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# Regulation of the Pool Size of Valine in *Escherichia coli* K-12

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Three mutations (*ilvH611*, *ilvH612*, and *ilvH613*) are described which make *Escherichia coli* K-12 resistant to valine inhibition and are located near *leu*. The expression of the *ilv* genes appears to be normal in these mutants since the isoleucine-valine biosynthetic enzymes are not derepressed relative to the wild type. The intracellular concentration of valine is, however, higher in the mutants than in the isogenic *ilvH*$^+$ strain. These mutants also excrete valine, probably because of the high intracellular concentration of this amino acid. The pool size of valine is regulated independently from that of isoleucine and leucine. The increased intracellular concentration of valine is due to a decreased feedback inhibition that valine exerts on its own biosynthetic pathway. In fact, acetolactate synthase activity assayed in extracts of *ilvH612* and *ilvH613* mutants is more resistant to valine inhibition than the activity assayed in the *ilvH*$^+$ isogenic strain. Two forms of acetolactate synthase activity can be separated from these extracts by adsorption and elution on hydroxylapatite. One of them is as sensitive to valine inhibition as that of the wild type, the other is more resistant to valine inhibition.

"Pool" has been defined as the total quantity of low-molecular-weight compounds that may be extracted from the cell under conditions such that the macromolecules are not degraded into low-molecular-weight subunits (4). One might think that it is an advantage for the cell to have very small pools with high turnover because in this way there is a low contribution to the intracellular osmotic pressure. On the other hand, it might be useful for the cell to keep relatively high pools for key intermediates, like amino acids. Indeed most intermediates in amino acid biosynthesis are undetectable unless there is a block in the metabolic pathway (30), whereas the amino acids themselves are detectable. The pool size of an amino acid is determined by at least two factors: (i) the activity of its aminoacyl-transfer ribonucleic acid synthetase and that of other utilization systems; (ii) its rate of production through the metabolic pathway, determined by several parameters, such as the repression level, the activity of the limiting enzyme, and the feedback inhibition that the amino acid exerts on the activity of the first enzyme of its metabolic pathway.

One peculiarity of *Escherichia coli* K-12 is the inhibition of the growth caused by the amino acid valine when this is added to a minimal medium (27). Valine inhibition is caused by inhibition of isoleucine biosynthesis (29) and it is reversed by addition of isoleucine to the growth medium. Valine, in fact, inhibits acetolactate synthase (acetolactate pyruvate-lyase [carboxylating], EC 4.1.3.18) activity which has been shown to be required for isoleucine and valine biosynthesis (10).

Mutants resistant to valine inhibition (Val$^r$) have been shown to occur spontaneously (8) with a frequency of about $5 \times 10^{-7}$. Resistance to valine can be accompanied by an increased rate of isoleucine biosynthesis. Mutants of this type are regulatory mutants and an operator gene mutant was found in this class (21-23). Other Val$^r$ mutants have described, which are altered in one or more transport systems for isoleucine, leucine, and valine (11-13). Resistance caused by loss of sensitivity of acetolactate synthase activity to valine inhibition has been reported (16, 19, 20, 23). Leavitt and Umbarger (16) showed a direct correlation between the pool size of valine and end-product inhibition of acetolactate synthase activity.

In the present paper we describe mutations causing valine resistance that decrease the sensitivity of acetolactate synthase activity to valine inhibition and permit the separation of two forms of this enzyme. This phenotype is due to the absence of the *ilvH* (previously *brnP*; 13) gene product. A consequence of the decreased feedback inhibition of acetolactate synthase activity is a specific increase in the intracellular concentration of valine. Although the biosynthesis of the branched chain amino acids is catalized by common enzymes, the pool size of

valine is regulated independently from that of isoleucine and leucine.

A preliminary account on these results has been reported (M. De Felice, J. Guardiola and M. Iaccarino, Lunteren Lectures on Molecular Genetics, Atti Ass. Genet. Ital. vol. XIX, p. 144-145, 1974).

## MATERIALS AND METHODS

**Bacterial strains.** Table 1 lists the strains of *E. coli* used.

**Reagents and media.** Reagents were of the highest purity commercially available. Amino acids used were all L-form. Dansyl chloride was purchased from Sigma Chemical Co., St. Louis, Mo. and [$^3$H]dansyl chloride was purchased from the Radiochemical Centre, Amersham, England. Micropolyamide plastic sheets were obtained from Ernst Schütt Jr., Göttingen, Germany. Hydroxylapatite (Bio Gel HTP) was purchased from Bio Rad Laboratories, Richmond, Calif.

The minimal medium used was "minimal citrate" (31). Usual supplements, when required, were: glucose, 0.4%; tryptophan, 25 μg/ml; arginine, 100 μg/ml; other amino acids, 10 μg/ml; nucleosides, 50 μg/ml; and thiamine 10 μg/ml. Rich media were L broth (17) and nutrient broth (5 g of NaCl and 8 g of nutrient broth (Difco), in 1 liter of distilled water).

**Transduction.** Transductions were performed either with *P1kc* or with *P1CMclr100* (24) as described previously (12).

