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Regulation of Branched-Chain Amino Acid Biosynthesis in *Salmonella typhimurium*: Isolation of Regulatory Mutants

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5',5',5'-Trifluoro-DL-leucine inhibited the activity of α-isopropylmalate synthetase (the initial enzyme unique to leucine biosynthesis) as well as the growth of *Salmonella typhimurium*. Mutants of *S. typhimurium* resistant to the analogue were isolated and characterized. In most cases, they overproduced and excreted leucine or leucine, valine, and isoleucine as a result of an alteration in the regulation of branched-chain amino acid biosynthesis. Biochemical and genetic tests allowed the mutants to be grouped into three classes: I, a moderately large group (13%) which had high, constitutive leucine biosynthetic enzyme levels and mutant sites linked to the leucine operon (operator constitutive); II, a single mutant in which the mutant site was linked to the leucine operon and in which α-isopropylmalate synthetase was not inhibited by leucine (feedback negative); III, a majority type which had constitutive levels of leucine, valine, and isoleucine biosynthetic enzymes and mutant sites unlinked to the leucine operon. Mutants of class I provide important evidence for the concept of an operon organization of genes involved in leucine biosynthesis. The properties of class III mutants indicate that there is some element involved in regulation which is common to the three pathways.

The biosynthetic pathways leading to leucine, valine, and isoleucine are complex and interrelated (Fig. 1). The pathways to valine and isoleucine are parallel in the sense that four intermediates in these pathways are homologous, differing only by a methylene group (26). Moreover, the reaction of each pair of homologues is catalyzed by a single enzyme (13). The pathway to leucine branches from the valine pathway, α-ketoisovalerate being the intermediate common to both pathways (3). As might be expected, the regulation of such a network of reactions is complex, and in *Salmonella typhimurium* both end product inhibition and end product repression serve as control mechanisms (10, 27). This paper describes the isolation and characterization of mutant strains of *S. typhimurium* which are resistant to an analogue of leucine, 5',5',5'-trifluoro-DL-leucine (fluoroleucine; 20), and which have altered regulatory properties.

MATERIALS AND METHODS

Bacterial strains. Strains in this report were derived from *S. typhimurium* strains LT2 *ara-9* or *ara-9 gal-205*. All strains isolated in the laboratory of the senior author carry the strain designation CV as a prefix.

Media. Nutrient broth and nutrient agar were used as complete media. A minimal salts solution (SSA) contained, per liter of distilled water: K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate dihydrate, 0.97 g; $MgSO_4$, 0.05 g. SSA supplemented with 0.2% glucose or 0.2% glucose and 1.5% agar served as liquid and solid minimal media, respectively (M medium, M plates). Enriched minimal agar contained 0.01 (EM) or 0.02% (EM 2; w/v) dehydrated nutrient broth. Plates for the assay of leucine excretion (SL plates) were prepared as follows. A 20-ml amount of solid M medium was poured and allowed to solidify in petri dishes. A 10-ml portion of the same agar, cooled to 47 C and containing 4×10^8 cells of strain *leuBCD39* per ml, was then layered on top. Plates for the assay of valine, isoleucine, and tryptophan excretion were prepared in the same way except that strain *leuBCD39* was replaced by, respectively, strains *ilvD27*, *ile-212*, and *trpC110*.

Chemicals. The following chemicals were obtained commercially: bovine serum albumin (Mann Research Laboratories, New York, N.Y.); *N*-acetyl-DL-leucine, L-ornithine, L-homoserine, L-α,β-diaminopropionic acid, *S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, D-leucine

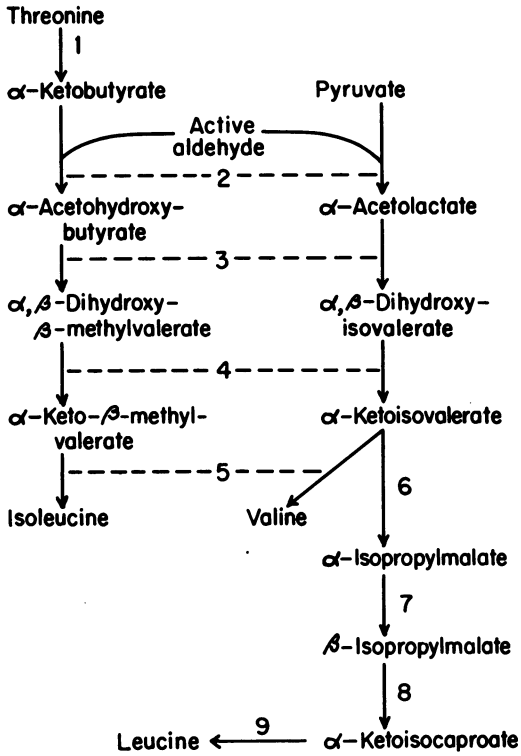

FIG. 1. *Pathways leading to the branched-chain amino acids.*

(Calbiochem, Los Angeles, Calif.); L-leucic acid, 2-methyl-DL-leucine, α-aminoisobutyric acid, L-α-amino-*n*-butyric acid, DL-norvaline, DL-norleucine, L-2,4-diaminobutyric acid, DL-C-allylglycine (Nutritional Biochemicals Corp., Cleveland, Ohio); L-α-chloroisocaproic acid, DL-α-amino-β-methoxypropionic acid, leucinol (K and K Laboratories, Plainview, N.Y.). 3-Cyclohexene-DL-alanine and cyclopentyl-DL-alanine were gifts from W. Shive and methallylglycine was donated by F. W. Dunn. Fluoroleucine, obtained initially as a gift from O. Rennert, was synthesized by the procedure of Rennert and Anker (20).

