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Metabolic Basis for the Isoleucine, Pantothenate or Methionine Requirement of *ilvG* Strains of *Salmonella typhimurium*

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Salmonella typhimurium strain DU501, which was found to be deficient in acetohydroxy acid synthase II (AHAS II) and to possess elevated levels of transaminase B and biosynthetic threonine deaminase, required isoleucine, methionine, or pantothenate for growth. This strain accumulated α-ketobutyrate and, to a lesser extent, α-aminobutyrate. We found that α-ketobutyrate was a competitive substrate for ketopantoate hydroxymethyltransferase, the first enzyme in pantothenate biosynthesis. This competition with the normal substrate, α-ketoisovalerate, limited the supply of pantothenate, which resulted in a requirement for methionine. Evidence is presented to support the conclusion that the ambivalent requirement for either pantothenate or methionine is related to a decrease in succinyl coenzyme A, which is produced from pantothenate and which is an obligatory precursor of methionine biosynthesis. The autointoxification by endogenously produced α-ketobutyrate could be mimicked in wild-type *S. typhimurium* by exogenously supplied α-ketobutyrate or salicylate, a known inhibitor of pantothenate biosynthesis. The accumulation of α-ketobutyrate was initiated by the inability of the residual AHAS activity provided by AHAS I to efficiently remove the α-ketobutyrate produced by biosynthetic threonine deaminase. The accumulation of α-ketobutyrate was amplified by the action of transaminase B, which decreased the isoleucine pool by catalyzing the formation of α-keto-β-methylvalerate and aminobutyrate from isoleucine and α-ketobutyrate; this resulted in release of threonine deaminase from end product inhibition and unbridled production of α-ketobutyrate. Isoleucine satisfied the auxotrophic requirement of the AHAS II-deficient strain by curtailing the activity of threonine deaminase. Additional lines of evidence based on genetic and physiological experiments are presented to support the basis for the autointoxification of strain DU501 as well as other nonpolarigenic *ilvG* mutant strains.

Analysis of the isoleucine-valine biosynthetic enzymes in a series of L-isoleucine auxotrophic strains of *Salmonella typhimurium* LT2 showed that one of them, DU501, possessed no obvious basis for its nutritional requirement. Isoleucine auxotrophy in *S. typhimurium* results from loss of either L-threonine deaminase or transaminase B; both of these enzymes are present in high levels in strain DU501. Further analysis of the isoleucine-valine biosynthetic enzymes revealed that this strain lacked the valine-insensitive acetohydroxy acid synthase II (AHAS II) but possessed the valine-sensitive synthase (AHAS I). It was subsequently shown that the isoleucine requirement of DU501 could be replaced by methionine or pantothenate. The lesion leading to this unusual phenotype was shown by deletion mapping to lie in the *ilvG* gene (4). The absence of AHAS II in strain DU501 was further demonstrated by the ability of valine to inhibit its growth in the presence of methionine or pantothenate. The presence of only valine-sensitive AHAS activity in strain DU501, as in the case of *Escherichia coli* K-12 (3), results in a conditional isoleucine auxotrophy by virtue of the ability of valine to effectively inhibit AHAS I, which participates in both valine and isoleucine biosynthesis (15, 16). The work described in this paper is aimed at determining the basis for the auxotrophic pattern of *ilvG* mutations.

MATERIALS AND METHODS

Bacterial strains and culture media. Table 1 describes the *S. typhimurium* LT2 strains used. Strains DU11 and DU12 were selected by plating dilutions of an overnight culture of strains DU501 onto minimal medium. The minimal medium used in all experiments was that described by Davis and Mingioli (10), which

TABLE 1. *S. typhimurium* strains

Strain	Genotype	Pertinent phenotype	Source
LT2	Wild type	Prototroph	Laboratory collection
DU501	*ilvG236 ara gal*	Ileu⁻, Met⁻, or Pan⁻	P. Margolin via M. Freundlich
DU502	*ilvG236 ilvA207*	Met⁻ or Pan⁻	From DU501
DU5125	*ilvG236 ilvA207 sup*(?)	Prototroph	From DU502
DU5025	*ilvG236 ilvA*	Ileu⁻	From DU502
DU608	Δ(*ilvGEDA*)	Requires leucine, isoleucine, and valine	(5)
DU6080	Δ(*ilvEDA*)/pDU5	Requires pantothenate, or isoleucine and methionine	This paper
DU61	*ilvA210*	Ileu⁻	(4)
DU401	*ilvE4*	Ileu⁻	(4)
DU23	*ilvA207 ilvD18*	Requires isoleucine and valine	Laboratory collection
DU11	*ilvG236 ara gal ilvE184*	Valine-sensitive prototroph	From DU501
DU12	*ilvG236 ara gal pan-187*	Valine-sensitive prototroph	From DU501
MF1680	*ilvG236 ara-7*	Ileu⁻, Met⁻, or Pan⁻	P. Margolin

was modified by omitting the citrate and raising the concentration of glucose to 0.5%. Solid medium was prepared by adding 1.5% agar (Difco Laboratories). The concentration of potassium phosphate and ammonium sulfate was routinely doubled in the medium used for growth experiments and in batch cultures to be used for the preparation of cell-free extracts.

