MICROBIOLOGICAL REVIEWS, Mar. 1979, p. 42-58
0146-0749/79/03-0042/17$02.00/0 Vol. 43, No. 1

Growth Inhibition as a Consequence of Antagonism Between Related Amino Acids: Effect of Valine in *Escherichia coli* K-12

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INTRODUCTION

Growth of a number of microorganisms is inhibited in minimal medium containing some natural amino acids; such inhibition is usually reversed by other amino acids. Gladstone (42) found that in *Bacillus anthracis* either valine or leucine alone inhibits growth, but no inhibition is observed when both are present. He also showed that an analogous, though less marked, relationship exists between serine and threonine. Similar observations were reported in the subsequent years by several authors. Tatum (103) showed that *Escherichia coli* K-12 fails to grow in minimal medium containing valine and that isoleucine counters this inhibition. Beerstecher and Shive (9) reported that growth of an *E. coli* strain is inhibited by tyrosine and that phenylalanine reverses this effect. Antagonistic phenomena among amino acids were also noticed in studies with *B. subtilis* (93), *Streptococcus bovis* (114), and some lactic acid bacteria (15, 67). A comprehensive formal analysis of these phenomena was performed by Rowley (94, 95) on a great number of *E. coli* strains. In Table 1 we show the pattern of amino acid inhibition, and in Table 2 we show a summary of the examples of complete prevention of amino acid inhibition by other amino acids described by Rowley.

More recent reports demonstrate that *relA* mutants of *E. coli*, in experiments of downshift from nutrient to minimal medium, are more sensitive than wild-type strains to growth inhibition by amino acids (3, 4, 111, 112). This is at least in part a consequence of the impaired expression of biosynthetic enzymes in *relA* mutants (38, 40, 70, 99, 100, 119).

Growth inhibition by natural amino acids is not limited to procaryotes: it has been observed also for a wide range of plants and plant tissues and cells in culture (1a, 10, 13, 14, 17, 51, 68, 96). In many of these cases reversion of the inhibition by other amino acids has been reported. For example, valine inhibits growth of barley embryos (68) and tobacco in vitro cultures of tissue, cells, and protoplasts (13, 14, 96); as in *E. coli* K-12, in these plant materials the effect is reversed by isoleucine.

These findings show that growth inhibition by amino acids may occur as a physiological consequence of a competition at the level of either biosynthesis or utilization among amino acids having structural, biosynthetic, and regulatory relationships.

Among the numerous cases observed, only the effect of valine in *E. coli* K-12 has been studied in detail, probably because this inhibition is severe, occurs in the most commonly studied microorganisms, and makes possible the isolation of valine-resistant (Val^r) mutants, which provides a precious tool for genetic studies on several metabolic processes. The aim of this article is to analyze the extensive literature existing on this subject, which has never been

TABLE 1. *Growth inhibition by amino acids among 356 E. coli strains*[a]

Amino acid tested	No. of strains inhibited
α-Aminobutyric acid	4
Aspartic acid	13
Cystine	40
Histidine	2
Lysine	3
Methionine	4
Norleucine	333
Norvaline	261
Serine	42
Threonine	2
Tyrosine	2
Valine	3

[a] Adapted from reference 94.

TABLE 2. *Prevention of amino acid growth inhibition by other amino acids in various E. coli strains*[a]

No. of strains tested	Inhibition by:	Prevented by:
1	Aspartic acid	Lysine
1	Aspartic acid	Valine
8	Cystine	Methionine
3	Lysine	Methionine
1	Methionine	Leucine or lysine
12	Norleucine	Methionine
5	Norvaline	Leucine or methionine
4	Serine	Glycine
1	Valine	Isoleucine or leucine
1	Valine	Isoleucine or leucine or methionine

[a] Adapted from reference 94.

reviewed in this framework. We intend to show that the work on the phenomenon of valine inhibition has made possible the elucidation of a typical process of antagonism between physiologically related metabolites. We also wish to emphasize that this work has substantially contributed to the understanding of active transport and regulation of biosynthesis of branched-chain amino acids, which represents, for reasons which will be focused on in this article, a unique model system in microbial molecular biology. From the ideas emerging from these studies we will derive, in Conclusions, a general interpretation of growth inhibition by natural amino acids.

MECHANISM OF VALINE INHIBITION

In 1946 Tatum (103) observed that valine and its keto acid precursor α-ketoisovaleric acid inhibit the growth of *E. coli* K-12 and that isoleucine counters the inhibition. This effect was confirmed and further investigated by Bonner (12). Rowley (94) reported that growth restriction of *E. coli* K-12 by valine is bacteriostatic. A further investigation was accomplished by Glover in 1962 (43). This author reported that, whereas *E. coli* B is insensitive to valine (up to 10 mg/ml), *E. coli* K-12 is inhibited at any concentration above 0.4 μg/ml. He also observed that leucine (20 μg/ml) does not affect the response to valine (40 μg/ml), whereas isoleucine antagonizes the inhibition when it is present at approximately 3% of the valine concentration. Finally, Glover reported that reversion can also be accomplished by some isoleucine precursors but not by some valine precursors.

The ability of isoleucine and some of its precursors to antagonize the growth inhibition exerted by valine led the early investigators to believe that the effect of valine is to restrict the biosynthesis of isoleucine.

Adelberg and Umbarger (2) showed that the presence of valine restricts the accumulation of α-ketoisovalerate in a strain which is unable to transform this intermediate into valine. Therefore, they concluded that valine inhibits its own biosynthesis. These results stimulated the idea that growth inhibition occurs at the level of a biosynthetic reaction common to both isoleucine and valine, and Umbarger and Brown (107) proposed that the common enzyme inhibited by valine might be isomeroreductase (EC 1.1.1.86). A few years later, more insight was gained into the details of the biosynthesis of isoleucine and valine (Fig. 1). Umbarger and Brown (108) studied the formation of the first five-carbon precursor of valine, α-acetolactate, and found that this synthesis is inhibited by valine. A short time later, in the same laboratory, a microbiological assay for the first six-carbon precursor of isoleucine, α-aceto-α-hydroxybutyrate, was devised (58, 59). The availability of this method made it possible to perform a comparative analysis of the synthesis of the acetohydroxy acid precursors of isoleucine and valine. Leavitt and Umbarger (59, 60) found that (i) the syntheses of α-acetolactate and α-aceto-α-hydroxybutyrate are characterized, in vitro, by the same kinetic parameters and by the same patterns of sensitivity (in *E. coli* K-12) or resistance (in *E. coli* W and in a valine-resistant *E. coli* K-12 mutant) to valine inhibition; (ii) the rate of α-acetolactate synthesis is reduced when the reaction mixture contains, in addition to pyruvate, α-ketobutyrate, and therefore α-aceto-α-hydroxybutyrate is also produced; (iii) the intracellular concentration of valine is higher in *E. coli* W and in a Val^r *E. coli* K-12 mutant than in wild-type *E. coli* K-12, whereas there is no difference in valine uptake by the two organisms. From these data, it