Incubation procedures. All bacteria were grown aerobically at 37 C. Aeration was provided by bubbling filtered air through tubes containing 10 ml of medium or by shaking cultures in Erlenmeyer flasks in a New Brunswick rotary shaker.

Transduction techniques. Transduction was mediated by PLT22 phage. The media and methods used were those reported by Margolin (15).

Assay for amino acid excretion. Excretion of leucine by mutants of *S. typhimurium* was determined qualitatively by an auxanographic technique. Colonies were transferred with sterile toothpicks to the surface of SL agar plates, care being exercised to avoid puncturing the surface of the agar. Fifty colonies could be conveniently tested on one SL plate. After incubation for 48 hr, leucine excretion was detected as growth of strain *leuBCD39* within the agar, beneath and surrounding the colonies. For recombinants resulting from transductions, a positive indication of leucine excretion was seen as a clear ring surrounding the colony on the SL plate. This was caused by lysis of the phage-sensitive *leuBCD39* indicator bacteria as they grew in response to the excreted leucine. Examples of these tests are shown in Fig. 2. Valine, isoleucine, and tryptophan excretion was determined in a similar fashion with plates seeded with the appropriate auxotrophs.

Isolation of mutants resistant to fluoroleucine. In isolations from solid medium, M plates containing 50, 250, and 500 μg of fluoroleucine per ml were spread with suspensions of *S. typhimurium* containing from 10^5 to 10^7 bacteria. After incubation for 48 and 72 hr, colonies of various sizes were picked and purified. Some of the colonies picked were obviously feeding the background lawn of cells. In isolations from liquid medium, tubes containing 2 ml of nutrient broth were each inoculated from a separate single colony, and after overnight growth the resulting cultures were centrifuged, washed, and resuspended in an equal volume of SSA solution. A 0.1-ml sample of each suspension (ca. 4×10^8 cells) was transferred to a tube containing 2 ml of selective medium composed of 0.2 g of sodium citrate dihydrate and 20 mg of fluoroleucine, in 100 ml of SSA solution. When the parental strain was an auxotroph, the selective medium also contained the appropriate growth factor. The tubes were incubated with aeration, and when growth became apparent in any particular tube (4 to 8 days) that culture was streaked for single colonies on nutrient agar. Ten single colonies were transferred to a nutrient agar master plate and to an SL plate to test for leucine excretion. Only a single fluoroleucine-resistant mutant from each tube was isolated and purified. In some cases, fluoroleucine-resistant mutants were isolated after treatment of the parent with 2-aminopurine. Isolated colonies of the parent were grown overnight in nutrient broth containing 0.5 mg of 2-aminopurine per ml, and these cultures were used to inoculate the fluoroleucine-containing medium.

Determination of growth rates. Erlenmeyer flasks (250 ml) containing 30 ml of M medium and inoculated with a log-phase suspension of cells were shaken in a water bath, and the absorbancy was determined at 30-min intervals. Absorbancy was converted to cell numbers (determined by direct microscopic count) by a standard curve.

Enzyme assays. Cells used for the preparation of extracts were grown aerobically in 2-liter Erlenmeyer flasks containing 300 ml of the indicated medium. The procedures for the harvesting of cells and the preparation of extracts have been described previously (2). The following enzymes in the pathways leading to valine, isoleucine, and leucine were assayed by published procedures except as otherwise noted: threonine deaminase (11), α-acetohydroxy acid synthetase (23), dihydroxyacid dehydrase (10), β-isopropylmalate (β-IPM) dehydrogenase (3), and α-isopropylmalate (α-IPM) synthetase by a modification (J. M. Calvo et al., Anal. Biochem., *in press*) of the fluorometric assay of Strassman and Ceci (24). Protein was deter-

mined by the method of Lowry et al. (14) with bovine serum albumin as the standard. In the experiments in which the extent of inhibition of α-IPM synthetase by leucine was determined, crude extracts were passed through a column (1.8 by 44 cm) of G25 Sephadex. A sample of extract was mixed with one-half volume of 40% (w/v) glucose, and a 7-ml portion was layered at the Sephadex-buffer interface. The column was buffered and eluted with 0.05 M potassium phosphate buffer, *p*H 6.8. After a volume corresponding to the void volume had passed, a 10 ml sample was collected for use as a source of ezyme.

In vitro analogue studies. In testing analogues for their effect upon α-IPM synthetase, a nitroprusside assay was used (2). Assay mixtures contained the following components in a total volume of 0.4 ml: tris(hydroxymethyl)aminomethane (*p*H 7.5), 50 μmoles; KCl, 50 μmoles; α-ketoisovalerate, 2.5 μmoles; and acetyl coenzyme A, 1.25 μmoles. Analogue (1 μmole)and analogue (1 μmole) plus L-leucine (0.25 μmole) were added to the assay mixture to test for the effect of the analogue on the enzyme and upon inhibition by leucine, respectively. The enzyme preparation was an extract of *S. typhimurium* treated with protamine sulfate (0.15 mg of protamine sulfate per mg of protein; *p*H 6.0) and ammonium sulfate (cut from 42 to 60% saturation).