Analysis of nutritional requirements and growth rate measurements. Nutritional requirements of DU501 and its derivatives were determined by diluting overnight cultures 20-fold into saline, inoculating 0.05 ml of this suspension into 1 ml of liquid minimal media, and incubating at 37°C with aeration. Metabolic intermediates and end products were added to liquid or solid media at the following concentrations: 100 μg/ml for leucine, valine, α-ketopantoate, pantoate, threonine, and succinate; 50 μg/ml for isoleucine, methionine, homoserine, cystathionine, and aspartate; and 20 μg/ml for calcium pantothenate, lysine, and diaminopimelate. Inhibition of growth by valine was scored on solid medium containing valine (1 mg/ml). Growth rates were determined by measuring the increase in the optical density with a Klett-Summerson colorimeter equipped with a no. 42 (blue) filter; cultures were incubated in sidearm flasks at 37°C with shaking.

Strain construction. P22-mediated transduction was performed as described by Margolin (26), except that the HT105/4 mutant derived by Schmieger (34) was used. Strains containing the *ilvG236* mutation together with a mutation in *ilvA* (*ilvA207*) which renders threonine deaminase insensitive to inhibition by isoleucine were constructed by preparing phage lysates on strain DU501 and using these to transduce strain DU1402 (*ilvA207 ilvD18*) to *ilvD*⁺ on medium supplemented with methionine and diaminopimelate; transductants which contain both *ilvG236* and the *ilvA* mutation grow on methionine-supplemented medium but, unlike strain DU501, do not grow on isoleucine-supplemented medium. Alternatively, the *ilvA* alleles which specify production of isoleucine-insensitive threonine deaminase can be introduced into strain DU501 by transduction with phage prepared on appropriate strains and selecting recombinants capable of growing on threonine as the sole source of nitrogen (7). In either case, the presence of the appropriate alleles was confirmed by enzyme assay.

DU6080 was constructed by transforming DU608 with purified pDU5 DNA (5); ampicillin-resistant transformants were selected on nutrient agar containing ampicillin (20 μg/ml). The transformation procedure of Lederberg and Cohen for *S. typhimurium* was used (22).

Preparation of cell extracts for enzyme assay. Strains in which the isoleucine-valine enzyme levels were to be measured were grown in 250-ml batch cultures at 37°C with aeration. Extracts were prepared as previously described except that pyridoxal phosphate was left out of the resuspending buffer (4).

Enzyme assays. The assays of the isoleucine-valine biosynthetic enzymes were performed as previously described: threonine deaminase (6), dihydroxy acid dehydrase (13), transaminase B (12), AHAS (37), and acetohydroxy acid isomeroreductase (1). AHAS II activity was measured in synthase reaction mixtures containing 1 mM valine. Since α-acetolactate was occasionally used as a substrate for the isomeroreductase, specific activities were normalized to those expected from α-acetohydroxybutyrate (17). β-Isopropylmalate dehydrogenase was assayed by the method of Parsons and Burns (31).

Ketopantoate hydroxymethyltransferase was assayed by measuring the formation of [α-^{14}C]ketopantoate by the method of Teller et al. (38) with some modifications. Hydroxymethyltransferase reactions were terminated by the addition of 0.3 ml of 1 M sodium acetate pH 4.5. [α-^{14}C]ketopantoate was then isolated by extracting the remaining H^{14}CHO as a dimedone complex into toluene: 0.2 ml of 0.4% HCHO and 0.5 ml of 0.4 M dimedone were added to stopped reaction mixes; this mixture was boiled for 5 min and cooled. Toluene (5 ml) was added (to extract the dimedone-HCHO complex), and the contents were mixed by blending for 3 min; phases were then separated by centrifugation at 5,000 rpm for 5 min. [α-^{14}C]ketopantoate remained in the aqueous phase; 0.05-ml portions were dispensed into 10 ml of scintillation cocktail and counted in Beckman model LS-233 scintillation counter.

Analysis of α-keto acid and α-amino acid in culture filtrates. Culture filtrates were prepared by centrifuging 50 ml of cultures in late log phase at 10,000 rpm for 10 min and carefully decanting the spent medium. α-Ketocarboxylic acids were detected by reacting cul-

ture filtrates with 2,4-dinitrophenylhydrazine; the resulting dinitrophenylhydrazones were identified by descending paper chromatography. Two milliliters of filtrate was added to tubes containing 2 ml of 25 mM 2,4-dinitrophenylhydrazine (prepared in 2 N HCl) and 5 ml of toluene; the phenylhydrazones were extracted as they formed by blending for 5 min. Portions (10 to 20 μl) of the toluene extract were spotted onto Whatman no. 3 filter paper along with the phenylhydrazones of α-ketobutyrate and α-ketoglutarate. Two solvent systems were used to develop chromatograms: (i) 1-butanol–water–ethanol (50:40:10) and (ii) 1-propanol–28% NH_4OH–water (60:30:10) (27). α-Aminobutyrate was identified and quantified by automated amino acid analysis. Culture filtrates were prepared for analysis by adjusting the pH to 2 with 0.01 N HCl. Five milliliters of acidified supernatant was pipetted onto a small column of Bio-Rad AG 50W-X8 in the hydrogen form and washed with 25 ml of deionized water. Amino acids were eluted with 5 ml of 1 N NH_4OH and evaporated to dryness. The residue was suspended in 1% formic acid and evaporated to dryness (twice). The residue was dissolved in an appropriate amount of sample buffer and analyzed.