**Mutagenesis.** For nitrosoguanidine mutagenesis a culture was grown overnight in a minimal medium, centrifuged, washed in unsupplemented minimal medium, resuspended in the same medium at the original density, and shaken for 30 min at 37 C in the presence of 30 μg of the mutagen per ml. A 10-fold dilution of this suspension was grown overnight in appropriately supplemented medium.

Ultraviolet mutagenesis was performed by irradiating a bacterial suspension at a dose that kills about 95% of the bacteria.

**Test for amber mutations.** To check if the phenotype of a mutant was due to an amber mutation, a given strain was lysogenized either with *φ80psu3* (prepared from strain 594) or with *φ80*, as a control. The lysogens were immune to both phages but sensitive to *φ80vir*; the *φ80* lysogens supported the growth of phage T4 but not of a phage T4 containing the mutation *amB17* in gene *23* (obtained from A. Coppo and J. F. Pulitzer). The *φ80psu3* lysogens supported the growth of both phages. The relevant phenotype of the strain to be tested as well as that of the corresponding *φ80psu3* lysogen was determined. When the phenotype of a mutation changed after lysogenization with *φ80psu3* the mutation was classified as an amber mutation.

**Enzyme assays and preparation of cell extracts.** Threonine deaminase, the *ilvA* gene product (L-threonine hydrolyase [deaminating], EC 4.2.1.16) was assayed by measuring the rate of formation of α-ketobutyrate as described (15). Acetolactate synthase was assayed by determining the rate of acetolactate formation (26). Acetohydroxy acid isomeroreductase, the *ilvC* gene product, was assayed as previously

TABLE 1. *Bacterial strains*[a]

| Strains | *ilvH* | Genotype | Origin |
|---|---|---|---|
| 594 | +[b] | *phoA, trp, φ80psu3* | From E. Gallucci (1) |
| AT739 | + | *thr-10, pyrA53, thi-1*, λ-HfrH | From A. L. Taylor |
| AW206 | + | *ilv0603, thr-10, pyrA53, thi-1*, λ$^-$, HfrH | Spontaneous Val$^r$ from AT739 (15) |
| Ca85 | + | *thi-1, his, lac*$^{am}$, HfrH | From J. Beckwith |
| X478 | + | *thi-1, leuB, metE, proC, purE, trp, lysA, ara, xyl, lacZ, azi, str, tonA, tsx*, F$^-$ | From P. Berg |
| MI149h | + | *thi-1*, HfrH | Thr$^+$, Pyr$^+$ transductants of AT739 |
| MI167 | *ilvH612* | *ara, thi-1*, λ$^-$, HfrH | See Results |
| MI167h | *ilvH612* | *thi-1*, λ$^-$, *ara*, HfrH | Thr$^+$, Pyr$^+$, Val$^r$ transductant of AT739 with P1 grown on MI167 |
| MI168 | *ilvH612* | *thi-1*, λ$^-$, HfrH | Ara$^+$ transductant of MI167 |
| MI183 | *ilvH611* | *thi-1*, HfrH | This Laboratory (13) |
| MI183a | *ilvH611* | *thi-1, metE, proC, purE, trp, lysA, ara, xyl, lacZ, azi, str, tonA, tsx*, F$^-$ | Leu$^+$, Val$^r$ transductant of X478 |
| MI183h | *ilvH611* | *thi-1*, λ$^-$, HfrH | Thr$^+$, Pyr$^+$, Val$^r$ transductant of AT739 with P1 grown on MI183 |
| MI219 | *ilvH613* | *thi-1, his, lac*$^{am}$, HfrH | From Ca85 by nitrosoguanidine mutagenesis |
| MI219h | *ilvH613* | *thi-1*, λ$^-$, HfrH | Thr$^+$, Pyr$^+$, Val$^r$ transductant of AT739 with P1 grown on MI219 |
| MI226 | + | *thr-10, pyrA53, thi-1, ilv0603, ilvG605*, λ$^-$, HfrH | From AW206 (Iaccarino, Favre, and Freundlich, manuscript in preparation) |
| MI244 | *ilvH613* | *thr-10, thi-1, ilv0603, ilvG605*, λ , HfrH | Pyr$^+$ Val$^r$ transductant of MI226 with P1 grown on MI219 |

[a] Symbols for genetic markers are those used by Taylor and Trotter (28).

[b] Wild-type allele.

described (13). Specific activities are expressed as nanomoles of product formed per minute per milligram of protein.

Bacteria grown under the desired conditions were harvested by centrifugation at 4 C and then frozen as a pellet at −20 C. To 100 to 150 mg of frozen cell pellet, 1 ml of cold extraction solution was added, and the cells were resuspended. The extraction solution was 0.05 M potassium phosphate, pH 7.4, containing 0.1 mM L-isoleucine, 0.5 mM ethylenediaminetetraacetic acid, and 0.5 mM dithiothreitol (5). When only acetolactate synthase activity was to be assayed, the extraction solution was 0.1 M potassium phosphate, pH 8.0. The suspension was treated twice for 45 s with a 1-min interval for cooling with an MSE sonic oscillator, and then it was centrifuged for 20 min at 15,000 rpm in a Sorvall SS-34 rotor. The extracts contained between 5 and 15 mg of protein per ml. Proteins were determined by the method of Groves et al. (9) with crystalline bovine plasma albumin as a standard.