Chemostat experiments. A Harvard peristaltic pump was used to transfer medium into the growth chamber (500-ml polycarbonate cylinder fitted with a gas dispersion tube for aeration), and a constant volume was maintained by aspiration through a tube extending part way into the cylinder from the top. The culture volume (250 ml) was replaced over various time periods by fresh minimal medium supplemented with 15 mg of L-leucine per liter At equilibrium, a constant absorbancy of 0.620 (12.7-mm light path) was maintained. In a typical experiment, the growth chamber was inoculated with strain *leuA124* (5×10^9 log-phase bacteria), and after incubation with vigorous aeration for 4 hr the pump was started. The bacteria were allowed to undergo at least three doublings before sampling for enzyme determinations.

RESULTS

Inhibition of α-IPM synthetase by analogue of leucine. α-IPM synthetase, the first enzyme unique to leucine biosynthesis, is inhibited by leucine (27). To investigate the specificity of this inhibition, a number of compounds having at least some structural features in common with leucine were tested for their effect upon this enzyme. The following compounds neither inhibited α-IPM synthetase nor prevented inhibition of the enzyme by leucine: L-valine, L-alanine, *N*-acetyl-DL-leucine, L-leucic acid, D-leucine, 2-methyl-DL-leucine, α-aminoisobutyric acid, glycine, L-α-chloroisocaproic acid, L-α-amino-*n*-butyric acid, DL-norvaline, L-glutamic acid, L-ornithine, L-homoserine DL-norleucine, L-2,4-diaminobutyric acid, L-methionine, L-aspartic acid, DL-α-amino-β-methoxypropionic acid, L-α,β-diamino-propionic acid, *S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, L-cysteine sulfinic acid, DL-C-allylglycine, L-phenylalanine, L-histidine, L-leucinamide, leucinol, and methallylglycine. A number of compounds such as L-valine and DL-norleucine, although not inhibiting α-IPM synthetase themselves, enhanced the inhibition caused by leucine by 30%. The following compounds had an inhibitory effect upon the enzyme: L-isoleucine, 3-cyclohexene-DL-alanine, cyclopentyl-DL-alanine, and fluoroleucine. Some representative data for various types of interactions are shown in Table 1.

Isolation of mutants resistant to fluoroleucine. The analogues (other than the standard amino acids) were tested for the ability to inhibit the growth of *S. typhimurium*. The only compound which had a significant effect upon the growth rate was fluoroleucine; concentrations as low as 3 μg/ml caused a lag of ca. 24 hr in liquid medium. At higher concentrations of fluoroleucine (>250 μg/ml), the inhibition was greater and was almost completely reversed by L-leucine (10 μg/ml) but not by the other 19 amino acids, adenine, guanine,

TABLE 1. *Effect of various leucine analogues upon the activity of α-IPM synthetase*

Compound	Concn	Amt of SH formed in 10 min	Per cent inhibition
	M	*μmoles*	
None		0.430	
L-Leucine	6.0×10^{-4}	0.255	41
L-Alanine	2.5×10^{-3}	0.425	1
L-Valine	2.5×10^{-3}	0.445	0
L-Leucine + L-alanine	6.0×10^{-4} + 2.5×10^{-3}	0.260	39
L-Leucine + L-valine	6.0×10^{-4} + 2.5×10^{-3}	0.175	59
None		0.420	
L-Leucine	6.0×10^{-4}	0.235	44
L-Isoleucine	2.5×10^{-3}	0.330	22
L-Leucine + L-isoleucine	6.0×10^{-4} + 2.5×10^{-3}	0.100	76
None		0.407	
L-Leucine	6.0×10^{-4}	0.220	46
Cyclopentylalanine	2.5×10^{-3}	0.265	35
L-Leucine + cyclopentylalanine	6.0×10^{-4} + 2.5×10^{-3}	0.145	64
None		0.555	
L-Leucine	6.0×10^{-4}	0.240	57
Fluoroleucine	1.25×10^{-3}	0.340	39
L-Leucine + fluoroleucine	6.0×10^{-4} + 1.25×10^{-3}	0.145	74

uracil, or a mixture of vitamins (pantothenic acid, riboflavine, thiamine, nicotinic acid, biotin, pyridoxine). The sensitivity of *S. typhimurium* to fluoroleucine was much greater than that reported for *Escherichia coli* (20). For *E. coli*, Rennert and Anker only observed a 3-hr lag with concentrations of analogue approaching 1 mg/ml.

Mutants resistant to fluoroleucine could be isolated from either solid or liquid medium. Good results were obtained with isolations from solid M medium with fluoroleucine concentrations greater than 250 μg/ml and 10^8 cells. With less fluoroleucine or more cells, a heavy background growth made the isolation of mutants difficult. Isolation of fluoroleucine-resistant mutants from liquid culture was facilitated by omitting glucose from the medium and by employing a citrate concentration of 0.2%. Tubes containing this medium supplemented with 200 μg of fluoroleucine per ml and inoculated to give a final titer of 10^7 cells per ml became turbid only after incubation for 3 to 10 days. A total of 225 fluoroleucine-resistant mutants were isolated in this study.

Excretion of branched-chain amino acids. Most of the fluoroleucine-resistant mutants that were isolated appeared to have lost some degree of control over the production of branched-chain amino acids. Wild-type *S. typhimurium* did not excrete detectable amounts of leucine (or isoleucine) after incubation for 2 days (Fig. 2). The control over valine production, however, was not as tight, and valine excretion by the wild type was detected within this time period. As judged by auxanographic tests of the type illustrated in Fig. 2, some fluoroleucine-resistant mutants excreted leucine, whereas others excreted leucine, valine, and isoleucine. That these mutants did not have a defect resulting in an excretion of amino acids in general was demonstrated by an experiment which tested for the excretion of an amino acid biosynthetically unrelated to leucine, valine, and isoleucine; neither the wild type nor the mutants excreted tryptophan. (It should be mentioned that, as used here, the term "excretion" is not meant to imply a physiological or active process but rather is intended to mean only that a metabolite is released into the medium.)