Chemicals and enzymes. Sodium α-acetolactate and sodium α-aceto-α-hydroxybutyrate were prepared by the saponification of their methyl esters; these esters and purified transaminase B were the generous gifts of Frank Armstrong. Purified threonine deaminase was prepared according to previously described procedures (7). α,β-Dihydroxyisovalerate was obtained from Reef Laboratories. *O*-Succinyl-DL-homoserine and *O*-acetyl-DL-homoserine were the generous gifts of Ronald Greene. Cystathionine and *O*-succinyl-L-homoserine were obtained from Calbiochem-Behring Corp. All other compounds, including D-pantoyllactone, DL-pantoyllactone, and calcium D-pantothenate, were obtained from Sigma Chemical Co. α-Ketopantoyllactone (α-keto-β,β-dimethyl-γ-butyrolactone) was prepared from DL-pantoyllactone by the method of Lipton and Strong (23) and dried over $CaCl_2$; the whitish-yellow crystalline product melted between 67 and 69°C. Then 100 mM Na^+-α-ketopantoate was prepared by dissolving 0.128 g of α-ketopantoyllactone in 9.37 ml of deionized water and titrating to pH 7 with 0.63 ml of 1.5 N NaOH. D-Pantoyllactone was converted to the free acid (Na^+ salt) as described by King et al. (19). Calcium D-pantothenate was converted to its sodium salt by passing 5 ml of a 200 mM solution over a 1 by 5-cm column of Bio-Rad AG50-X8 in the sodium form. All optically active compounds used in growth nutrition studies were in the L form except D-pantoic acid, D-pantothenate, and diaminopimelate, which was a mixture of DD, DL, LD, and LL forms. $H^{14}CHO$ was obtained from New England Nuclear Corp. β-Isopropylmalate was prepared by the method of Calvo and Gross (9).

RESULTS

Nutritional requirements and growth properties of DU501. Strain DU501 was among a series of isoleucine auxotrophs provided to us by M. Freundlich in 1973. It originated from the collection of P. Margolin and was derived after mutagenesis with nitrosoguanidine. In addition to the *ilvG* mutation, which we have designated *ilvG236* after the original number designation of the strain, it has been shown to contain a non-*supX* suppressor of *leu-500* (R. Weinberg, personal communication). The properties of strain DU501 described in this paper are attributable solely to the *ilvG* mutation; strains constructed by crossing *ilvG236* into them possess the pertinent properties of strain DU501. Also, none of the properties to be described in this paper are separable by appropriate genetic analysis.

Strain DU501 appeared to be a strict auxotroph on agar plates, but when washed cells were inoculated into minimal liquid medium, they underwent three doublings regardless of inoculum size. This observation suggests the presence of an endogenous nutrient which the organism is unable to synthesize in minimal medium. Evidence will be presented below to suggest that this compound is succinyl coenzyme A (succinyl-CoA).

Addition of isoleucine, methionine, or pantothenate to minimal medium permitted the normal growth of DU501. A number of metabolic intermediates relating to methionine and pantothenate biosynthesis also supported the growth of strain DU501. Homoserine, cystathionine, homocysteine, α-ketopantoate, and pantoate were as effective as isoleucine, methionine, or pantothenate in stimulating growth. *O*-acetylhomoserine, a known surrogate for *O*-succinylhomoserine in the cystathionine synthase reaction (28), could also support the growth of DU501. Threonine was slightly stimulatory. Succinate, aspartate, glutamate, β-alanine, leucine, diaminopimelate, or lysine failed to stimulate growth. Table 2 presents growth rate constants of strain DU501 on minimal medium containing various supplements.

Level of the isoleucine-valine enzymes in strain DU501. The levels of the isoleucine-valine biosynthetic enzymes were measured in cells cultivated in methionine-supplemented medium and in the same medium containing excess branched-chain amino acids. The *ilvEDA* and *ilvB* enzyme levels were elevated in strain DU501 compared with the wild-type strain and, with the exception of AHAS I, remained at relatively high levels under repressing conditions (Table 3). No detectable AHAS II was produced when isoleucine was limiting and leucine and valine were in excess (Table 3, line 6). These results suggested that the *ilvG236* mutation, in addition to causing a loss of AHAS II, also caused increased expression of the conjoined *ilv* genes. The relatively high level of AHAS I in the nonrepressed culture of strain DU501 suggested that either leucine or valine was somewhat limiting under these conditions. The expression of *ilvB*, which is located on the *S. typhimurium*

TABLE 2. Exponential growth rate constants (generations/hour) of DU501 and DU502 in the presence of various nutrients

Nutrient	DU501	DU502
Minimal	0.00 (0.43)[a]	0.00 (0.33)
Isoleucine	0.78	0.00 (0.36)
Methionine	1.0	0.72
Methionine + diaminopimelate	0.98	0.80
Threonine	0.00 (0.67)	0.00 (0.17)
Isoleucine + α-ketoisovalerate	0.47	0.69
α-Ketopantoate	0.77	0.45
Pantoate	0.77	0.75
Pantothenate	0.83	0.82
Leucine	0.00 (0.88)	0.00 (0.46)
Succinate	0.00 (0.32)	0.00 (0.86)

[a] The numbers in parentheses are the exponential growth rate constants before cessation of growth (three generations); see text for details and Materials and Methods for concentration of supplements.

chromosome some distance from the *ilvGEDA* cluster (Weinberg, personal communication; 35), is divalently repressed by leucine and valine (29). The α-acetohydroxy acid isomeroreductase levels in strain DU501 grown on isoleucine- or methionine-supplemented medium approached the repressed levels observed in wild type; this indicated that the synthesis of the α-aceto-α-hydroxy acid intermediates, the inducers of isomeroreductase (1), was curtailed in spite of the derepressed levels of AHAS I.