When extracts were prepared for hydroxylapatite columns, the extraction solution was 42 mM potassium phosphate, pH 7.1, containing 5 mM $MgCl_2$, 20 μg of flavin adenine dinucleotide per ml, 200 μg of thiamine pyrophosphate per ml and 20% glycerol. Three milliliters of this solution was added to 1.5 to 2.5 g (wet weight) of cells. The suspension was treated three times for 1 min each with sonic oscillation and then centrifuged. The pellet was resuspended in extraction fluid and sonically disrupted, and the two supernatant fluids were combined.

A similar extraction solution, containing 10 mM potassium phosphate, was used for the heat inactivation experiment of Fig. 3.

**Measurement of intracellular concentration of amino acids.** Cultures of the appropriate strains were grown in minimal medium and centrifuged while still in late exponential phase (0.8 to 1 × $10^9$ cells/ml). The packed cells were weighed, 5 ml of 75% ethanol (vol/vol) per g was added, and the suspension was mixed occasionally for 30 min at 0 C. After centrifugation the supernatant was collected, divided into two portions, and lyophilized. One portion was then dissolved with 0.2 mM norleucine in 0.01 N HCl, and the concentration of acidic and neutral amino acids was determined with a Technicon amino acid autoanalyzer using norleucine as a standard. Another portion was dissolved in 0.05 M $NaHCO_3$, pH 9.6, mixed with an equal volume of [$^3$H]dansylchloride (500 mCi/mmol; 0.5 mg/ml in acetone), and incubated at 37 C for 2 h. The dansyl derivatives were separated by two-dimensional chromatograms which were developed according to Hartley (14) in (i) benzene-acetic acid (45:5) and (ii) water-formic acid (97:3). They were detected by using an ultraviolet lamp, cut, and the radioactivity was determined in Bray (2) scintillation fluid in a liquid scintillation analyzer. Appropriate standards were used to locate the position of the various dansyl-amino acids on the chromatograms.

## RESULTS

**Isolation of mutants and their Val$^r$ phenotype.** The isolation, mapping, and preliminary characterization on the *ilvH611* mutation of strain MI183 has been described (13). The symbol *brn* had been chosen for the gene altered in this mutant because it shows alterations in the uptake of branched chain amino acids (13). The possibility was discussed, however, that the transport alterations in this mutant were secondary to other alterations and, since this appears to be the case, we have changed gene designation *brnP* to *ilvH*, and the *brnP1* mutation of strain MI183 to *ilvH611*.

Strain MI167 is a spontaneous Val$^r$ mutant derived from strain X478 during an attempt to isolate a mutant altered at a different locus, namely *val-r-D* that is located between *thr* and *pyrA* (8). A suspension of strain X478 was diluted to a density of approximately $10^8$ cells per ml in a flask containing supplemented minimal medium plus 50 μg of valine per ml, selecting, in this way, the Val$^r$ mutants present in the population. After full growth (about 2 × $10^9$ cells per ml) had occurred at 37 C with shaking, the culture was diluted 50-fold into the same medium and after growth, phage P1kc was grown on a portion of it. Strain AT739 (*thr*, *pyrA*) was treated with this phage and Thr$^+$ and Pyr$^-$ transductants were selected on minimal agar containing thiamine as the only supplement. About 1% of the transductants were Val$^r$ and one of them, strain MI167, was purified twice by single colony isolation and retained for further study. The mutation causing resistance to valine in this strain will be called *ilvH612*.

Strain MI219 was isolated from strain Ca85 (*lac*$^{am}$) by nitrosoguanidine-induced mutagenesis and plating on medium supplemented with valine. Four independent Val$^r$ mutants were isolated. After purification they were infected with *ϕ80psu3*, and lysogens were selected by plating on minimal medium supplemented with lactose. Lac$^+$ colonies were purified and tested for their Val$^r$ phenotype. One of the strains had become Val$^s$ and therefore it contains an amber mutation (which will be called *ilvH613*) causing resistance to valine. This strain was called MI219.

Strain MI183 is resistant to valine (0.2 mM) only if leucine (0.5 mM) is also present. Under these conditions the duplication time in minimal medium was equal (50 to 55 min) to that of the parental *ilvH*$^+$ strain. It has been shown that the leucine action is due to a competition at the transport level (7, 12). Strains MI167 and MI219 are resistant to valine (up to 100 μg/ml) even in the absence of leucine, and they are also resistant to glycylvaline. The duplication time of these strains in minimal medium supplemented with 0.2 mM valine or glycylvaline was

identical (50 to 55 min) to that of the parental *ilvH*$^+$ strain. Upon lysogenization with *ϕ80psu3* strains MI167 was still Val$^r$, whereas strain MI219 became Val$^s$.

**Chromosomal location.** The location on the chromosome of the *ilvH611* mutation of strain MI183 has already been described (13).

