IpOHA required the addition of isoleucine as well. This suggests that at inhibiting concentrations of IpOHA there was still sufficient uninhibited KARI activity to support flux to isoleucine. Addition of isoleucine, leucine, and pantothenate together, on the other hand, apparently could not free sufficient enzyme activity for valine synthesis. From the total amino acid content of *S. typhimurium* (unpublished results), one can calculate that the addition of valine would spare about 80% of the total flux through KARI required for branched-chain amino acid synthesis, while addition of isoleucine and leucine would spare about 60%. It seems unlikely that this difference could explain the absolute dependence on valine in the presence of IpOHA and complete lack of dependence on isoleucine. A further indication of the apparent specificity of IpOHA for inhibition of the valine branch is the fact that intracellular ketomethylvalerate levels did not decrease, even in the presence of very high concentrations of IpOHA, while ketoisovalerate levels dropped below the limit of detection (Table 2).

One explanation for this effect which immediately suggested itself to us is the same effect of ketobutyrate on the flux through AHAS to acetolactate discussed above. However, the increase of more than 10-fold in the intracellular levels of acetolactate upon inhibition of cells by IpOHA (Table 1) argues against a flux-limiting role for AHAS under these conditions.

We considered the possibility that some other enzyme activity with a higher specificity for acetohydroxybutyrate could be responsible for the observed relief of *S. typhimurium* from IpOHA inhibition by valine alone. In an early report, Ratzkin et al. (38) described the existence of a constitutive KARI activity in an *E. coli* W mutant allowing the synthesis of isoleucine only. However, we found that an *ilvC* mutant, strain TV191, which lacks KARI activity was unable to grow on glucose minimal medium in the presence of valine alone (data not shown). The possible formation of 2,3-dihydroxy-3-methylvalerate from acetohydroxybutyrate in these *Salmonella* strains by any enzyme other than KARI can thus be excluded.

We can also rule out the possibility that the dependence of IpOHA-inhibited cultures on valine but not on isoleucine could be due to the specificity of KARI for one of its alternative substrates. The relative rates of two competing reactions of the same enzyme should be in the ratio $[S]k_{cat}/K_m$ for the two substrates. The $k_{cat}$ values of KARI are 1,100 and 4,700 $min^{-1}$, and the $K_m$ values are 0.3 and 0.79 mM for acetolactate and acetohydroxybutyrate, respectively (36). From the data of Table 1 on intracellular acetohydroxy acid concentrations, KARI would thus favor synthesis of 2,3-dihydroxyisovalerate over synthesis of 2,3-dihydroxy-3-methylvalerate by about fivefold under control conditions in strain TV105. After addition of IpOHA, acetolactate levels increase much more than acetohydroxybutyrate levels (Table 1), so that KARI should favor synthesis of dihydroxyisovalerate (on the way to valine) by some 80-fold.

After exclusion of the alternative explanations discussed above, we are tempted to propose that the apparently adequate rate of synthesis of isoleucine in the presence of IpOHA reflects a nonhomogeneous spatial organization of the branched-chain amino acid pathway. For instance, our observations could be explained by a hypothetical complex of threonine deaminase, AHAS isozyme II, and KARI molecules responsible for much of the isoleucine synthesis in strain TV105, if it were relatively less sensitive to IpOHA inhibition than is free KARI, or if the high total intracellular acetolactate (Table 1) is not available to compete effectively with acetohydroxybutyrate formed within the enzyme complex.

The observed recovery of strain TV105 from growth inhibition in the presence of IpOHA (Fig. 2) might be caused by displacement of the reaction intermediate analog IpOHA from KARI by the accumulating acetohydroxy acid substrates of KARI, by induction of synthesis of the enzyme by the accumulating substrates (38), or by metabolic inactivation of the inhibitor. We believe that competition of the acetohydroxy acids with the inhibitor can be excluded as the cause for recovery from growth inhibition. Cellular acetolactate and acetohydroxybutyrate were significantly increased after brief incubation with IpOHA (Table 1), even with 0.2 μM IpOHA (data not shown), but growth inhibition persisted (Fig. 2B) for a relatively long period after acetohydroxy acids had accumulated. It has been shown in vitro that the KARI substrates can compete with IpOHA for the active site on the enzyme if their addition precedes that of IpOHA. However, once the enzyme-inhibitor complex has been formed, inhibition is virtually irreversible in the presence of the acetohydroxy acids (9, 41). Induction of enzyme synthesis and inactivation of the inhibitor seem to be the likely mechanisms for the observed recovery from growth inhibition: the observed growth recovery was accompanied by a simultaneous increase in KARI activity (Fig. 3) and by disappearance of the inhibitory effect of IpOHA from the medium.