Excretion of α-aminobutyrate and α-ketobutyrate by DU501. DU501 was observed to feed DU61 (*ilvA210*) but not DU401 (*ilvE4*). This feeding pattern indicated that DU501 excreted α-ketobutyrate or α-aminobutyrate but not isoleucine. Supernatant fluids of pantothenate- or methionine-supplemented cultures of DU501 and LT2 were analyzed by thin-layer and ion-exchange chromatography. The presence of large quantities of α-ketobutyrate (630 nmol/ml) and α-aminobutyrate (41 nmol/ml) was confirmed; the wild type had very low levels of these compounds (1 to 10%). We show below that α-ketobutyrate inhibited the growth of wild-type *S. typhimurium*. The presence of isoleucine in the growth medium of DU501 prevented the accumulation of these compounds in the culture filtrates. This observation suggested a basis for the ability of exogenously added isoleucine to satisfy the growth requirement; i.e., isoleucine effectively inhibits the activity of L-threonine deaminase (8) and curtails production of the toxic compounds. Methionine and pantothenate must reverse the effects of the toxic accumulation in a transitive fashion, since they had no effect on the activity of threonine deaminase.

Mechanism of α-ketobutyrate and α-aminobutyrate accumulation in strain DU501. The level of α-ketobutyrate in strain DU501 is high for two reasons. (i) The α-ketobutyrate which is normally converted to α-aceto-α-hydroxybutyrate by AHAS II is not efficiently removed by AHAS I; it is known that AHAS I is relatively inefficient

TABLE 3. Specific activities of the isoleucine-valine enzymes and β-isopropylmalate (β-IPM) dehydrogenase in LT2, DU501, DU5025, and DU5125

Strain	Growth conditions	Sp act (nmol/min per mg[a])						
		AHAS I (*ilvB*)	AHAS II (*ilvG*)	Transaminase B (*ilvE*)	Dihydroxy acid dehydrase (*ilvD*)	Threonine deaminase (*ilvA*)	Acetohydroxy acid isomeroreductase (*ilvC*)	β-IPM dehydrogenase (*leuB*)
LT2	Methionine[b]	3.80	3.05	130	99.0	255	69.4	61.7
	Leucine, isoleucine, and valine[c]	2.35	1.15	92.0	23.2	111	16.0	NA
DU501	Isoleucine[d]	86.0 (8.35)	0.0	679	335	1,200	41.6	146
	Methionine	57.5 (10.6)	0.0	625	536	1,580	41.9	132
	Leucine, isoleucine, and valine	0.55 (0.15)	0.0	263	387	506	9.60	NA
	Limiting isoleucine[e]	1.65 (0.20)	0.0	1,140	473	1,580	1.54	NA
DU5025	Isoleucine	374 (26.8)	0.0	763	NA	ND	NA	NA
DU5125	Isoleucine	12.5 (1.50)	0.0	218	NA	366	NA	NA

[a] The numbers in parentheses represent the level of AHAS activity when assayed in the presence of 1.0 mM L-valine. This quantity of valine-resistant activity resides in AHAS I and is routinely seen in crude extracts. *ilvB ilvG* mutant strains totally lack AHAS activity. NA, not assayed; ND, not detectable.

[b] Medium supplemented with methionine (50 μg/ml).

[c] Medium supplemented with isoleucine (50 μg/ml), leucine (100 μg/ml), and valine (100 μg/ml).

[d] Medium supplemented with isoleucine (50 μg/ml).

[e] Medium supplemented with isoleucine (5 μg/ml), leucine (100 μg/ml), valine (100 μg/ml), and pantothenate (20 μg/ml).

in catalyzing the formation of α-aceto-α-hydroxybutyrate (35). As a consequence, the isoleucine pool is lowered and α-ketobutyrate accumulates. The level of isoleucine is further diminished by the accumulation of α-ketobutyrate which drives the transaminase B-catalyzed transamination of isoleucine to yield α-keto-β-methylvalerate and α-aminobutyrate. (ii) The low level of isoleucine allows the unbridled production of α-ketobutyrate by releasing the intrinsically high levels of threonine deaminase from allosteric inhibition. The release of threonine deaminase from inhibition and the concomitant overproduction of α-ketobutyrate represent a "short circuit" in the biosynthesis of isoleucine (depicted in Fig. 1).