The Val$^r$ mutation of strain MI167 is linked, by P1 transduction, to *thr* and *pyrA*. Phage P1 grown on strain MI167 was used to transduce strain AT739 and either Thr$^+$, or Pyr$^+$, or Thr$^+$ and Pyr$^+$ transductants were selected. The frequency of cotransduction of the unselected Val$^r$ phenotype was 3% (2/64), 21% (20/95), and 10% (10/96), respectively, thus indicating that the *ilvH612* mutation is located on the side of *pyrA* opposite to *thr*. For this reason an Ara$^+$, Val$^r$ transductant (strain MI168) was prepared and a P1 phage grown on this strain was used to transduce strain X478 with selection of Ara$^+$ transductants. This transduction (Table 2) shows that the *ilvH612* mutation is located on the *azi* side of *leu*. Eighty-eight per cent (103/117) of the unselected Leu$^+$ transductants were *ilvH612*.

The location of the *ilvH613* mutation of strain MI219 was established by a three-point cross analogous to that reported for strain MI183 (13). The *ilvH613* mutation is also located on the *azi* side of *leu* and shows a cotransduction frequency with *leu* comparable to that shown for the *ilvH611* and *ilvH612* mutation.

**Intracellular concentration of isoleucine-valine biosynthetic enzymes.** Since resistance to valine has been correlated with a derepression of biosynthetic enzymes (see, for example, 21), the activity of these enzymes was examined. It has been reported (13) that in strain MI183a (*ilvH611*) there is a normal level of biosynthetic enzymes as compared to the isogenic strain X478 (*ilvH*$^+$). The experiment of Table 3 shows that threonine deaminase, acetolactate synthase, and isomeroreductase are not derepressed in strains MI167h and MI219h. Since coordinate expression for the *ilvADE* operon has been reported (21, 22), we assume that the formation of the *ilvD* and *ilvE* gene products is also not derepressed. Thus, the *ilvH* mutations described do not affect the level of the isoleucine-valine biosynthetic enzymes.

**Intracellular concentration of valine.** As shown by Guardiola and Iaccarino (13), strain MI183 shows alterations in the uptake of branched chain amino acids; strain MI167 (experiments not reported) also shows uptake alterations as compared to its isogenic *ilvH*$^+$ strain. To determine whether these alterations are a direct or secondary consequence of the *ilvH* mutation, the intracellular concentration of valine and other related amino acids was measured. To this purpose, isogenic strains carrying different *ilvH* alleles and no mutations causing an amino acid auxotrophy were prepared. After growth in minimal medium the intracellular amino acids were extracted and determined. A preliminary experiment with an amino acid auto analyzer showed no alterations in the intracellular concentration of the acidic and neutral amino acids in the *ilvH* strains as compared to the *ilvH*$^+$ parental strain except for valine. Since detection of some amino acids with this method was not sensitive enough, the concentrations of isoleucine, leucine, and valine in the same samples were determined by measuring their dansyl-derivatives separated by thin-layer chromatography (14). The experiment shown in Table 4 shows that the intracellular concentration of valine is increased in those strains which carry an *ilvH* mutation as compared to the isogenic *ilvH*$^+$ strain, whereas this does not happen in the case of isoleucine and leucine.

TABLE 2. *Transduction of strain X478 with P1 phage grown on strain MI168 (ilvH612) and selection of Ara$^+$ transductants*

| *leu* | *ilvH* | *azi* | Frequency (%) |
|---|---|---|---|
| + | − | + | 12.5 (20/160) |
| + | − | − | 52 (83/160) |
| + | + | + | <0.6 (0/160) |
| + | + | − | 9 (14/160) |
| − | − | + | <0.6 (0/160) |
| − | − | − | 3 (5/160) |
| − | + | + | <0.6 (0/160) |
| − | + | − | 24 (38/160) |

| | | | | | |
|---|---|---|---|---|---|
| | | + | + | − | + |
| | P1 (MI168) | *ara* | *leu* | *ilvH* | *azi* |
| X478 | | − | − | + | − |

TABLE 3. *Specific activity[a] of the isoleucine and valine biosynthetic enzymes in strains carrying different ilvH alleles*

| Strain | *ilvH* | Sp act | | |
|---|---|---|---|---|
| | | Threonine deaminase | Acetolactate synthase | Reducto isomerase |
| MI149h | + | 17 | 33 | 1.9 |
| MI183h | *611* | 10 | 36.5 | 2.5 |
| MI167h | *612* | 8 | 17 | 1.5 |
| MI219h | *613* | 4.5 | 34 | 2.2 |

[a] Nanomoles of product formed per minute per milligram of protein.

TABLE 4. *Intracellular amino acid concentration measured by analysis of the dansyl-derivatives in strains carrying different ilvH alleles*

| Strain | *ilvH* | Free amino acid content (wet wt) of cells (nmol/g [wet wt]) | | |
|---|---|---|---|---|
| | | Isoleucine | Leucine | Valine |
| MI149h | + | 67 | 106 | 367 |
| MI183h | *611* | 39 | 76 | 700 |
| MI167h | *612* | 62 | 92 | 1800 |
| MI219h | *613* | 95 | 92 | 1005 |

Strains carrying the *ilvH611*, the *ilvH612*, or the *ilvH613* mutations also show an appreciable excretion of valine as compared to an isogenic *ilvH*$^+$ strain. Analysis by microbiological methods of culture fluids in which these strains had been grown showed that excretion of isoleucine and leucine was undetectable for all strains, whereas excretion of valine (undetectable in the culture fluids of *ilvH*$^+$ strains) was evident in strains carrying the *ilvH611*, *ilvH612*, or *ilvH613* mutations. Analysis of the dansyl-derivatives isolated by thin-layer chromatography from the culture fluids in which bacteria had grown to a concentration of $8 \times 10^8$/ml gave the following results: $< 0.01$ μg/ml of valine for an *ilvH*$^+$ strain, 0.10 μg/ml for an *ilvH611* strain, and 0.40 μg/ml for an *ilvH612* or *ilvH613* strain. We presume that the excretion of valine is a consequence of the increase in its intracellular concentration.