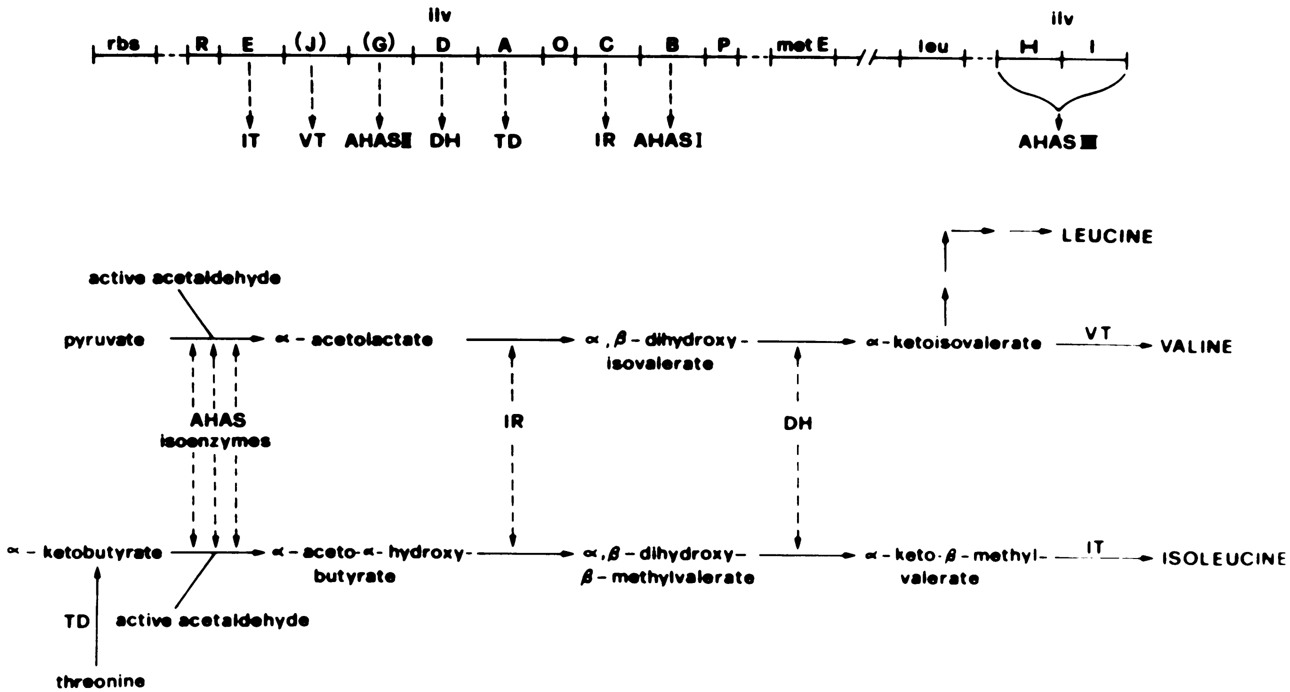

FIG. 1. *Biosynthetic pathway for isoleucine and valine and the corresponding ilv genes. The enzymes are abbreviated as follows: TD, threonine deaminase; AHAS, acetohydroxy acid synthase; IR, isomeroreductase; DH, dehydrase; VT, valine transaminase; IT, isoleucine transaminase. Genetic designation and map structure are adapted from Bachmann et al. (7). The ilvO and ilvR sites are cis-acting regulatory loci affecting the expression of the ilvEDA structural genes. The ilvJ (44) and ilvG (36, 98) loci are in parentheses because their location is not exactly known (see text for further details). All these genes are located on the E. coli K-12 map at 83 min, with the exception of ilvH and ilvI, which are located at 2 min.*

was possible to conclude that the synthesis of the two acetohydroxy acids is catalyzed by a common enzyme activity, that this activity is the target of valine inhibition, and that valine regulates its intracellular pool through feedback control of the first step of its own biosynthesis. The work of several investigators has confirmed the findings of Umbarger and his collaborators and has substantiated their model later (28, 33, 36, 39, 82, 90, 105).

Cohen (20) reported that in *E. coli* K-12 exogenous valine is incorporated into proteins in the place of other amino acids, thus producing faulty proteins. Further investigation (105) suggests that this phenomenon does not account for the growth inhibition. Other authors have shown (73) that valine inhibits the synthesis of isoleucyl-transfer ribonucleic acid (tRNA) by forming a complex with isoleucyl-tRNA synthetase. Val^r mutants altered in isoleucyl-tRNA synthetase have not been described, which indicates that this effect is not the primary cause of valine inhibition. However, we cannot exclude that these phenomena might contribute to the severity of valine action. Also, the α-ketoisovalerate inhibition of isoleucine synthesis from α-keto-β-methylvalerate (12, 104; see Fig. 1) could contribute to valine inhibition.

At present, the idea that valine inhibition is mainly the consequence of a block of α-aceto-α-hydroxybutyrate synthesis is commonly accepted. However, the biochemical details of the mechanism of inhibition still need to be clarified. A biochemical assay is available for the synthesis of α-acetolactate (101), whereas α-aceto-α-hydroxybutyrate synthesis is only studied by means of a microbiological method (58, 59). For this reason, most of the work on acetohydroxy acid synthase (AHAS; EC 4.1.3.18) activity has been performed on the first of these two reactions, and no evidence has been presented as to whether essential differences exist in the kinetics of the two reactions. Furthermore, the total AHAS activity is composed of isoenzymes (see below), which are difficult to separate physically and to purify, and, therefore, most biochemical studies have been performed on crude extracts. Relevant information is mainly based on genetic manipulations.