Because fluoroleucine was not readily available in large amounts, the property of excretion as measured by auxanographic tests was adopted as a phenotype which could be conveniently measured in genetic experiments. Studies in this paper all relate to strains which excreted one or more branched-chain amino acids.

Growth rates. The generation times of 40 fluoroleucine-resistant strains were determined. The growth rates of some of the strains were identical with that of the wild type, whereas others had growth rates slightly reduced (generation times elevated by 2 to 12 min; Table 2). The growth rate of strain CV123 was not increased when the medium was supplemented with a mixture of L-leucine, L-valine, and L-isoleucine.

Linkage of mutant sites to the leucine operon. Strains were tested for linkage of their mutant sites to the leucine operon by transductional analysis. Phage grown on the fluoroleucine-resistant strains were used to transduce a leucine auxotroph to prototrophy and the recombinants were scored for the leucine excretion phenotype (Table 3). A control cross with phage grown on

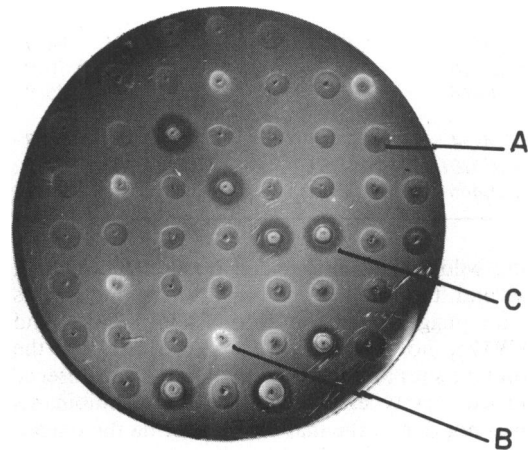

FIG. 2. *Auxanographic test for leucine excretion. (A) Wild-type colony; no excretion of leucine is detectable. (B) CV112 colony; brightness is due to light scattered from bacteria seeded within the agar. (C) Recombinant from a transduction having the CV112 mutation which results in leucine excretion. The clear halo surrounding the colony is caused by lysis of the phage-sensitive indicator bacteria seeded within the agar.*

TABLE 2. *Growth rates of some fluoroleucine-resistant strains*

Strain	Generation time (min)[a]
ara-9 gal-205 (wild type)	46.4 ± 1.5 (23)[b]
CV6	47.0 ± 1.3 (7)
CV112	47.2 ± 1.2 (6)
CV191	48.3 ± 2.4 (8)
CV19	49.9 ± 1.4 (8)
CV123	58.8 ± 1.9 (9)
CV20	53.1 ± 2.7 (7)
CV21	47.4 ± 1.8 (7)

[a] Time required for the cells to double in number.

[b] Number of determinations preceded by the standard deviation.

TABLE 3. *Linkage of mutant sites to the leucine operon*

Cross		No. of colonies analyzed	No. with excreter phenotype	Percentage
Recipient	Donor			
leuA124	Wild type (LT2)	632	0	0
leuA124	CV4	1,157	0	0
leuA124	CV19	200	0	0
leuBCD39	CV125	200	0	0
leuA124	CV6	830	816	98.4
leuC5101	CV6	200	160	80.0
leuD466	CV6	735	497	67.7
thrA201	CV6	51	0	0
leuA124	CV177	460	454	98.9
leuC5101	CV177	200	140	70.0
leuD466	CV177	200	130	65.0
leuA124	CV191	399	378	95.7
leuC5101	CV191	200	169	84.5
leuD466	CV191	200	161	80.5

the wild-type strain yielded no excreters among several thousand recombinants. In the crosses with phage grown on strains CV4, CV19, and CV125, no linkage was observed between the mutant sites and the leucine operon. The absence of excreter types from among the recombinants was not due to the inability of scoring the phenotype in these transductions, because in crosses in which the recipient carried the fluoroleucine resistance marker (selection being made for *ara*$^+$ or *gal*$^+$) all of the recombinants were excreters. Strains CV6, CV177, and CV191, donors in the last three groups of crosses in Table 3, are representative of a group of mutants with lesions linked to the leucine operon. The very high proportion of excreters found among the recombinants suggests that these mutant sites are located within or very close to the leucine operon. The lesions *leuA124*, *leuC5101*, and *leuD466* lie at the far left, middle, and far right of the leucine operon, respectively (Fig. 3). The results suggest that the mutant sites of strains CV6, CV177, and CV191 are located on the left side of the leucine operon with the sites of strains CV6 and CV177 being very closely linked to *leuA124*. Of 126 strains examined, 90 excreted one or more branched-chain amino acids, and of these 17 had mutant sites that were linked to the leucine operon by transduction.

Leucine, valine, and isoleucine enzyme levels. The specific activities of a number of enzymes involved in the biosynthesis of leucine, valine, and isoleucine were determined (Fig. 1, Table 4). Strains were grown under two different conditions, in minimal medium (partially derepressed) and in minimal medium supplemented with L-valine, L-isoleucine, and L-leucine (repressed). The levels of enzymes from the wild type were only two to three times higher in cells grown in minimal medium than in those grown in supplemented medium (Table 4). More highly derepressed enzyme levels could be induced by growing auxotrophs in a medium containing a limiting amount of growth factor in a chemostat (Fig. 4). At doubling times greater than 3 hr, the specific activity of β-IPM dehydrogenase from strain *leuA124* was 85, a value some 60 times greater than the repressed level.