The short-circuit reactions could be reproduced in vitro by using either crude extracts of DU501 or a combination of purified transaminase B and threonine deaminase. Figures 2A (crude extract) and 2B (purified enzymes) show what appears to be an autocatalytic release of the isoleucine-imparted inhibition of threonine deaminase; the rate of α-ketobutyrate synthesis by threonine deaminase was initially slow, but gradually increased as isoleucine was converted to α-keto-β-methylvalerate by the action of holotransaminase B. The release from inhibition in both the crude extract and the purified system required the presence of added pyridoxal 5'-monophosphate. This simply reflects the differential requirement of threonine deaminase and transaminase B for added cofactor; i.e., pyridoxal 5'-monophosphate is tightly bound to threonine deaminase (8), whereas it readily dissociates from transaminase B (D. Primerano and R. O. Burns, unpublished data). Isoleucine corrects the short circuit and allows DU501 to grow by inhibiting threonine deaminase, thus reducing the level of α-ketobutyrate. This view is supported by the inability of isoleucine to support the growth of a derivative of DU502 which contains an *ilvA* mutation rendering threonine deaminase insensitive to inhibition by isoleucine (8). Table 2 shows the response of this strain, DU502 (*ilvG236 ilvA207*), to various compounds. DU502 grew in minimal medium supplemented with methionine or pantothenate. Unlike its parent, DU501, it did not grow in the presence of isoleucine because the production of α-ketobutyrate could not be checked; however, it grew on medium containing a combination of isoleucine and either valine or α-ketoisovalerate (Table 2). It appeared from the growth pattern of strain DU502 that α-ketoisovalerate could overcome the autointoxification. Valine was effective by serving as a source of α-ketoisovalerate. An alternate view, that valine simply spared α-ketoisovalerate (the synthesis of which was somewhat curtailed), was ruled out by the observation that leucine (which is also produced from α-keto-isovalerate) did not stimulate the growth of DU502 (Table 4). The basis for the ability of α-ketoisovalerate to overcome the intoxification by endogenously produced α-ketobutyrate is explained below.

Inhibition of pantothenate biosynthesis by α-ketobutyrate. Powers and Snell have observed that, in *E. coli* K-12, α-ketobutyrate is an alternate substrate for ketopantoate hydroxymethyltransferase, the first enzyme specific to pantothenate biosynthesis (32). We have confirmed this observation in *S. typhimurium*. Figure 3 shows the results of a kinetic analysis of ketopantoate hydroxymethyltransferase with α-ketoisovalerate and α-ketobutyrate as substrates; the apparent K_m values for α-ketoisovalerate, the normal substrate, and α-ketobutyrate were 1.15 and 1.30 mM, respectively. These results taken with the foregoing observations strongly

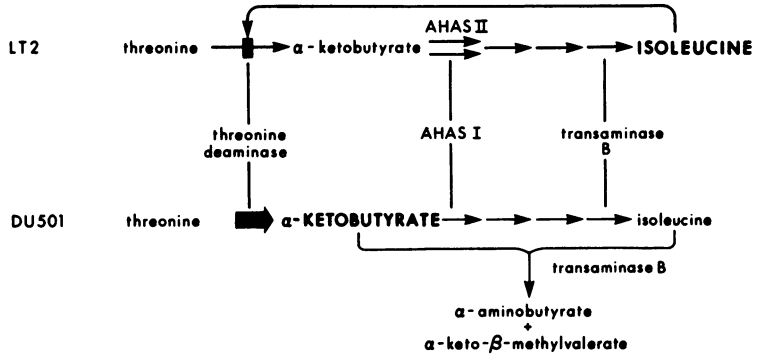

FIG. 1. Transaminase B- and α-ketobutyrate-mediated removal of L-isoleucine in an AHAS II-deficient strain of *S. typhimurium*. In with wild-type strain, α-ketobutyrate is efficiently converted to isoleucine, which regulates the flow of intermediates by inhibiting threonine deaminase. In the absence of AHAS II (strain DU501), α-ketobutyrate accumulates and drives the conversion of isoleucine to α-keto-β-methylvalerate. This reaction releases threonine deaminase from allosteric inhibition and allows the unbridled production of α-ketobutyrate.

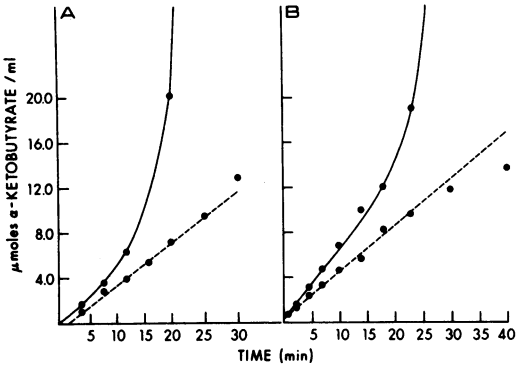

FIG. 2. Release of isoleucine-imparted inhibition of threonine deaminase in the presence of active transaminase B. One-milliliter reaction mixtures contained 0.1 M Tris-hydrochloride, pH 8.0, 0.1 M NH_4Cl, 0.08 M L-threonine, and 0.75 mM L-isoleucine; (----) without pyridoxal 5'-monophosphate; (——) with 0.08 mM pyridoxal 5'-monophosphate. (A) Reactions initiated at 37°C with crude extract containing 100 U of threonine deaminase and 150 U of transaminase B. (B) Reaction initiated at 37°C with a mixture containing 100 U of pure threonine deaminase and 170 U of pure transaminase B.