SITE OF INHIBITION: PRODUCTION OF THE ACETOHYDROXY ACIDS

The biosynthesis of isoleucine, leucine, and valine (Fig. 1) occurs through a branched pathway in which three steps are common to each end product. Mutants lacking threonine deaminase (EC 4.2.1.16) or isoleucine transaminase activity are auxotrophic for isoleucine (Ile^-). Mutants lacking valine transaminase activity should be auxotrophic only for valine, but their growth also requires isoleucine and leucine, because of the block of acetohydroxy acid synthesis determined by valine inhibition of the total AHAS activity. Mutants lacking isomeroreductase and dehydrase (EC 4.2.1.9) activity are auxotrophic for both isoleucine and valine (Ilv^-). The presence of leucine is not required for growth of such strains, because α-ketoisovalerate, the first leucine precursor, is produced by transamination of valine. Mutants lacking AHAS activity are, for the same reason, auxotrophic for isoleucine and valine.

The AHAS reactions represent the first biosynthetic step common to the three products. They are catalyzed by isoenzymes and are the target of end-product inhibition by valine. The sum of these properties determines a unique pattern, which gives to this step a central role in the carbon flow through the whole pathway.

The biochemical features of the synthesis of the acetohydroxy acid precursors of isoleucine, leucine, and valine in *E. coli* K-12 have been studied by Umbarger and his collaborators, as well as by other investigators (58–60, 88 [and references quoted therein], 108, 109). The reactions consist of a condensation of active acetaldehyde (derived from pyruvate) with either α-ketobutyrate or pyruvate to yield α-aceto-α-hydroxybutyrate (a precursor of isoleucine) or α-acetolactate (a precursor of leucine and valine), respectively (Fig. 1).

In the last few years, an extensive genetic and biochemical analysis of the properties of this step, mainly performed in our laboratories, has provided the possibility of uncovering essential biological features of the phenomenon of valine inhibition and of gaining some insight into *ilv* regulation. In this section, we will review this information.

AHAS Isoenzymes and the Problem of *ilvG* Gene Expression

The property of *E. coli* K-12 of sensitivity to growth inhibition by valine is unique among the commonly studied *Enterobacteriaceae*; this is due to the severity of valine inhibition on the total AHAS activity in *E. coli* K-12 as compared with other organisms (60).

Work from different laboratories showed that in *Salmonella typhimurium* and *E. coli* W there are two AHAS activities, one sensitive (Val^s) and one resistant to valine, the latter being absent in *E. coli* K-12 (11, 36, 74). It was therefore suggested that the Val^s phenotype of *E. coli* K-12 is due to the absence of the Val^r activity. Recently, it has been shown that three distinct

AHAS activities, one of which is Val^r, can be separated chromatographically from *E. coli* 9723 extracts (117). An analysis in *E. coli* K-12 (26, 28, 33, 36, 46, 49) has demonstrated that this strain contains the structural genes for three AHAS activities, designated AHAS I, AHAS II, and AHAS III isoenzymes (see Fig. 1). AHAS I and AHAS III are both inhibited by valine (49), whereas AHAS II is resistant to it (36). However, AHAS II is not expressed (33) except in the presence of *cis*-dominant mutations in either the *ilvO* or the *ilvR* locus (Fig. 1). Such mutants, because of the expression of AHAS II, are insensitive to growth inhibition by valine (36; C. Squires, M. Levinthal, M. Levinthal, P. Abrescia, and M. De Felice, submitted for publication). Thus, the peculiar sensitivity of *E. coli* K-12 to valine inhibition is a consequence of a specific regulatory pattern of the *ilv* gene expression, resulting in lack of expression of the *ilvG* gene.

An analysis of the properties of the AHAS isoenzymes in extracts of *E. coli* K-12 strains, each containing one isoenzyme, has been performed (32; Squires et al., submitted for publication). The data summarized in Table 3 show that the three enzymes are significantly different in many respects.

The *ilvB* gene codes for a polypeptide required for AHAS I activity. A mutation in this gene causes lack of AHAS I activity (32, 49) and confers an Ilv^- phenotype in an *ilvH ilvI* background (46). A temperature-sensitive mutation in *ilvB* causes the appearance of a temperature-sensitive AHAS I activity and confers a temperature-sensitive Ilv^- phenotype in an *ilvH ilvI* background (49). The location of *ilvB* on the *E. coli* K-12 linkage map reported in Fig. 1 is based on genetic evidence existing in the literature (46, 49, 92). However, more recent results suggest that *ilvB* is located at 81 min between *pyrE* and *bgl* (T. Newman and M. Levinthal, unpublished data). No other genes in addition to *ilvB* are known to code for a polypeptide involved in AHAS I activity. Expression of the *ilvB* gene is under the control of a *cis*-dominant regulatory element, *ilvP* (91, 92), closely linked to it (Fig. 1).

TABLE 3. *Comparison of the biochemical properties of the AHAS isoenzymes of E. coli K-12*[a]

Enzyme	Affinity (K_m, μM):		Inhibition by valine(%)		pH optimum
	Pyruvate	Flavin adenine dinucleotide	50 μM	1.5 mM	
AHAS I	1,300	0.16	10	85	7.3
AHAS II	2,900	30	~0	~0	8.6
AHAS III	7,600	~0	70	85	8.9

[a] The data reported are summarized from reference 32 and from Squires et al., (submitted for publication). They derive from studies on crude extracts of cells of various strains, each containing one isoenzyme. For AHAS II, we report values derived from experiments in which an *ilvO603* strain was used. These values were identical to those obtained with an *ilvR264* strain.

AHAS III is the product of two structural genes, *ilvH* and *ilvI*, both closely linked to the leucine operon (85% cotransduction frequency). The *ilvI* gene product is a polypeptide subunit responsible for the catalytic activity of AHAS III: amber and temperature-sensitive mutations in this gene have been studied (26). The *ilvH* gene product is a polypeptide subunit distinct from the *ilvI* product and responsible for the valine sensitivity of AHAS III, as suggested by studies on *ilvH* missense and amber mutations (28). Evidence that the *ilvH* and *ilvI* gene products are distinct polypeptides derives from an in vitro complementation analysis (26). The details of the interaction between the two subunits are unknown. Recessive mutations in a nonadjacent locus cause derepression of AHAS III (M. V. Ursini and M. De Felice, unpublished data).