Our results suggest that if recovery from growth inhibition could be avoided or bypassed, IpOHA or a derivative would be an even more effective inhibitor of cell growth than SMM. The MIC of IpOHA is significantly lower than that of SMM (0.2 μM) (Fig. 2), and accumulation of the potentially toxic metabolite ketobutyrate, together with a significant decrease in the

ketoisovalerate/ketobutyrate ratio, occurs at lower concentrations of IpOHA than of SMM (Table 2). It is important to note that because of the mechanism-based nature of inhibition by IpOHA (41), mutants resistant at the enzyme level are far less likely than are extraneous-site inhibitors of AHAS. Further experiments to investigate the mechanism for recovery of bacterial cells from inhibition by IpOHA are thus desirable.

Although the experiments described here were done with a particular *S. typhimurium* mutant, we believe they have considerable relevance to the metabolic effects of inhibitors of AHAS and KARI, in general. Since the relative amounts of the three branched-chain amino acids are similar in almost all organisms, the partitions between the alternative pathways are likely to be similar in different organisms. Ketobutyrate is apparently a minor metabolite relative to pyruvate in most prototrophic organisms, as would be expected from the problems which arise from the competition between ketoisovalerate and ketobutyrate on a number of enzymes (19, 25, 35). The potentiation of the inhibitory effect of an AHAS inhibitor by the competition between the two reactions catalyzed by AHAS is thus likely to be an important element in the efficacy of these inhibitors in all sensitive organisms, including plants. The eventual explanation for the recovery of strain TV105 from IpOHA inhibition may also shed light on the unexpectedly poor herbicidal activity of this KARI inhibitor (50).

Recently, leucine auxotrophs of the pathogenic bacterium *Mycobacterium bovis* have been found to lose their pathogenicity and ability to grow in vivo (27). It would be interesting to see whether the inhibitors we have studied here would similarly affect mycobacteria and thus have antibiotic activity. Experiments of the kind performed in this study might serve as a guide to the development of effective antibacterial agents acting on the branched-chain amino acid pathway in these important pathogens.