**Acetolactate synthase activity in extracts.** An increase in the intracellular concentration of valine without derepression of the biosynthetic enzymes might conceivably be a consequence of a decreased feedback inhibition by valine of acetolactate synthase. Acetolactate synthase activity was assayed in extracts of various strains (Fig. 1). It is clear that extracts of strains MI219h (*ilvH613*) and MI167h (*ilvH612*) contain an acetolactate synthase activity more resistant to valine inhibition than that of strains MI183h (*ilvH611*) or MI149h (*ilvH*$^+$). This result suggests that strains MI167h and MI219h either contain actolactate synthase molecules more resistant to valine or contain two populations of molecules: one resistant and one normally sensitive. No alteration is observed with extracts of strain MI183, but results reported below indicate that the *ilvH* gene product is still partially active in this strain.

A mutation (*ilvO603*) at another locus has been described which also causes an increased resistance to valine of acetolactate synthase activity. The effect of this mutation (M. Iaccarino, R. Favre, and M. Freundlich, manuscript in preparation) is suppressed by a mutation in another gene, *ilvG605*. The possibility therefore existed that the *ilvG605* mutation also suppressed the resistance to valine caused by mutations in the *ilvH* gene.

To test this possibility, strain MI226 (*pyrA53*, *ilvO603*, *ilvG605*, *ilvH*$^+$) was treated with P1 phage grown on strain MI219 (*pyr*$^+$, *ilvH613*), and Pyr$^+$ transductants were selected. The *ilvH* gene shows 21% cotransduction frequency with *pyrA* (see above) and therefore if the *ilvH613* mutation is expressed not only in an *ilvG*$^+$, but also in an *ilvG*$^-$ background, about 21% of the transductants should be Val$^r$. This was actually the case (10%, 5/48). One of these transductants was purified and designated strain MI244. In Fig. 2 the acetolactate synthase activity of strain MI244 and of strains isogenic to it as a function of valine concentrations is shown. In the presence of 1.5 mM valine strain AT738 (wild type) showed 11% activity; strain AW206 (*ilvO603*) showed 22% activity, the difference being due to the expression of an acetolactate synthase resistant to valine inhibition (Iaccarino, Favre, and Freundlich, manuscript in preparation); strain MI226 (*ilvO603*, *ilvG605*) showed only 11% activity because of the muta-

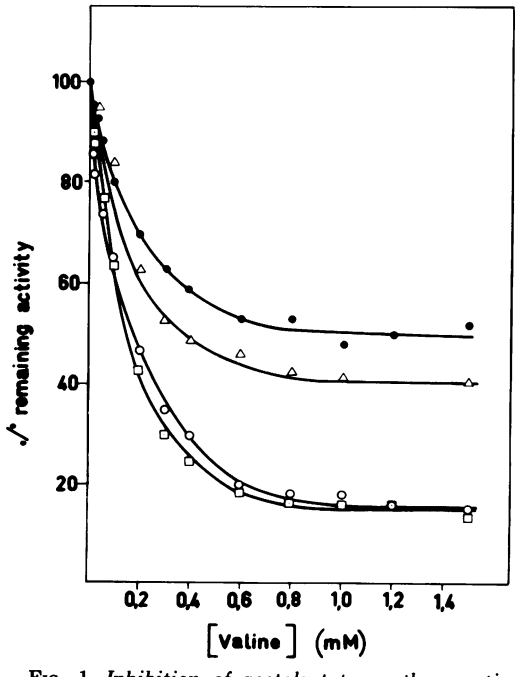


FIG. 1. *Inhibition of acetolactate synthase activity by valine in crude extracts of strains MI149h (ilvH*$^+$; ○), *MI183h (ilvH611;* □), *MI167h (ilvH612;* ●) *and MI219h (ilvH613;* △). *Specific activities in the absence of valine were 33 (nmol/min per mg) for strain MI149h, 49 for strain MI183h, 48 for strain MI167h, and 41 for strain MI219h.*