The mutation in strain CV6 resulted in a derepression of the enzymes involved in leucine biosynthesis (Table 4). The increase in specific activity relative to the wild type (values in parentheses in Table 4) was rougly the same for α-IPM synthetase and β-IPM dehydrogenase, indicating coordinate derepression. Growth of strain CV6 in a medium supplemented with leucine, valine, and isoleucine resulted in only a slight, though reproducible, decrease in the specific activities of the leucine biosynthetic enzymes. The isoleucine-valine biosynthetic enzyme levels of strain CV6, however, did not differ significantly from those of the wild type. The results above for strain CV6 were, with one exception, characteristic of fluoroleucine-resistant strains that excreted leucine, but not valine or isoleucine, and in which the mutant sites were linked to the leucine operon by transduction.

The results of enzyme determinations in extracts of strains CV20, CV4, CV5, and CV19 are also given in Table 4. They were characteristic of many of the fluoroleucine-resistant mutants studied. Both the leucine and the isoleucine-valine biosynthetic enzymes were derepressed above the level found in the wild-type extracts. The leucine-forming enzymes were coordinately derepressed and the addition of isoleucine, valine, and leucine to the growth medium did not cause significant repression. Two of the enzymes involved in isoleucine and valine biosynthesis, threonine deaminase and dihydroxyacid dehydrase, were derepressed over a much narrower range (1.5- to 10-fold) but the derepression was also coordinate.

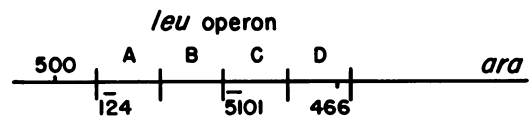

FIG. 3. *Organization of the leucine operon in S. typhimurium.*

TABLE 4. *Levels of some enzymes involved in leucine, valine, and isoleucine biosynthesis*

Strain	Growth conditions[a]	Specific activity[b] of enzymes involved in the biosynthesis of				
		Leucine		Isoleucine and valine		
		α-IPM synthetase	β-IPM dehydrogenase	Threonine deaminase	Dihydroxyacid dehydrase	α-Acetohydroxy acid synthetase
Wild type	Minimal + ilvaleu	0.30	0.89	7.50	2.50	0.53
Wild type	Minimal	0.82	2.80	12.5	4.70	1.50
CV6	Minimal + ilvaleu	11.2 (37)[c]	44.1 (49)[c]	8.40	2.20	0.70
CV6	Minimal	13.9 (17)	54.0 (19)	11.2	3.10	2.50
CV20	Minimal + ilvaleu	1.55 (5.2)	5.42 (6.1)	18.0 (2.4)	7.00 (2.8)	5.30 (10)
CV20	Minimal	2.18 (2.7)	8.17 (2.9)	22.7 (1.8)	11.1 (2.4)	7.80 (5.2)
CV4	Minimal + ilvaleu	8.10 (27)	27.7 (31)	18.5 (2.5)	6.00 (2.4)	27.1 (51)
CV4	Minimal	8.16 (10)	28.4 (10)	21.4 (1.7)	7.40 (1.6)	21.0 (14)
CV5	Minimal + ilvaleu	10.1 (34)	35.0 (40)	23.7 (3.2)	7.80 (3.1)	36.5 (69)
CV5	Minimal	11.3 (14)	42.7 (15)	36.0 (2.9)	15.0 (3.2)	37.7 (22)
CV19	Minimal + ilvaleu	16.1 (54)	60.2 (68)	65.0 (8.7)	38.0 (15)	48.0 (91)

[a] Strains were grown in either minimal medium (SSA plus 0.2% glucose) or in minimal medium supplemented with (ilvaleu) L-leucine and L-isoleucine (each at 50 μg/ml) and L-valine (100 μg/ml).
[b] Amount (μmoles) of product formed per hour per milligram of protein.
[c] Values in parentheses represent the increase in specific activity relative to the wild type.

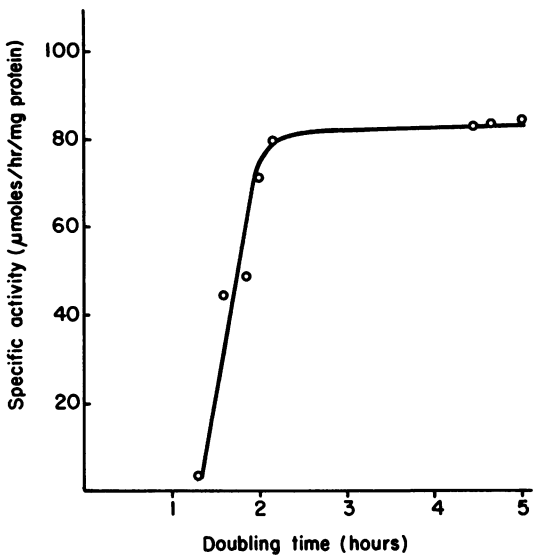

FIG. 4. *Specific activity of β-IPM dehydrogenase from leuA124 as a function of doubling time. A chemostat with leucine as the growth factor was used to control the doubling time.*

α-Acetohydroxy acid synthetase levels were not coordinate with any of the others and, like the two leucine-forming enzymes, were derepressed to a level many times above the wild-type repressed level (50- to 90-fold in some cases). There did not seem to be any great differences between these strains in their pattern of enzyme levels; rather, the major difference between them was a quantitative one. When the repressed α-IPM synthetase levels of strains CV20, CV4, CV5, and CV19 were compared to the repressed levels of that enzyme in the wild type, it was found that the mutants were 5.2-, 27-, 34-, and 54-fold derepressed, respectively. Nearly the same percentage increases occurred in the corresponding levels of β-IPM dehydrogenase and α-acetohydroxy acid synthetase (Table 4). On the other hand, the amount of derepression of threonine deaminase and the dehydrase were nearly the same in strains CV20, CV4, and CV5 grown under repressed conditions. However, the levels of these two enzymes were higher in strain CV19.