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## REFERENCES

1. **Abell, L. M., and J. V. Schloss.** 1991. Oxygenase side reactions of acetolactate synthase and other carbanion-forming enzymes. Biochemistry **30:**7883–7887.
2. **Aulabaugh, A., and J. V. Schloss.** 1988. The kinetic mechanism of ketol-acid reductoisomerase. J. Cell Biol. **107:**402A.
3. **Aulabaugh, A., and J. V. Schloss.** 1990. Oxalyl hydroxamates as reaction-intermediate analogues for ketol-acid reductoisomerase. Biochemistry **29:** 2824–2830.
4. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Burns, R. O.** 1971. L-Threonine deaminase-biosynthetic *Salmonella typhimurium*. Methods Enzymol. **17B:**555–560.
6. **Danchin, A., L. Dondon, and J. Daniel.** 1984. Metabolic alterations mediated by 2-ketobutyrate in *Escherichia coli* K-12. Mol. Gen. Genet. **193:**473–478.
7. **Daniel, J., L. Dondon, and A. Danchin.** 1983. 2-Ketobutyrate, a putative alarmone of *Escherichia coli*. Mol. Gen. Genet. **190:**452–458.
8. **Daniel, J., E. Joseph, and A. Danchin.** 1984. Role of 2-ketobutyrate as an alarmone in *Escherichia coli* K-12: inhibition of adenylate cyclase activity mediated by the phosphoenol pyruvate:glucose phosphotransferase transport system. Mol. Gen. Genet. **193:**467–472.
9. **Dumas, R., C. Cornillion Bertrand, P. Guigue Talet, P. Genix, R. Douce, and D. Job.** 1994. Interactions of plant acetohydroxy acid isomeroreductase with reaction intermediate analogues: correlation of the slow, competitive, inhibition kinetics of enzyme activity and herbicidal effects. Biochem. J. **301:** 813–820.
10. **Epelbaum, S., D. M. Chipman, and Z. Barak.** 1990. Determination of products of acetohydroxy acid synthase by the colorimetric method, revisited. Anal. Biochem. **191:**96–99.
11. **Gollop, N., Z. Barak, and D. M. Chipman.** 1987. A method for simultaneous determination of the two possible products of acetohydroxy acid synthase. Anal. Biochem. **160:**323–331.
12. **Gollop, N., Z. Barak, and D. M. Chipman.** 1988. Assay of products of acetolactate synthase. Methods Enzymol. **166:**234–240.
13. **Gollop, N., B. Damri, Z. Barak, and D. M. Chipman.** 1989. Kinetics and mechanism of acetohydroxy acid synthase isozyme III from *Escherichia coli*. Biochemistry **28:**6310–6317.
14. **Goodsell, D. S.** 1991. Inside a living cell. Trends Biochem. Sci. **16:**203–206.
15. **Holms, W. H.** 1986. The central metabolic pathways of *Escherichia coli*: relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate. Curr. Top. Cell. Regul. **28:**69–105.
16. **Johnsen, K., S. Molin, O. Karlström, and O. Maaløe.** 1977. Control of protein synthesis in *Escherichia coli*: analysis of an energy source shift-down. J. Bacteriol. **131:**18–29.
17. **Julliard, J. H.** 1994. Purification and characterization of oxopantoyl lactone reductase from higher plants: role in pantothenate synthesis. Bot. Acta **107:**191–200.
18. **Kishor, G. M., and D. M. Shah.** 1988. Amino acid biosynthesis inhibitors as herbicides. Annu. Rev. Biochem. **57:**627–663.
19. **Kisumi, N., M. Siguira, and I. Chibata.** 1976. Biosynthesis of norvaline, norleucine, and homoisoleucine in *Serratia marcescens*. J. Biochem. **80:**333–339.
20. **Klingenberg, M., and E. Pfaff.** 1967. Means of terminating reactions. Methods Enzymol. **10:**680–684.
21. **Krampitz, L. O.** 1948. Preparation and determination of 2-acetolactic acid. Arch. Biochem. **17:**81.
22. **Landstein, D., S. Epelbaum, S. Arad (Malis), Z. Barak, and D. M. Chipman.** 1995. Metabolic response of *Chlorella emersonii* to the herbicide sulfometuron methyl. Planta **197:**219–224.
23. **LaRossa, R. A., and J. V. Schloss.** 1984. The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. J. Biol. Chem. **259:**8753–8757.
24. **LaRossa, R. A., and T. K. Van Dyk.** 1989. Leaky pantothenate and thiamin mutations of *Salmonella typhimurium* conferring sulfometuron methyl sensitivity. J. Gen. Microbiol. **135:**2209–2222.
25. **LaRossa, R. A., T. K. Van Dyk, and D. R. Smulski.** 1987. Toxic accumulation of α-ketobutyrate caused by inhibition of the branched-chain amino acid biosynthetic enzyme acetolactate synthase in *Salmonella typhimurium*. J. Bacteriol. **169:**1372–1378.
26. **Levitt, G.** 1983. Agricultural sulfonamides. U.S. patent 4,394,506.
27. **McAdam, R. A., T. R. Weisbrod, J. Martin, J. D. Scuderi, A. M. Brown, J. D. Cirillo, B. R. Bloom, and W. R. Jacobs.** 1995. In vivo growth characteristics of leucine and methionine auxotrophic mutants of *Mycobacterium bovis* BCG generated by transposon mutagenesis. Infect. Immun. **63:**1004–1012.
28. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–356. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Muhitch, M. J., D. L. Shaner, and M. A. Stidham.** 1987. Imidazolinones and acetohydroxyacid synthase from higher plants. Properties of the enzyme from maize suspension culture cells and evidence for the binding of imazapyr to acetohydroxyacid synthase *in vivo*. Plant Physiol. **83:**451–456.
31. **Neidhardt, F. C.** 1987. Chemical composition of *Escherichia coli*, p. 3–6. *In* J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
32. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
33. **Palmer, D. T., P. H. Blum, and S. W. Artz.** 1983. Effects of the *hisT* mutation in *Salmonella typhimurium* on translation elongation rate. J. Bacteriol. **153:** 357–363.
34. **Payne, S. M., and B. N. Ames.** 1982. A procedure for rapid extraction and high-pressure liquid chromatographic separation of the nucleotides and other small molecules from bacterial cells. Anal. Biochem. **123:**151–162.
35. **Powers, S. G., and E. E. Snell.** 1976. Ketopantoate hydroxymethyltransferase. 2. Physical catalytic and regulatory properties. J. Biol. Chem. **251:** 3786–3793.
36. **Primerano, D. A., and R. O. Burns.** 1983. Role of acetohydroxy acid isomeroreductase in biosynthesis of pantothenic acid in *Salmonella typhimurium*. J. Bacteriol. **153:**259–269.
37. **Putnam, S. L., and A. L. Koch.** 1975. Complications in the simplest cellular enzyme assay: lysis of *Escherichia coli* for the assay of β-galactosidase. Anal. Biochem. **63:**350–360.
38. **Ratzkin, B., S. Arfin, and H. E. Umbarger.** 1972. Isoleucine and valine metabolism in *Escherichia coli*. XVIII. Induction of acetohydroxy acid isomeroreductase. J. Bacteriol. **112:**131–141.
39. **Ray, T. B.** 1984. Site of action of chlorosulfuron. Plant Physiol. **75:**827–831.
40. **Schloss, J. V., and A. Aulabaugh.** 1990. Acetolactate synthase and ketol-acid reductoisomerase: targets for herbicides obtained by screening and *de novo* design. Z. Naturforsch. **45c:**544–551.