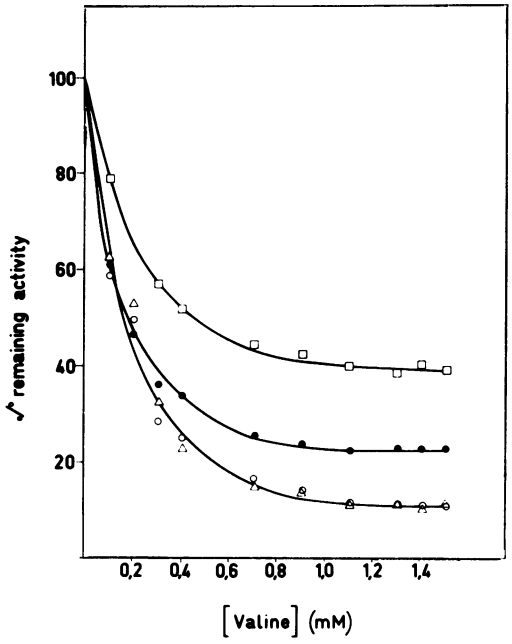


FIG. 2. *Inhibition of acetolactate synthase activity by valine in crude extracts of strains AT739 (ilvO⁻, ilvG⁺, ilvH⁺;* ○*), AW206 (ilvO603, ilvG⁺, ilvH⁺;* ●*), MI226 (ilvO603, ilvG605, ilvH⁻;* △*), and MI224 (ilvO603, ilvG605, ilvH613;* □*). Specific activities in the absence of valine were 69 (nmol/min per mg) for strain AT739, 65 for strain AW206, 59 for strain MI226, and 52 for strain MI244.*

tion in the *ilvG* gene; the triple mutant strain MI244 (*ilvO603, ilvG605, ilvH613*) showed 40% activity. In conclusion, the fraction of acetolactate synthase activity resistant to valine inhibition of the *ilvH613* mutant is present not only in an *ilvO⁻, ilvG⁺* background (Fig. 1, strain MI219h), but also in an *ilvO603, ilvG605* background. Other experiments, not reported, have shown that also the *ilvH612* mutation expresses a Val$^r$ phenotype in an *ilvO603, ilvG605* background.

**Two forms of acetolactate synthase activity.** Mutations in the *ilvH* gene (*ilvH612* and *ilvH613*) make acetolactate synthase activity more resistant to valine inhibition (Fig. 1). However, in the mutants examined, the acetolactate synthase activity was still partially sensitive to valine inhibition and the inhibition observed seemed to approach a constant level. It is possible to explain this result by assuming that the acetolactate synthase molecules of the *ilvH612* and *ilvH613* strains can still interact with valine, but are more active than the molecules of the wild-type strains. However, another possibility is that there are, in the *ilvH* strains, two populations of acetolactate synthase molecules, one more sensitive and another more resistant to valine inhibition. If two populations do exist it is possible that their rate of inactiviation under specific conditions is different. The experiment of Fig. 3 showed that when an extract of strain MI244 (*ilvH613*) was incubated at 40 C and at different times the activity was assayed, this decreased as a function of time. The rate of inactivation was higher if the assay was performed in the presence of valine. Although other interpretations are possible, this again suggests a heterogeneity of acetolactate synthase molecules. These molecules would be different not only in their sensitivity to valine, but also in their rate of inactivation when incubated in the experimental conditions used. We have succeeded in separating these two forms of acetolactate synthase by adsorption on hydroxylapatite as shown in Fig. 4. Data taken from these columns are summarized in Table 5. Under the conditions used acetolactate synthase activity of the *ilvH⁺*, strain MI226, is not adsorbed to the column and no activity is eluted by raising the ionic strength of the buffer (Fig. 4A). The same experiment was repeated with crude extracts of strain MI183h (*ilvH611*), MI167h (*ilvH612*), and MI244 (*ilvH613*). Whereas one component of acetolactate synthase activity was obtained with the *ilvH⁺* strain, two were obtained with strains containing different *ilvH* mutations (Fig. 4 A-D). In the

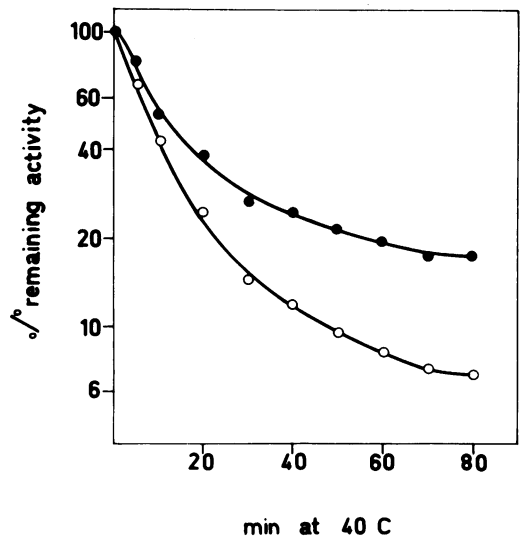


FIG. 3. *Rate of inactivation of a crude extract of strain MI244 at 40 C. Protein concentration was 45 mg/ml and the specific activity was 55 nmol/min per mg. The extract was put at 40 C and at different times samples were withdrawn and put at O C. Activity was assayed without* (●) *or with* (○) *1.5 mM valine.*

case of strains MI167h (*ilvH612*) and MI244 (*ilvH613*), the activity not adsorbed at low ionic strength was more sensitive to valine inhibition than the activity of the crude extract, whereas that eluted at higher ionic strength was more resistant. This shows that the extract of these strains were more resistant to valine inhibition because two forms of acetolactate synthase were present, one of which partially resistant to valine inhibition under the experimental conditions used. When a strain contains the *ilvH611* mutation it is resistant only to low concentrations of valine and its acetolactate synthase activity is as sensitive to valine inhibition as the activity of extracts of a wild-type strain (Fig. 1). In agreement with this result, Table 5 shows that the resistance to valine of the extracts of strains MI183h (*ilvH611*) and MI226 (*ilvH*$^+$) is the same. However, as with strains carrying the *ilvH612* of the *ilvH613* mutation, strain MI183h also yielded two components of acetolactate synthase. The second component in this case was only slightly more resistant to valine inhibition than the first one. The discrepancy of this result with the result obtained with the other two strains, MI167h (*ilvH612*) and MI244 (*ilvH613*), suggests that the structure of the form of acetolactate synthetase eluting at higher ionic strength is different from that of the *ilvH*$^+$ strain.