TABLE 4. Reversal of α-ketobutyrate-inhibited growth of *S. typhimurium*

Addition to medium	Growth rate constant (generation/h) at given concn (mM) of α-ketoisovalerate, valine, pantothenate, or methionine			
	0	0.1	1.0	10.0
None	1.0			
50 mM α-ketobutyrate + α-ketoisovalerate	0.037	0.038	0.048	0.40
50 mM α-ketobutyrate + valine	0.037	0.039	0.050	0.25
50 mM α-ketobutyrate + pantothenate	0.037	0.22	0.29	0.29
50 mM α-ketobutyrate + methionine	0.037	0.45	0.51	NT[a]

[a] Not tested.

suggested that the α-ketobutyrate which accumulates in strain DU501 curtailed the production of pantothenate by competing with the normal substrate in the hydroxymethyltransferase reaction. This leaves us to explain the basis for the ability of methionine to satisfy the auxotrophy of strain DU501. The sole apparent connection between pantothenate and methionine biosynthesis is succinyl-CoA; pantothenate is a precursor of CoA, and succinyl-CoA serves as a substrate for homoserine succinyltransferase, the initial enzyme for methionine biosynthesis. The pantothenate or methionine auxotrophy of strain DU501 revealed the methionine biosynthetic pathway as the most sensitive of the pathways utilizing CoA or succinyl-CoA. We show below that a more severe curtailment of pantothenate biosynthesis revealed the role of succinyl-CoA in diaminopimelate and lysine biosynthesis.

Effect of α-ketobutyrate and salicylate on the growth of wild-type *S. typhimurium*. Our contention, that the auxotrophy of strain DU501 is explained by the inhibition of pantothenate biosynthesis by accumulated α-ketobutyrate, was supported by the observation that inhibition of

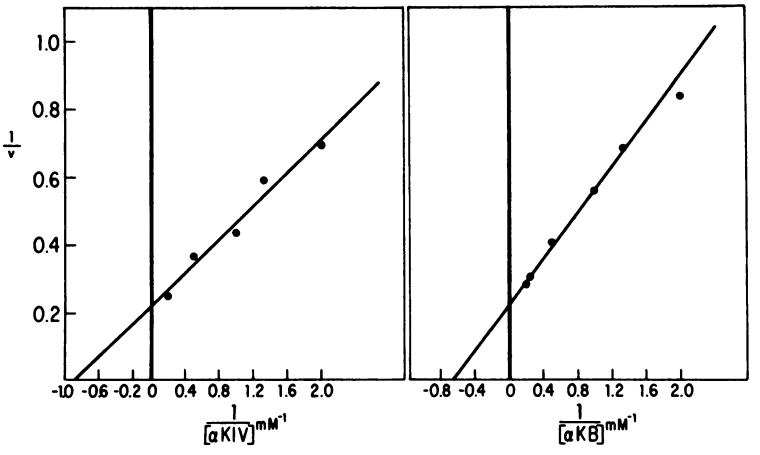

FIG. 3. Kinetic analysis of ketopantoate hydroxymethyltransferase in crude extract of *S. typhimurium*. The reciprocal of the initial velocity (nanomoles/minute per milligram of protein) is plotted against the reciprocal of the concentrations of α-ketoisovalerate (left) and α-ketobutyrate (right). The kinetic constants derived from this analysis are: for α-ketoisovalerate, K_m = 1.15 mM and specific activity = 4.60 nmol/min per mg; for α-ketobutyrate, K_m = 1.30 mM and specific activity = 4.2 nmol/min per mg. The assay procedure is described in Materials and Methods.

the growth of wild-type *S. typhimurium* by exogenously added α-ketobutyrate could be overcome by methionine or pantothenate but not by isoleucine. Furthermore, the growth rates presented in Table 4 showed that α-ketoisovalerate and valine overcame the toxic effect of 50 mM α-ketobutyrate in a competitive manner, whereas panthothenate and methionine reversed inhibition in a noncompetitive manner. These results are consistent with the observation that α-ketobutyrate served as a competitive substrate for ketopantoate hydroxymethyltransferase and support the view that this enzyme is its primary target.

At higher concentrations of α-ketobutyrate (100 mM), either diamino-pimelate or lysine enhanced the recovery from inhibition in the presence of methionine; in addition, diaminopimelate or lysine stimulated the growth of DU502 in methionine-supplemented medium (Table 2). These observations are best explained in terms of a starvation for succinyl-CoA which is involved in methionine as well as diaminopimelate-lysine biosynthesis in enteric bacteria (39). This proposal is supported by results obtained with salicylate, a known inhibitor of pantothenate biosynthesis. Maas has shown that salicylate exerts a pantothenate-reversible inhibition of the growth of *E. coli* W and that salicylate exerts its effect on the synthesis of pantoate (25). We have extended these results to *S. typhimurium*. Although kinetic parameters were not quantified, we found that salicylate was an effective inhibitor of α-ketopantoate hydroxymethyltransferase in crude extracts of *S. typhimurium*; 2.5 mM salicylate resulted in 81% inhibition of enzyme activity in the standard assay. Salicylate had no effect on either ketopantoate reductase or α-ketoglutarate dehydrogenase.