The hypothesis that major regulatory relationships occur between *ilvH ilvI* and the *leu* operon in *E. coli* K-12 has been considered in the past. Recent data permit us to rule out this possibility. In fact, the two gene clusters are separated by a minimum of 1,000 base pairs of unrelated deoxyribonucleic acid and are transcribed from opposite strands (S. Wessler, M. De Felice, and J. M. Calvo, unpublished data). Furthermore, although regulation of both gene clusters is controlled by leucine, the two mechanisms of expression are very different. In leucine-containing minimal medium the *ilvH ilvI* gene expression is completely blocked (30), whereas expression of the *leu* operon is only repressed two- to threefold (24, 25). A limitation for leucine causes a two- to threefold derepression of AHAS III (F. Lacara, C. Squires, and M. De Felice, unpublished data), whereas in the same conditions the leucine biosynthetic enzymes are derepressed about 50-fold (25).

S. typhimurium may synthesize an enzyme equivalent to the *E. coli* K-12 AHAS III isoenzyme: a restriction fragment of *E. coli* K-12 deoxyribonucleic acid containing information for AHAS III hybridizes with an *S. typhimurium* deoxyribonucleic acid fragment located very close to the *leu* operon, apparently in the same position in which the *E. coli* K-12 *ilvH ilvI* genes are located (Wessler et al., unpublished data).

For AHAS II, as for AHAS I, only one structural gene, *ilvG*, has been reported. The function of this gene has been elucidated through the study of amber and temperature-sensitive mutations (36, 46, 49). As mentioned above, in wild-type *E. coli* K-12, this gene is unexpressed. We believe that the regulatory block to *ilvG* gene expression, which is strictly related to the molecular aspects of the control of the *ilvREJGDAO* cluster, is the central problem in *ilv* regulation, and therefore we will discuss it in detail.

Favre et al. showed, by means of a three-factor cross, that the *ilvG605* mutation is located between *ilvE* and *ilvD* (36). Shortly thereafter, Umbarger and his collaborators, from studies on the polarity of mutations determined by Mu phage insertion, proposed that *ilvG* lies between *rbs* and *ilvR* (98). Because of these controversial conclusions, in Fig. 1 we report *ilvG* in parentheses, as a marker that has not been definitively mapped. In vivo and in vitro studies are in progress in several laboratories, from which conclusive information on this problem and an explanation for the discrepancies existing in the literature should be derived. However, the ideas presented herein are independent from the exact location of *ilvG*. The *ilvREJGDAO* cluster is not referred to as an "operon" (56) in this review, because it has several features which are atypical in the framework of the common view of an operon. Two regulatory regions, *ilvO* and *ilvR*, are present on each side of the cluster, and *cis*-dominant mutations have been found in both regions (22, 36, 61, 90, 91, 92; Squires et al., submitted for publication). The polarity appears to be from *ilvE* to *ilvA* (19, 98). Therefore, the *ilvR* locus could bear the properties of an operator, whereas the *ilvO* locus, downstream from the point of transcription initiation, could be involved in some novel regulatory functions. For example, *ilvO* could contain information for stability or translatability of messenger RNA or could affect the secondary structure and subsequent translatability of messenger RNA (63). *ilvO* could also contain information for a *cis*-acting protein (35), as has been proposed (98). However, it cannot be excluded that the *ilvREJGDAO* cluster is a genetic unit regulated in an unusual way, in which both *ilvO* and *ilvR* would be new types of regulatory elements. Additional features of the *ilvREJGDAO* cluster are: (i) the *ilvA* gene product, threonine deaminase, is part of the regulatory mechanism (45, 61, 62); (ii) the effect of mutations in the *rho* gene suggests the presence of an attenuator (1, 98, 115); (iii) the control is at least in part transcriptional (18, 64, 113); (iv) gene expression is multivalently controlled by isoleucine, leucine, and valine (110).

Expression of the *ilvG* gene, and therefore appearance of the Val^r AHAS II isoenzyme, occurs only when a derepressed synthesis of the *ilvEDA* gene products is promoted by *ilvR* or *ilvO* mutations (36; Squires et al., submitted for publication) and not when the same extent of derepression is caused by corepressor starvation (34) or by a *rho* mutation (1). At the present time, there is no explanation for this phenomenon.

The Val^r AHAS activity of *S. typhimurium* (see above) appears to have a role similar to that of AHAS III of *E. coli* K-12. Its structural gene, *ilvG*, has been reported to be located on the *S. typhimurium* chromsome in a position analogous to the *E. coli* K-12 *ilvG* gene (75).

An extensive discussion on the regulation of *ilv* gene expression is reported in a specific review (55).

Physiological Role of the AHAS I and AHAS III Isoenzymes

Multiple enzymes for the catalysis of the first reaction occur in several diverging biosynthetic pathways (16). The biosynthesis of both the aspartate family (21) and the aromatic group (66) of amino acids in *E. coli* K-12 is characterized by a correlation between isoenzymes and terminal products, each isoenzyme being specifically inhibited by one end product. Therefore, carbon flow through the pathway cannot be completely inhibited unless all the end products are present. For the branched-chain amino acids, the first common biosynthetic step is catalyzed, in wild-type *E. coli* K-12, by two isoenzymes, AHAS I and AHAS III (Fig. 1). Experiments performed with extracts of two isogenic strains, each containing only one isoenzyme, show that they are distinct from both a genetic and a biochemical point of view (Table 3). However, both enzymes are severely inhibited by valine (85% inhibition at 1.5 mM valine for both in crude extracts; see Table 3), moderately inhibited by isoleucine (40% inhibition at 3.0 mM isoleucine for both), and insensitive to leucine (30, 32). Therefore, the presence of AHAS I and AHAS III does not prevent growth inhibition of *E. coli* K-12 by valine. In fact, the appearance of a third isoenzyme (AHAS II) is required to circumvent the problem of valine sensitivity. The similar sensitivity to valine of AHAS I and AHAS III in vitro suggests that they should have a physiological role different from that illustrated in the cases of the aspartate family (21) and the aromatic group (66) of amino acids.