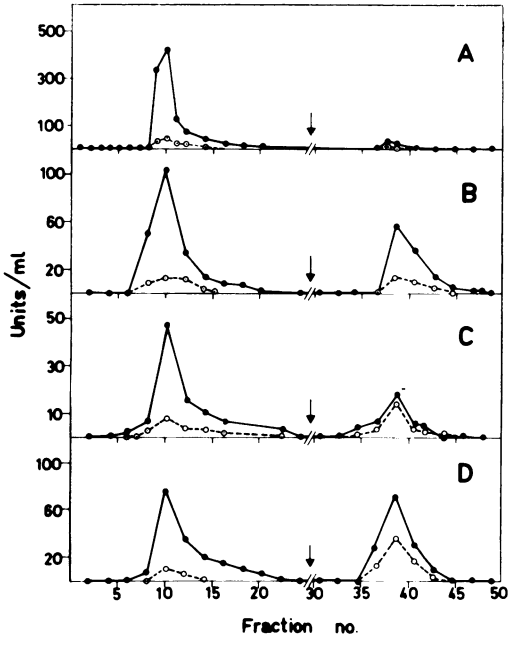


FIG. 4. *Hydroxylapatite separation of acetolactate synthase activities from strains MI226 (A), MI183h (B), MI167h (C) and MI244 (D). The crude extracts, prepared as described, were layered on a column (1.1 by 30 cm) of hydroxylapatite previously washed with the extraction solution. The column was then eluted with 100 ml of this solution. A second elution with 100 ml of the same solution containing 100 mM potassium phosphate (pH 7.1) was started as indicated by arrows. Fractions of 3.5 ml were collected and 0.1-ml samples were assayed with (○) and without (●) 1.5 mM valine for acetolactate synthase activity. Units are expressed in nanomoles of product formed per minute. The specific activities of the crude extracts were 54 (nmol/min per mg) for strain MI226, 43 for strain MI183h, 50 for strain MI167h, and 52 for strain MI244.*

The sensitivity to valine of the two forms of acetolactate synthase obtained from strain MI244 (*ilvH613*) was analyzed in greater detail in the experiment of Fig. 5. The most active fractions of each of the two forms (Fig. 4) were pooled separately and their activities were analyzed as a function of valine concentration. The experiment confirms that two forms of acetolactate synthase are present in extracts of strain MI244, one more sensitive to valine inhibition, the other less sensitive. The experiment also shows that the sensitive form is not completely sensitive whereas the resistant form is not completely resistant.

In conclusion, the experiments of Fig. 3–5 show that the Val$^r$ phenotype of the *ilvH* strains

TABLE 5. *Summary of data taken from the experiments of Fig. 4*

| Strain | Total units[a] | | | | Resistance to valine (%)[b] | | |
|---|---|---|---|---|---|---|---|
| | Column input | Component I[c] | Component II[c] | Yield (%)[d] | Extract | Component I[c] | Component II[c] |
| MI226 (*ilvH*$^+$) | 4,375 | 4,100 | < 180 | 95 | 13 | 10 | |
| MI183h (*ilvH611*) | 4,660 | 1,493 | 836 | 50 | 13 | 14 | 23 |
| MI167h (*ilvH612*) | 1,750 | 1,100 | 350 | 82 | 46 | 14 | 70 |
| MI244 (*ilvH613*) | 3,100 | 1,880 | 950 | 91 | 31 | 6 | 50 |

[a] Nanomoles of product formed per minute.

[b] Activity remaining when assayed in the presence of 1.5 mM valine.

[c] Component I is the one not adsorbed, whereas component II is that eluted with 100 mM potassium phosphate.

[d] [(Component I + component II)/column input] × 100.

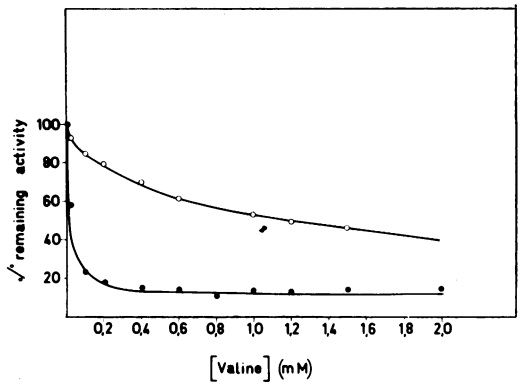


FIG. 5. *Inhibition of acetolactate synthase activity by valine in a pool of the most active fractions of component I (●) and component II (○) eluted from strain MI244.*

is due to the presence of an acetolactate synthase more resistant to valine inhibition than that present in the wild type. This acetolactate synthase might be absent in the *ilvH*$^+$ strain or it might be present as a Val$^s$ form, unseparated from the remaining activity. We have reported elsewhere (10) that the acetolactate synthase activity eluting at low ionic strength is absent in a strain carrying an *ilvB* mutation. We have also found (unpublished data) that *ilvB* is the structural gene for a specific acetolactate synthase.