The enzyme levels of strain CV191 (Table 5) were not significantly different from those of the wild type. This observation, plus two other properties of strain CV191, excretion of leucine (but not of either isoleucine or valine) and linkage of its mutant site to the leucine operon, distinguished it as a unique type of fluoroleucine-resistant mutant. Thus far, of several hundred mutants that have been screened, strain CV191 was the only one found that had these properties.

End product inhibition of α-IPM synthetase. Figure 5 shows the effect of L-leucine upon the

TABLE 5. *Enzyme levels of strain CV191*

Strain	Growth conditions[a]	Specific activity of enzymes involved in the biosynthesis of				
		Leucine		Isoleucine and valine		
		α-IPM synthetase	β-IPM dehydrogenase	Threonine deaminase	Dihydroxyacid dehydrase	α-Acetohydroxy acid synthetase
Wild type	Minimal + ilvaleu	0.11[b]	1.92[b]	5.2	2.6	0.8
Wild type	Minimal	0.27	5.20	11.8	4.8	1.8
CV191	Minimal + ilvaleu	0.21	3.8	6.4	3.6	1.2
CV191	Minimal	0.27	4.0	8.4	4.2	3.6

[a] Same as footnote *a* in Table 4.

[b] These values differ from those in Table 3 partly because a different protein was used to prepare the protein standard curve.

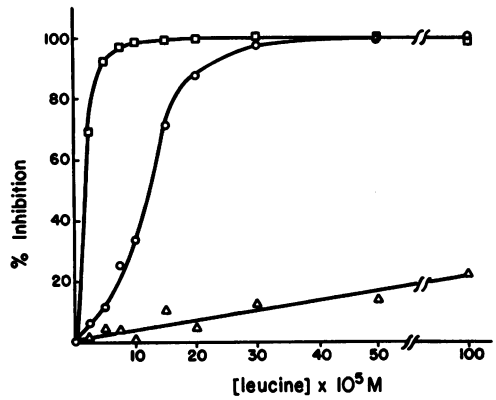

FIG. 5. *Effect of* L-*leucine upon the activity of α-IPM synthetase in extracts from several strains. Symbols:* ○, *ara-9 gal-205 (wild type);* □, *CV19;* △, *CV191. Each point represents the average of four determinations (duplicate determinations of two separate cultures).*

activity of α-IPM synthetase in extracts from several different strains. L-Leucine inhibited the wild-type enzyme 50% at a concentration of ca. 1.2×10^{-4} M. The synthetase from strain CV191, on the other hand, was not sensitive to inhibition caused by leucine except at high concentrations where a slight inhibition was observed. The possibility that extracts of strain CV191 contained some component which reduced the sensitivity of α-IPM synthetase to inhibition by leucine was eliminated by the results of a "mixing experiment"; extract from strain CV191 did not diminish the inhibition of the wild-type synthetase by leucine.

It was anticipated that α-IPM synthetase from other fluoroleucine-resistant strains (e.g., CV19, CV112) would be as sensitive to inhibition by leucine as the enzyme from the wild type. Unexpectedly, the synthetases from strains CV19, CV112, and CV123 seemed more sensitive than that from the wild type, 50% inhibition occurring at 1.5×10^{-5} M L-leucine. (In Fig. 5, only the curve for strain CV19 is shown; the curves for strains CV112 and CV123 can be superimposed on the CV19 curve.) All extracts had been passed through Sephadex before being assayed; thus, the difference cannot be explained by assuming that the extract prepared from strain CV19 contained more leucine than the extract derived from the wild type. The results of the following experiment suggested that the synthetases from strain CV19 and the parent are not fundamentally different. Strain *leu-5057* is isogenic with the wild type except that it has a multisite mutation which covers the *leu A* cistron and thus produces no α-IPM synthetase. Cells of strains *leu-5057* and CV19 were mixed in a proportion such that the specific activity of α-IPM synthetase in an extract prepared from the mixture was similar to that from the wild type. The sensitivity of the synthetase in the latter mixed extract to inhibition by leucine was less than that for the enzyme in the extract prepared from strain CV19 alone (50% inhibition occurred at 6×10^{-5} M L-leucine). We conclude that the sensitivity of the synthetase to inhibition by leucine is a function of either the synthetase concentration or the ratio of synthetase to other protein. Thus, there is no evidence indicating that the mutation in strain CV19 resulted in any alteration in the structure of α-IPM synthetase.