Recent observations, based on the different affinities of AHAS I and AHAS III for their substrates, suggest that AHAS I is specialized for the biosynthesis of leucine and valine (through α-acetolactate) and that AHAS III is specialized for the biosynthesis of isoleucine (through α-aceto-α-hydroxybutyrate), although each enzyme is able to synthesize both precursors with different efficiencies (32; M. De Felice, unpublished data). It is also interesting to note that synthesis of AHAS I and AHAS III occurs through different regulatory patterns, the former being multivalently controlled by leucine and valine (31) and the latter being regulated by leucine alone (30). Isoleucine is not directly involved in the control of synthesis of either enzyme. However, this amino acid controls, through end-product inhibition of threonine deaminase (106), the synthesis of α-ketobutyrate, which is both a substrate for isoleucine synthesis and an inhibitor of leucine and valine synthesis (32). This allows isoleucine to affect the expression of both AHAS I and AHAS III by indirectly affecting the pool size of leucine and/or valine. We believe that this pattern of specialized activity and different regulation of synthesis of AHAS I and AHAS III allows the cell to exert a fine control of carbon flow through the pathway for branched-chain amino acid biosynthesis in a great variety of physiological situations, such as conditions determining unbalanced intracellular concentrations of intermediates or products. This control would confer to the pathway a regulatory elasticity which could have determined a selective advantage for the existence of the two isoenzymes.

Additional regulatory features of the AHAS isoenzymes of *E. coli* K-12 have been investigated. Freundlich (38) reports that normal expression of the total AHAS activity requires the *relA* gene and that cyclic adenosine 3′,5′-monophosphate can replace this requirement. There is also evidence that expression of the AHAS activity is sensitive to catabolite repression (116); this effect appears to be specifically related to AHAS I (M. Freundlich, personal communication).

The AHAS isoenzymes affect the synthesis of lysine from *meso*-diaminopimelate in a negative way. In fact, mutants lacking one or more AHAS synthesize lysine at an abnormally high rate. This deprives the cell of *meso*-diaminopimelate and, as a consequence, decreases the synthesis of peptidoglycan, the macromolecule responsible for the cell shape and integrity, in which *meso*-diaminopimelate is an essential component. Therefore, such mutants show morphological defects which are suppressible by the introduction of mutations in the *lysA* gene, the structural gene for *meso*-diaminopimelate decarboxylase (29). The molecular details of these cross-effects are unknown. A symmetrical finding is reported in the case of aspartokinase III, one of the three isoenzymes catalyzing the first step in the biosynthesis of the aspartate family of amino acids, which acts as a positive effector of *meso*-diaminopimelate decarboxylase synthesis (77). In conclusion, these two sets of isoenzymes generate regulatory connections between metabolic processes, suggesting that their presence might not only be informational for specific pathways, but also provide a more general contribution to the cell economy.

VALINE-RESISTANT MUTANTS

The failure of *E. coli* K-12 to grow in minimal medium containing valine provides the possibility of positively selecting Val^r mutants. This tool has been and still is widely used and has been essential in the understanding of the mechanism of valine inhibition. Furthermore, it has allowed a noticeable elucidation of the active transport and regulation of the biosynthesis of the branched-chain amino acids. This procedure is now used also for cell cultures of tobacco (*Nicotiana tabacum*), whose growth is inhibited by valine (14).

Manten and Rowley (65) showed that Val^r mutants occur spontaneously at a frequency of about 5×10^{-7} in a range of valine concentrations between 1 μg/ml and 1 mg/ml. The analysis of a Val^r mutant allowed these authors to recognize a genetic locus for valine resistance, which was shown to be closely linked, by conjugation experiments, to the *leu* operon, located at 1.5 min on the *E. coli* K-12 linkage map (7). Other mutants were described a few years later (20, 59, 60). The biochemical characterization of these strains contributed to the clarification of the mechanism of inhibition, but no genetic analysis was performed. In 1962 Glover published a paper (43) containing a systematic genetic analysis of Val^r mutants. This author isolated 62 spontaneous independent mutations causing resistance to 40 μg of valine per ml and defined their map position by crossing F^- strains with three Hfr strains having different origins and containing the mutations under study. The analysis was completed by P1 transductions. This work defined six genetic classes of Val^r mutations. These classes are: A, approximately located in the *ilv-metE* region (83 to 84 min) but unlinked by P1 transduction to either of these two markers; B, mapping very close to the *thr* operon (0 min), counterclockwise to it; C, mapping very close to the *leu* operon (1.5 min), clockwise to it; D, mapping between the *thr* and *leu* operons; E and F, both located close to the *ilv* cluster and possibly inside it, with a possibility of each being a mixture of more than one class. Class F is located closer than class E to the *ilv* mutation used. The latter class could be located clockwise to this mutation. Glover also studied the level of valine resistance of each class. Mutations of classes B and D give resistance up to 80 μg of valine per ml; mutations of class C give resistance up to 1 mg of valine per ml; and mutations of classes A, E, and F give resistance up to 5 mg of valine per ml.

The work of Glover stimulated further investigations. In fact, the surprisingly large number of different classes of mutants found by this author convinced other workers of the possibility of understanding the phenomenon of valine inhibition and its relationship to the physiology of *E. coli* K-12 through genetic analysis. A great number of new Val^r mutants have been isolated and characterized in the last 16 years. These fall into two classes: transport mutants and mutants altered in the inhibitory process.

Transport Mutants

Valine exerts its inhibition of *E. coli* K-12 growth whether it is present in the medium as a free amino acid or as a component of dipeptides and oligopeptides. The latter compounds are cleaved inside the cell by peptidases, thus liberating valine (79, 102). Mutants resistant to all of these compounds are altered in the intracellular inhibitory process. Mutants resistant to valine but sensitive to valine-containing dipeptides and oligopeptides are altered in the transport of the free amino acid. Mutants resistant to valine-containing oligopeptides but sensitive to valine and to valine-containing dipeptides, if not defective in the mechanism of peptide hydrolysis, are altered in the active transport of oligopeptides. Finally, uptake mutants resistant to valine-containing dipeptides but sensitive to valine and valine-containing oligopeptides should be altered in the transport of dipeptides. However, such mutants cannot be isolated, because the oligopeptide transport also involves uptake of dipeptides. Therefore, mutants lacking dipeptide transport can only be isolated in strains containing alterations in the oligopeptide transport. For details on this subject, the reader is referred to specific reviews (53, 54, 79, 102).