Growth inhibition by 140 μM salicylate was overcome by ketopantoate, pantoate, pantothenate, or a combination of lysine and methionine; if lower concentrations of the inhibitor were used growth inhibition was overcome by methionine alone. At concentrations greater than 280 μM, only pantothenate overcame inhibition. These experiments show that partial inhibition of pantothenic biosynthesis resulted in an exclusive perturbation of methionine biosynthesis (presumably by depleting the amount of *S*-succinyl-CoA available for the homoserine transsuccinylase reaction). This conclusion was supported by the observation that methionine and lysine spared pantothenate in a *panB* mutant strain of *S. typhimurium* LT2.

Prototrophic derivatives of strain DU501. When strain DU501 is spread onto minimal agar plates, colonies of various sizes appear. Several of these revertants have been characterized and have been categorized into two classes. Approximately one-half of these prototrophic derivatives (Class I) exhibit elevated levels of AHAS I and, in addition to being prototrophic, are resistant to valine inhibition. Apparently, the elevation of AHAS I causes removal of sufficient α-ketobutyrate so as to prevent the initiation of the transaminase B-catalyzed short circuit of isoleucine biosynthesis described earlier in this report. The remainder of these prototrophs (class II) are valine sensitive. One of these strains, DU12, has 10-fold higher levels of ketopantoate hydroxymethyltransferase than does the wild type or DU501. Preliminary genetic analysis has shown that the lesion (*pan-187*) giving rise to this elevated enzyme level is 90% linked to the *pan* gene cluster by P22 transduction. Strain DU12 has apparently bypassed the autointoxification by increasing the production of α-ketopantoate. A second class II prototroph, strain DU11, carries a suppressor mutation which is closely linked to or within the *ilvE* gene. We have shown that crude extracts of this strain are incapable of effecting the release of isoleucine inhibition as depicted for extracts of strain DU501, and we have tentatively concluded that the relevant mutation effectively decreases the ability of transaminase B to use α-ketobutyrate as an amino group acceptor. Prototrophic valine-sensitive revertants in which the suppression mechanism was not characterized also were obtained. The *ilvG236* mutation itself is very stable; this is demonstrated by the stability of the isoleucine-valine requirement of strains containing the *ilvG236* allele together with a stable *ilvB* mutation (Weinberg, unpublished data).

The role of threonine deaminase in the autointoxification of the *ilvG* mutant strain was further demonstrated by selecting variants of strain DU502 that grow in the presence of isoleucine. This strain is ideally suited for determining whether loss of threonine deaminase results in detoxification. As previously mentioned, this strain will not grow in the presence of isoleucine presumably because of the feedback-negative character of its altered threonine deaminase. When this strain is plated on minimal agar containing isoleucine, an equal number of large and small colony variants arise. Several of the large colonies (e.g., DU5025) were purified, and subsequent analysis showed that all required isoleucine and lacked biosynthetic threonine deaminase activity (Table 3, line 7). Some of the smaller colonies were shown to be prototrophic; the level of *ilvEDA* enzymes in one of these prototrophs, DU5125, was reduced to 25% of that in the parent strain (compare lines 3 and 8 in Table 3). It is apparent from the phenotypes of DU11, DU5125, and DU5025 that high levels of enzymatically active threonine deaminase and transaminase B together with a deficiency in

AHAS II are required for the observed autointoxification.

DISCUSSION

The foregoing results suggest that the unusual auxotrophic pattern of *ilvG* mutant strains of *S. typhimurium* is explained by an autointoxification of pantothenate biosynthesis by the accumulation of α-ketobutyrate as depicted in Fig. 1. We have concluded that the curtailment of pantothenate biosynthesis in these strains results in a decrease in the level of CoA and succinyl-CoA which is manifested as a requirement for methionine or, in some cases, methionine and lysine. The methionine-lysine requirement of strain DU502 is similar to that of *S. typhimurium* strains which lack α-ketoglutarate dehydrogenase (*sucA*). These strains, which are deficient in succinyl-CoA, have an ambivalent requirement for succinate or a combination of methionine and lysine (20). Succinyl-CoA is produced directly from succinate by succinyl-CoA synthetase in the *sucA* mutant strains (33). The difference between the *sucA* and *ilvG* mutant strains is that growth of the latter is not stimulated by succinate. This comparison suggests that the activity of succinyl-CoA synthetase is not sufficient to provide adequate amounts of succinyl-CoA from exogenously added succinate when the concentration of CoA is decreased.

The properties of *ilvG* and *sucA* mutant strains reveal the limited role of succinyl-CoA in biosynthesis. It is of interest that of the three prominent biosynthetic processes in *S. typhimurium* which utilize succinyl-CoA, namely, methionine (39), diaminopimelate-lysine (39), and δ-aminolevulinic acid (i.e., heme biosynthesis) (33), only the α-amino acid requirements are revealed in the *ilvG* and *sucA* mutants. This reflects the incomplete nature of the block in succinyl-CoA synthesis in these strains.

Shaw and Berg (35) have shown that high levels of α-ketobutyrate inhibit the growth of wild-type *S. typhimurium* and that this inhibition is overcome by valine. These authors concluded that α-ketobutyrate prevented growth by inhibiting the formation of the valine precursor, α-acetolactate. Although there is little doubt that α-ketobutyrate dampens the synthesis of valine to some degree, it appears from the results described here that the direct inhibition of ketopantoate synthesis more adequately explains the toxic effects of α-ketobutyrate. This view is supported by the observation that growth inhibition by α-ketobutyrate is overcome competitively by α-ketoisovalerate or valine and noncompetitively by pantothenate or methionine.