DISCUSSION

The detailed mechanisms by which fluoroleucine inhibits the growth of *S. typhimurium* are just now being studied. On a molar basis, it is almost as effective as leucine as an inhibitor of α-IPM synthetase. In *E. coli* grown under certain

conditions, fluoroleucine is incorporated into protein (20). It seems likely that the same is true in *S. typhimurium* since fluoroleucine protects leucine transfer ribonucleic acid (tRNA) from inactivation by periodate and the analogue allows protein synthesis to continue in a leucine auxotroph (M. Freundlich and J. Trela, Bacteriol. Proc. p. 132, 1968). Thus, the inhibition might be due to "false feedback" (16), to inhibition of the leucine-activating enzyme, or to the synthesis of fluoroleucine-containing proteins which do not function or which inhibit growth, or, of course, to some combination of the three.

The properties of some of the strains studied are summarized in Table 6. There is no apparent correlation between growth rates and the levels of enzymes involved in branched-chain amino acid biosynthesis. For example, strains CV6 and CV112 have high leucine biosynthetic enzyme levels and wild-type growth rates. Strain CV19 has very high levels of both leucine and isoleucine-valine biosynthetic enzymes and its doubling time is ca. 3.5 min longer than that of the wild type. In contrast, strain CV20 has only moderately elevated enzyme levels yet its doubling time is 9 min longer than that of the wild type. It has not yet been determined whether slow growth and elevated enzyme levels are the consequence of a single genetic change.

On the basis of the properties shown in Table 6, these strains may be divided into three classes. Mutants within two of these classes have altered sites which are linked by transduction to the cluster of four *leu* cistrons (Fig. 3). This cluster is thought to comprise a single operon on the basis of the following data: the enzymes determined by these cistrons vary coordinately under various conditions of derepression (2); extensive mapping studies indicate that the four cistrons are contiguous (15; Calvo, *unpublished data*); some mutant sites that lie in cistron A result in a decrease in the amount of cistron B product which is formed, whereas the reverse is not true (2); *leu-500*, a mutant site located at the left end or outside of cistron A, contains barely detectable amounts of enzymes specified by cistrons A, B, C, and D (15). The group of strains including CV6, CV112, and CV117 have mutant sites at the left end (as diagrammed in Fig. 3) of this cluster of genes and have high, constitutive leucine enzyme levels. More detailed mapping studies (Calvo, *in preparation*) place these sites to the left of cistron A sites but to the right of *leu-500*. The strains bearing these lesions are tentatively considered to be "operator-constitutive" mutants and they are assigned the genotype locus designation *leu0* (5). Confirmation that these strains are indeed "operator-constitutive" requires dominance tests, but such tests are not yet possible because an episome carrying the leucine operon is not available.

The α-IPM synthetase activity in crude extracts of strain CV191 is not significantly inhibited by high concentrations of L-leucine. Thus, this analogue-resistant mutant appears to lack a specific end product inhibition mechanism, a phenomenon first described by Moyed (16). That the mutation directly affected the structure of α-IPM synthetase is suggested both by the mixing experiments and the fact that the mutant site is located

TABLE 6. *Properties of several classes of fluoroleucine-resistant strains*

Class	Representative strains	Resistance to fluoroleucine	Excretion[a]		Linkage to *leu* operon	Growth rate	Enzyme levels		Sensitivity of α-IPM synthetase to leucine
			Leucine	Isoleucine			Leucine	Isoleucine-valine	
	ara-9 gal-205 (parent)	Sensitive	−	−		Normal	Normal (low)	Normal (low)	Normal (sensitive)
leu O	CV6 CV112 CV177	Resistant	+	−	Yes	Normal	High	Normal	Normal
leu A	CV191	Resistant	+	−	Yes	Slightly slow	Normal	Normal	Insensitive
flr	CV19 CV123 CV20 CV21	Resistant	+	+	No	Normal to slow	High	High	Normal

[a] Plus and minus refer to the presence or absence of excretion as determined in an auxanographic test.

within the leucine operon. Preliminary deletion mapping studies indicate that the mutant site in strain CV191 is located near the operator-distal end of cistron A. This site has been assigned the genotype designation *leuA2010* (5).

The third class of fluoroleucine-resistant mutants (strains CV19, CV123, etc.) have mutant sites unlinked to the leucine operon and have constitutive levels of both leucine and isoleucine-valine biosynthetic enzymes. These strains are tentatively assigned the genotype locus designation *flr* (acronym for fluoroleucine-resistance).

At least some of them have mutant sites which lie near the *gal* region of the genome (R. R. Alexander and J. M. Calvo, Genetics, *in press*). The properties of these strains strongly support the idea of a control element common to all three pathways. That the common element must act through the cytoplasm seems clear from the location of the genes in question. The genes (*ilv*) which specify the isoleucine-valine-forming enzymes are clustered in a region of the genome near the *metE* locus (minute 123 on the standard map of *S. typhimurium*, reference 21), whereas the genes involved in leucine biosynthesis are clustered near the arabinose operon (minute 4). In *E. coli*, the *ilv* cluster is comprised of at least two and probably three operons (19), and it is likely that the same is true in *S. typhimurium*. Thus, a mutation near *gal* (minute 17) affects the expression of several operons located in different parts of the genome. That genetic loci located in different parts of the genome fall under common control is known in a number of systems (1).

An earlier study by Freundlich, Burns, and Umbarger (10) revealed another common feature in the control of leucine, valine, and isoleucine biosynthesis, namely, leucine. They showed that, although leucine alone repressed the formation of enzymes unique to the leucine pathway, valine, isoleucine, *and leucine* were required for repression of the isoleucine-valine-forming enzymes. This phenomenon was termed multivalent repression.