The use of the positive selection described above has allowed a comprehensive analysis of the active transport of oligopeptides, dipeptides, and branched-chain amino acids. Several studies performed in our and in other laboratories have demonstrated that these compounds permeate the cell envelope by means of a multiplicity of active transport systems, each having a specific genetic pattern and typical Michaelis-Menten kinetics.

Oligopeptide uptake occurs through a transport system which is nonspecific, since it mediates the permeation of several oligopeptides and even dipeptides (78). A genetic locus for oligopeptide transport, *opp*, has been defined, and its position at 27 min on the *E. coli* K-12 map has been determined (8, 27). Evidence for a multiplicity of oligopeptide transport systems has been reported (71).

Mutants altered in the *opp* gene still transport dipeptides (8, 27). In fact, dipeptide incorporation occurs not only through the oligopeptide transport system, but also, and predominantly, through a dipeptide-specific transport system, which takes up a number of different dipeptides (27, 78). A genetic locus, *dpp*, responsible for the activity of this system has been defined in *E. coli* K-12. Its map position is at 5 min, distinct from that of the *opp* locus (7, 27).

The criteria described above have been successfully used to isolate mutants altered in the transport of valine (5, 48, 50). The analysis of these mutants has shown that valine uptake occurs through a multiplicity of transport systems, most of which are common to the three branched-chain amino acids. A summary of some properties of the different transport systems for the branched-chain amino acids is reported in Table 4. Table 5 lists the genetic markers thus far described for the transport of branched-chain amino acids and summarizes their relevant properties. Other authors have used alternative designations for some of the systems described in this paper (89, 118). The results reported by these workers are included in Tables 4 and 5, and the alternative designations are listed in Table 4. The isoleucine-leucine-valine transport systems are differentiated on the basis of different patterns of affinity for substrates and inhibition by some compounds (Table 4). Some of the loci reported in Table 5 are common to more than one transport system. The *brnR* gene product is a protein required for activity of both high-affinity 1 and high-affinity 2 transport systems (48). This protein appears to play a role analogous to that observed for the *hisP* gene product in the case of histidine transport in *S. typhimurium* (37); namely, it appears to represent a branching point to both high-affinity systems. In fact, whereas *brnQ* and *brnS* mutants only lack one transport system, high affinity 1 and high affinity 2, respectively, *brnR* mutants lack both of them. The *brnR* gene could also be involved in the expression or activity of the very high affinity transport system, since one of the mutations described [*brnR6*(Am)] has less than 10% of the activity of this system (48).

TABLE 4. *Transport systems for the branched-chain amino acids in E. coli K-12*

Transport system[a]	Substrates	K_m (M)	Relevant inhibitors	Alternative designation	References
VHA	Isoleucine	1.0×10^{-8}	Methionine, threonine, and alanine	LIV-I	47, 48, 89, 118
	Leucine	2.0×10^{-8}			
	Valine	1.0×10^{-7}			
HA-1	Isoleucine	2.0×10^{-6}		LIV-II	47, 48, 89, 118
	Leucine	2.0×10^{-6}			
	Valine	2.0×10^{-6}			
HA-2	Isoleucine	2.0×10^{-6}	Threonine	LIV-II	47, 48, 89, 118
	Leucine	2.0×10^{-6}			
	Valine	2.0×10^{-6}			
L-HA	Leucine	2.0×10^{-7}		Leucine-specific system	41, 47, 48, 89, 118
I-LA	Isoleucine	0.5×10^{-4}			47, 48
L-LA	Leucine	0.5×10^{-4}			47, 48
V-LA	Valine	0.5×10^{-4}			47, 48

[a] VHA, Very high affinity; HA-1 and HA-2, high affinity 1 and 2, respectively; L-HA, leucine-specific high affinity; I-LA, L-LA, and V-LA, isoleucine-specific, leucine-specific, and valine-specific low affinity, respectively.

TABLE 5. *Genetic loci for isoleucine, leucine, and valine transport in E. coli K-12*

Locus[a]	Transport system involved[b]	Map position (units)[a]	Gene Product	References
*brnQ**	HA-1	8	Unknown protein	48, 50
*brnR**	VHA, HA-1, and HA-2	8	Unknown protein	48
*brnS**	HA-2	1	Unknown protein	48
*brnT**	VHA and I-LA	61	Unknown	48, 53
*livH**	VHA	74	Unknown	5
*livJ**	VHA	74	LIV-binding protein (?)[c]	5
*livK**	L-HA	74	L-binding protein (?)[d]	5
livR	VHA	20	Regulatory locus	6
lstR	L-HA	20	Regulatory locus	6

[a] Genetic designations and map positions are as reported by Bachmann et al. (7) with more recent additions. Loci marked with an asterisk are those to which Val^r mutations have been assigned.

[b] See Table 4 for abbreviations.

[c] Binding protein for isoleucine, leucine, and valine.

[d] Leucine-specific binding protein.

A similar pleiotropic effect has been observed for mutations in the *brnT* locus, indicating that this gene is required for the activity of both the very high affinity and the isoleucine-specific, low-affinity transport systems (48, 53).

Some loci are listed in Table 5 as possible structural genes for two binding proteins, one specific for leucine and the other common to the three branched-chain amino acids. A third binding protein, specific for isoleucine, has also been found, but no genetic elements affecting it have been described (41, 80, 89). These proteins can be extracted by osmotic shock (52, 72) from *E. coli* K-12 cells and have been shown to be involved in active transport (76).

Multiplicity of transport systems has been found in several other cases (97). The regulation of isoleucine, leucine, and valine transport is currently under study, mainly by Oxender and his collaborators. These authors report that regulation of transport appears to be independent from regulation of the biosynthesis of these amino acids (87). They also show that leucine plays a central role in the control of active transport. They suggest that isoleucine and valine do not intervene directly in the process but exert some indirect effect, probably because of their role on the expression of the leucine biosynthetic enzymes (85). Finally, they present evidence that the *rho* factor (86), an intact leucyl-tRNA synthetase (83), and fully mature tRNA^{Leu} (84) are required for normal regulation of transport.