## DISCUSSION

In this paper the effects of *ilvH* mutations on the physiology of *E. coli* K-12 are described. One of these effects, as described previously (13), is an alteration in the uptake of branched chain amino acids, and for this reason the gene had been called *brnP*. The *ilvH612* mutation described in this paper causes even more profound transport alterations (experiments not reported) but we believe that they are a consequence of the increased pool size of valine and/or of the excretion of intracellular valine.

The mutants described in this paper seem to be altered in the locus *val-r-C* described by Glover (8). They show, in fact, the same frequency of cotransduction with *leu*. Strains carrying the *ilvH612* or the *ilvH613* mutation are resistant not only to the inhibitory action of valine but also to that of dipeptides containing valine. Since valine is transported by systems different from that for dipeptides (7) these mutants are not altered in transport. A strain carrying the *ilvH611* mutation is resistant to valine only if leucine is also present and is sensitive to glycylvaline. As discussed (11, 12), this does not necessarily mean that this mutant is altered in valine transport, but only that leucine is necessary to decrease valine transport as leucine and valine share common transport systems. We believe that this strain would be resistant to valine even in the absence of leucine provided that a lower concentration of valine is used. However, this is difficult to demonstrate because the valine concentration in the medium decreases owing to its utilization for protein synthesis.

Except for the close location and the similarity in phenotype, we show no evidence that the three mutations described are in the same gene. Complementation experiments cannot be done because only the *ilvH613* mutation is recessive and the other two are dominant (6). The experiment of Fig. 4 indicates that the same gene product is altered in the mutants and on this basis we prefer to keep the *ilvH* symbol for all of them.

Mutants altered in the *ilvH* gene product show the presence of an acetolactate synthase relatively resistant to valine inhibition. On the basis of this evidence the *ilvH* gene product might be a repressor of the specific acetolactate synthase which appears in the *ilvH* mutants. Another possibility is that the *ilvH* gene product is a subunit of an acetolactate synthase: when this subunit is present this specific acetolactate synthase is more sensitive to valine inhibition and cannot be separated from the remaining acetolactate synthase activity present in the *ilvH*$^+$ strain. More experiments bearing on this problem are reported in the accompanying paper (6). In any event, the experiment of Fig. 4 confirms the heterogeneity of acetolactate synthase activity previously reported (22). We have recently reported (10) that in *E. coli* K-12 three acetolactate synthase isoenzymes exist. One, sensitive to valine inhibition, is the product of the *ilvB* gene (10; unpublished data). A second, sensitive to valine inhibition, is the product of the *ilvH* and *ilvI* genes (6, 10). The third one is the product of the *ilvG* gene (10; unpublished data).

When a product exerts in vitro an inhibitory effect on the activity of the first enzyme of a metabolic pathway, it is usually inferred that this action in vivo contributes to the regulation of the pool size. Indirect evidence confirming this hypothesis has been reported in several systems as, for example, in the case of isoleucine (15). More direct evidence has been reported in the case of mutants that are resistant to feedback inhibition and excrete the terminal product of the metabolic pathway (see, for example, 25). Another example is that of the accumulation of high concentrations of uridine 5'-triphos-

phate in cells which are starved for the feedback inhibitor (18). In the present paper we report the presence of an increased intracellular concentration of valine in the *ilvH612* and *ilvH613* mutants. The increased concentration of valine is due to the appearance of an acetolactate synthase activity relatively resistant to feedback inhibition. This result confirms the role of feedback inhibition in the regulation of the pool size of valine previously shown (16). Furthermore, it is evident that, whereas several common gene products are involved in the biosynthesis of the branched chain amino acids, the pool size of valine is regulated independently from that of isoleucine and leucine.

The utilization of valine by *E. coli* can be estimated in a way analogous to that reported by Brenner and Ames (3) to be 5.15 $\mu$mol/g (dry weight) per min. From this figure a value of about $1.3 \times 10^6$ molecules of valine utilized per min and per cell can be estimated (assuming 1 g [dry weight] to be equal to $2.4 \times 10^{12}$ cells). In an analogous way it can be estimated that an *ilvH*$^+$ *E. coli* contains about $3.6 \times 10^5$ molecules of valine per cell (assuming 1 g [wet weight] to be equal to $0.6 \times 10^{12}$ cells). This means that wild-type *E. coli* contains about 0.3 min worth of valine. The difference in pool size of valine between the wild-type strain and the *ilvH* mutant is small. This is probably due to the fact that when the pool size increases an excretion of valine takes place. In fact, excretion of valine can already be detected for a mutant like *ilvH611* in which there is only a doubling of the pool size. Therefore, excretion of valine appears to be a consequence of the increased pool size of this amino acid.

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