Do amino acids themselves or some derivative thereof act most directly in repression? In some cases, it is clear that amino acid activation is required for repression (17), whereas, in others the amino acids themselves may function directly (6). In the case of the branched-chain amino acids, the following evidence indicates that their activation is required for repression of the isoleucine, valine, and leucine biosynthetic enzymes. (i) Szentirmai et al. isolated mutant strains of *E. coli* having derepressed levels of several isoleucine-valine enzymes, presumably as a result of an altered isoleucine-activating enzyme (25). (ii) A mutant of *E. coli* with a temperature-sensitive, valine-activating enzyme was shown by Eidlic and Neidhardt (8) to have derepressed levels of its isoleucine and valine enzymes when grown at a restrictive elevated temperature. (iii) Preliminary experiments suggest that some of the mutants analyzed in this study have an altered leucine-activating enzyme. Furthermore, Freundlich has evidence which suggests that, in addition to activation, repression requires that valine be charged to valine tRNA (9). However, the interpretation of his results is not completely unambiguous because an alternative explanation (that lowered enzyme levels result from false proteins rather than from repression) cannot be eliminated. Thus, although it seems likely that various types of charged tRNA are involved in repression, it is also possible that the activating enzyme itself or an activating enzyme-amino acid-tRNA complex is the repressor.

A model which assumes negative control and which is consistent with multivalent repression, the properties of *flr* strains, and the role of amino acid-activating enzymes is presented in Fig. 6. The product of a proposed regulator gene (assumed to be a protein; designated apo*R* in Fig. 6) is pictured as being activated for binding to the *leuO* region whenever there is an ample supply of leucine tRNA ($tRNA^{leu}$) which is charged with leucine (leu $tRNA^{leu}$). For repression of the *ilvO* regions, valine tRNA charged with valine (val $tRNA^{val}$) and isoleucine tRNA charged with isoleucine (ile $tRNA^{ile}$) must also be present in ample amounts. According to this model, the properties of *flr* mutant strains could be the result of either an alteration in the aporepressor or in the rate of charging of one or more $tRNA^{leu}$ species. The latter might be caused by an altered activating enzyme or an altered or missing species of tRNA. There is no direct evidence at present for the participation of a protein repressor molecule or a tRNA in the regulation of this system. However, the existence of regulator genes that give rise to protein products that have the properties of repressor molecules is now well documented in at least a few cases (12, 18) and there is direct evidence for the participation of $tRNA^{his}$ in the regulation of histidine biosynthesis (22).

In the valine-sensitive *E. coli* strain K-12, the control pattern is slightly more complicated, since studies with thiaisoleucine-resistant mutants have recently revealed that isoleucine is only required for the repression of three of the isoleucine-valine enzymes in that organism (7, 25). The model represented in Fig. 6 would be consistent with this additional data if an intermediate complex, R'_2, involving the aporepressor and charged $tRNA^{leu}$ and $tRNA^{val}$ species, is postulated. In this case, both R_2 and R'_2 would bind to the operator sites

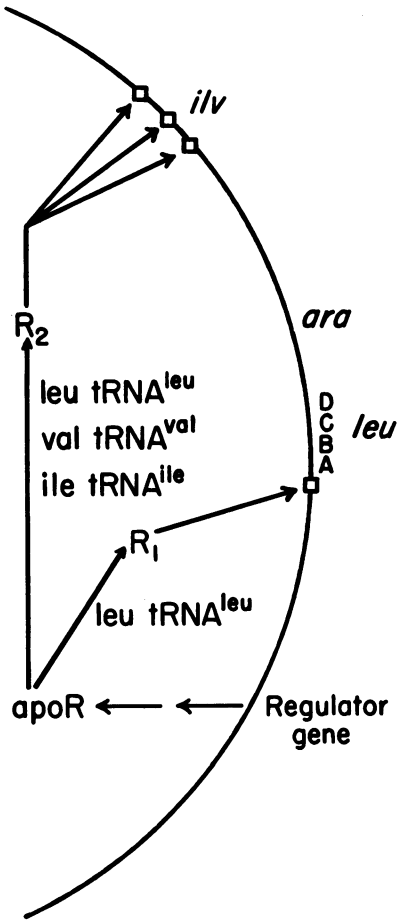

FIG. 6. *Model for the control of the synthesis of enzymes which form branched-chain amino acids. The squares represent operator regions; apoR, aporepressor; R_1 and R_2, active repressor molecules.*

within the *ilv* gene cluster concerned with the formation of acetohydroxyacid synthetase and isomeroreductase, whereas only R_2 would bind to the operator site(s) concerned with the other three isoleucine-valine-forming enzymes.

The three classes of mutants studied all seem to be resistant to fluoroleucine by virtue of the fact that they overproduce leucine. This is consistent with the observation that small amounts of exogenous leucine (10 μg/ml) overcome the inhibition caused by a larger concentration of fluoroleucine (200 μg/ml). The loss of either end product repression (classes 1 and III) or end product inhibition (class II) does not have an extensive adverse effect upon the growth of these strains. Apparently, the loss of a single control mechanism leads to the formation of sufficient excess leucine to overcome inhibition by fluoroleucine, but the operation of the remaining control mechanism prevents a serious imbalance from developing. As might be expected, the simultaneous absence of both types of control mechanisms does have a deleterious effect upon the organism. Calvo and Calvo (4) constructed, by genetic means, a double mutant that lacked both end product inhibition and repression of leucine biosynthesis. This strain grew slowly and converted ca. 50% of the glucose supplied as a carbon source to leucine.

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