The partial inhibition of α-acetolactate synthesis by α-ketobutyrate is not ruled out by these observations. The elevation of both AHAS I and β-isopropylmalate dehydrogenase in DU501 (Table 3) strongly suggests that the formation of α-ketoisovalerate is curtailed in this strain. A decrease in the level of α-ketoisovalerate could, in fact, exacerbate the inhibition of ketopantoate synthesis. The sensitivity of pantothenate biosynthesis to the action of α-ketobutyrate is probably based on the relatively high K_m of α-ketopantoate hydroxymethyltransferase for α-ketoisovalerate (32). Fultz et al. have constructed *S. typhimurium* strains which overproduce α- and β-isopropylmalate (14); they have proposed that it is primarily the biosynthesis of pantothenic acid which is disrupted by the decreased α-ketoisovalerate pool present in these strains (14). It would be of interest to determine whether methionine stimulates the growth of these mutant strains.

CoA and α-ketoisovalerate are substrates for α-isopropylmalate synthetase, the first enzyme in leucine biosynthesis (39). It is interesting that CoA, rather than α-ketoisovalerate, is competitive with respect to feedback inhibition by leucine (39). This regulatory phenomenon may represent a mechanism for controlling the flow of α-ketoisovalerate to pantothenic acid and leucine. If CoA is limiting, inhibition by leucine is more effective and α-ketoisovalerate is diverted to pantothenic acid or valine.

The growth requirements of strain DU501 also apply to other strains of *S. typhimurium* which lack AHAS II (2, 30). Strain MF1680 (*ilvG237*) described by O'Neill and Freundlich contains a temperature-sensitive form of AHAS II (29). At 42°C this strain is valine sensitive and requires isoleucine. We have found that methionine or pantothenate also supports the growth of MF1680 at 42°C. An extreme case of autointoxification can be demonstrated by introducing the pBR322 derivative, pDU5 (5), which contains *ilvEDAY* into strain DU608 [Δ(*ilvGEDA*)]; the expression of the *ilvEDA* enzymes in the resulting strain, DU6080, is 5- to 10-fold that seen in DU501. Strain DU6080 requires a combination of isoleucine and pantothenate or isoleucine, methionine, and lysine for full growth. Substantial growth is seen in the presence of pantothenate alone; neither isoleucine nor methionine alone stimulates growth. Apparently, the high activities of threonine deaminase and transaminase B in this *ilvG* organism cause the production of even greater levels of α-ketobutyrate which results in an exacerbation of the autointoxification. Targets other than α-ketopantoate hydroxymethyltransferase exist for the action of α-ketobutyrate. This is evident from the data presented in Table 4, which show that pantothenate is not totally effective in reversing the effects of added α-ketobutyrate. We have previ-

ously mentioned that α-ketobutyrate curtails the production of α-ketoisovalerate to some degree. Also, high levels of α-ketobutyrate have been shown to participate in a phosphoryl exchange reaction with phosphoenolpyruvate mediated by enzyme I of the phosphoenolpyruvate transport system (11).

The possible role of α-aminobutyrate in the autointoxification is revealed by the differential effects of salicylate and α-ketobutyrate. Inhibition of the growth of *S. typhimurium* by low concentrations of salicylate (72 μM) is overcome by methionine. At high concentrations of salicylate, only pantothenate is effective in reversing growth inhibition. Inhibition by 100 mM α-ketobutyrate, however, is reversed more effectively by methionine than by pantothenate. This pattern suggests that α-ketobutyrate or α-aminobutyrate may disrupt the biosynthesis of methionine in a direct manner. This view is supported by the observation that α-aminobutyrate inhibits homoserine dehydrogenase (Primerano, unpublished data).

E. coli K-12 and *S. typhimurium ilvG* strains are similar in that neither contains valine-insensitive AHAS activity, and the growth of each is inhibited by valine. *E. coli* K-12, however, does not display the growth requirements of *S. typhimurium ilvG* mutant strains. *E. coli* K-12 may be prototrophic for a number of reasons and, interestingly, two of these are recapitulated in the properties of the prototrophic revertants of DU501 and DU502. Teller et al. have shown that *E. coli* K-12 has a higher level of ketopantoate hydroxymethyltransferase than does *S. typhimurium* or *E. coli* W (38); this situation is similar to that seen in DU12, one of the class II suppressors. In addition, *E. coli* K-12 possesses intrinsically lower levels of biosynthetic threonine deaminase and transaminase B than does wild-type *S. typhimurium*; in this characteristic it is similar to the prototrophic revertant of DU502, strain DU5125, which has decreased *ilvEDA* enzyme levels. The absence of valine-insensitive AHAS activity and the decreased expression of *ilvEDA* in wild-type *E. coli* K-12 are explained by the presence of a frameshift mutation which generates a stop codon within the *ilvG* gene (21). *S. typhimurium ilvG* mutants generated by the insertion of Tn*10* represent an additional case of detoxification by decreased expression of transaminase B and threonine deaminase. These mutants are isoleucine bradytrophs which presumably do not overproduce α-ketobutyrate to the same extent as do nonpolar *ilvG* mutant strains (Primerano, unpublished data).

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