The evolutionary origin and physiological significance of the multiplicity of transport systems, which involves a great amount of genetic material, are not yet understood. Since the lipid bilayer of the cell membrane seems to be impermeable to many nutrients (see 53 for a discussion), active transport could be absolutely necessary for life. Therefore, multiplicity could be required to allow the cell to take up nutrients in a wide range of concentrations and in the presence of an excess of other nutrients or compounds negatively affecting the expression or activity of specific transport systems. Furthermore, transport systems could serve additional roles besides uptake, such as excretion or functions related to the cell envelope properties.

Mutants Altered in the Inhibitory Process

Mutants resistant to valine and to valine-containing peptides are altered in the intracellular target of valine inhibition. They will be grouped into the following classes: (i) structural modifications of AHAS I; (ii) regulatory mutations affecting AHAS I; (iii) modifications of AHAS III; (iv) regulatory mutations promoting the expression of AHAS II; (v) other mutations.

(i) Structural modifications of AHAS I. A mutation rendering total AHAS activity insensitive to valine inhibition and consequently conferring a Val^r phenotype to strains containing it was designated *ilvB197* (81, 92). A specific study of the effect of this mutation on AHAS I has not been performed. No other lesions of the same type are known.

(ii) Regulatory mutations affecting AHAS I. The *ilvP* locus (Fig. 1) has been defined as an operator for expression of the *ilvB* gene (91). A strain containing a *cis*-dominant mutation in this locus was isolated as resistant to 1 mM α-aminobutyric acid and was found to be also resistant to 0.2 mM valine. This phenotype appears to be due to the presence of high levels of total AHAS activity.

(iii) Modifications of AHAS III. As reported above, the genetic information for AHAS III is coded for by two genes, *ilvH* and *ilvI*. Mutations in *ilvI* cause absence of AHAS III activity and are responsible for an auxotrophy for isoleucine and valine in an *ilvB* background, even in the presence of the wild-type allele of the *ilvH* gene (26). No Val^r mutations altered in *ilvI* have been found. On the contrary, four *ilvH* mutants have been described, having a Val^r phenotype, in which the AHAS III activity is Val^r in vitro (28, 57). Two additional mutations, *ilv-660* and *ilv-661* (23), are probably located in the *ilvH* gene. Another Val^r mutation, unlinked to any *ilv* gene (the closest marker is *argH*), affects the sensitivity of AHAS III to valine, probably because it causes an enhanced production of an antagonist of the *ilvH* subunit (G. Grimaldi and J. Guardiola, unpublished data).

(iv) Regulatory mutations promoting the expression of AHAS II. In 1964, Ramakrishnan and Adelberg described a *cis*-dominant mutation mapping between the *ilvA* and *ilvC* genes and causing a derepressed synthesis of the *ilvA*, *ilvD*, and *ilvE* gene products (91, 92). At the time, the existence in *E. coli* K-12 of the *ilvG* gene was not yet known, and therefore the reason for the Val^r phenotype of that mutant was unclear. In 1976, Favre et al. (36) described a newly isolated mutation (*ilvO603*) giving rise to a Val^r phenotype and mapping between *ilvA* and *ilvC*, thus confirming the existence of a *cis*-dominant regulatory locus in this position. They also recognized and characterized the *ilvG* gene, which permitted the explanation of the Val^r phenotype of *ilvO603* mutants. One more mutation causing a Val^r phenotype, *ilv-2005* (61), maps between *ilvA* and *ilvC* and therefore has been proposed to be located in the *ilvO* locus. On the other hand, Cohen and Jones (22) showed that two *cis*-dominant Val^r mutations originally isolated but not mapped by Ramakrishnan and Adelberg (90) (now called *ilvR268* and *ilvR269*) are located on the *rbs* side of *ilvE* (Fig. 1). Almost simultaneously, the direction of transcription being from *ilvE* to *ilvA* (instead of the reverse, which had been assumed for many years) was proposed (19, 98). Smith et al. (98) introduced the designation *ilvR* for the region in which the two mutations mapped by Cohen and Jones are located. Two additional *cis*-dominant mutations causing a Val^r phenotype (*ilvR264* and *ilvR651*) and located in this region have been found recently (Squires et al., submitted for publication).

A comparison of the properties of AHAS in mutants containing the *ilvR264* and *ilvO603* mutations shows that the two lesions give rise to expression of activities with identical features (reported in Table 3), which confirms that alterations in either *ilvR* or *ilvO* promote the appearance of a single enzyme, AHAS II, as a consequence of expression of the *ilvG* gene.

(v) Other mutations. A mutation causing a Val^r phenotype, *ilvF465*, has been described and shown to be unlinked to any *ilv* gene (82). The total AHAS activity is insensitive to valine in strains carrying this mutation. It is not known whether this mutation expresses an additional Val^r AHAS or affects in some way the expression or activity of one of the three isoenzymes. In Table 6 we report a list of the genetic loci involved in the process of valine inhibition.

Resistance to valine inhibition arises also as a secondary effect of alterations in the structural genes for leucyl-tRNA synthetase (*leuS*) (M. Iaccarino, unpublished data) and for valyl-tRNA synthetase (*valS*) (120). This phenotype could be due to an enhanced synthesis of AHAS I and/or AHAS III indirectly caused by the mutations.

TABLE 6. *Genetic loci involved in the process of valine inhibition in E. coli K-12*

Locus^a	Map position (units)^a	Function	Phenotype of mutants	References
ilvB	83	Structural gene for AHAS I	Ilv$^-$ in an *ilvI* background	46, 91
ilvF	54	Unknown	Val^r; appearance of a pH 6 AHAS activity; total AHAS is Val^r	82
ilvG	83	Structural gene for AHAS II	Ilv$^-$ in an *ilvO ilvB ilvI* background	33, 36
ilvH	2	Structural gene for a regulatory subunit of AHAS III	Val^r; appearance of a Val^r form of AHAS III	28
ilvI	2	Structural gene for a catalytic subunit of AHAS III	Ilv$^-$ in an *ilvB* background	26
ilvO	83	*cis*-Dominant regulatory effects for synthesis of the *ilvEJDA* gene products and for expression of *ilvG*	Val^r; appearance of the Val^r AHAS II	36, 61, 91, 92
ilvP	83	*cis*-Dominant regulatory effect on *ilvB* expression	Resistance to α-aminobutyric acid and valine	91, 92
ilvR	83	Same as for *ilvO*	Same as for *ilvO*	22, 98; Squires et al., submitted for publication

^{a} Genetic designations and map positions are adapted from Bachmann et al. (7).