41. **Schloss, J. V., and A. Aulabaugh.** 1990. Acetolactate synthase and ketol-acid reductoisomerase: a search for reason and a reason for search, p. 329–356. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH, Weinheim, Germany.
42. **Schulz, A., P. Spoenemann, H. Koecher, and F. Wengemayer.** 1988. The herbicidally active experimental compound Hoe 704 is a potent inhibitor of the enzyme acetolactate reductoisomerase. FEBS Lett. **238:**375–378.
43. **Shaner, D. L., and B. K. Singh.** 1993. Phytotoxicity of acetohydroxyacid synthase inhibitors is not due to accumulation of 2-ketobutyrate and/or 2-aminobutyrate. Plant Physiol. **103:**1221–1226.
44. **Singer, P. A., M. Levinthal, and L. S. Williams.** 1984. Synthesis of the isoleucyl- and valyl-tRNA synthetases and the isoleucine-valine biosynthetic enzymes in a threonine deaminase regulatory mutant of *Escherichia coli* K-12. J. Mol. Biol. **175:**39–55.
45. **Tse, J. M. T., and J. V. Schloss.** 1993. The oxygenase reaction of acetolactate synthase. Biochemistry **32:**10398–10403.
46. **Van Dyk, T. K., and R. A. LaRossa.** 1986. Sensitivity of a *Salmonella typhimurium aspC* mutant to sulfometuron methyl, a potent inhibitor of acetolactate synthase II. J. Bacteriol. **165:**386–392.
47. **Van Dyk, T. K., and R. A. LaRossa.** 1990. Prevention of endogenous 2-ketobutyrate toxicity in *Salmonella typhimurium*, p. 123–130. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched chain amino acids. VCH, Weinheim, Germany.
48. **Wang, Z.-J., K. Zaitsu, and Y. Ohkura.** 1988. High-performance liquid chromatographic determination of 2-ketoacids in human serum and urine using 1,2-diamino-4,5-methylenedioxybenzene as a precolumn fluorescence derivatisation reagent. J. Chromatogr. **430:**223–231.
49. **Westerfeld, W. W.** 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. **161:**495–502.
50. **Wittenbach, V. A., A. Aulabaugh, and J. V. Schloss.** 1991. Examples of extraneous site inhibitors and reaction intermediate analogs: acetolactate synthase and ketol-acid reductoisomerase, p. 151–160. *In* H. Frehse (ed.), Pesticide chemistry. VCH, Weinheim, Germany.
51. **Zaritzky, A., C. L. Woldringh, C. E. Helmstetter, and N. B. Grover.** 1993. Dimensional rearrangement of *Escherichia coli* B/r cells during a nutritional shift down. J. Gen. Microbiol. **139:**2711–2714.