It is worthwhile to interpret the collection of Glover's Val^r mutants (see above) in the light of the work accomplished in the last 16 years. Glover defined the existence of at least three classes of mutants, A, E, and F, in the *ilv-metE* region. Presently, we know that mutations in three different genes located in this region, *ilvR*, *ilvO*, and *ilvP*, give rise to a Val^r phenotype and could correspond to Glover's three classes or part of them. Mutants in Glover's class B (mapping close to the *thr* operon at 0 min) have not yet been described. It is unlikely that this class is equivalent to mutations in *valS* (95 min). Structural alterations in the *ilvH* and/or *ilvI* genes or regulatory mutations affecting expression of these genes could identify Glover's class C, mapping very close to the *leu* operon, clockwise to it. Finally, Glover's class D mutants are likely to be altered in the *brnS* gene. Unfortunately, the use of Glover's mutants for further investigations is not possible, since these mutants have been lost (S. W. Glover, personal communication).

Figure 2 shows a linkage map of *E. coli* K-12 in which all the loci relevant to the phenomenon of valine inhibition are reported.

CONCLUSIONS

The occurrence of numerous cases of growth inhibition by natural amino acids in procaryotes and eucaryotes and the reversion of such inhibition by other natural amino acids suggest the existence of antagonism between metabolites in the cell.

Valine inhibition in *E. coli* K-12, being the most dramatic of these phenomena in the most commonly studied microorganism, has been analyzed in detail and provides a typical mechanism of antagonism between structurally related amino acids sharing common features of active transport, biosynthesis, and physiological regulation. A large body of literature demonstrates that the major cause of valine inhibition is the block that this amino acid exerts on the synthesis of the acetohydroxy acid precursors of the branched-chain amino acids, which generates a restriction of isoleucine biosynthesis. An analogous mechanism has been proposed for growth inhibition by valine in barley embryos (68) and in tobacco cell lines (14). Similarly, in some physiological conditions tryptophan inhibits growth of *B. subtilis* as a consequence of its inhibitory effect on the activity of prephenate dehydratase, a phenylalanine biosynthetic enzyme (93). It is likely that many other examples of growth inhibition by natural compounds are the result of similar connections in the cell's processes of biosynthesis and utilization of related metabolites.

The occurrence of these inhibitory phenomena indicates that they escape the limits of the cell's regulatory elasticity. We think that living organisms have not selected biological functions to circumvent this antagonism because conditions of growth in the presence of a severe excess of one compound as compared to a related one do not occur normally in natural environments. The studies on valine inhibition suggest that in the extreme situations in which there is growth inhibition, mutations giving rise to a resistant phenotype occur spontaneously in several different genetic loci. Therefore, the problems caused by antagonism are minor consequences of a cellular organization in which structurally related

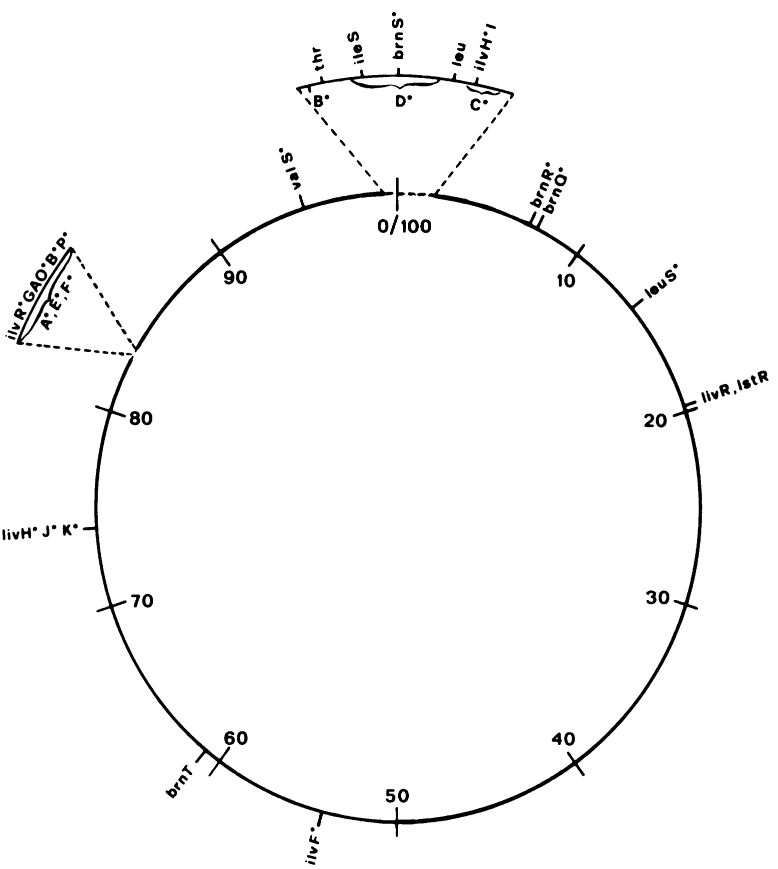

FIG. 2. *E. coli K-12 linkage map (adapted from reference 7) showing the loci relevant to valine inhibition. Inside the circle are reported Glover's classes of Val^r mutants. Loci marked with an asterisk are those to which Val^r mutations have been assigned.*

compounds are also physiologically related, which presumably causes a major energy advantage.

In addition to leading to these general considerations, studies on valine inhibition have provided insight into specific physiological problems, such as regulation of gene expression and active transport.

We have presented the concept that valine inhibits growth because in *E. coli* K-12 the *ilvG* gene, the structural gene for the Val^r AHAS II isoenzyme, is not expressed, although structurally unaltered, and therefore exogenous valine blocks the synthesis of isoleucine through feedback inhibition of AHAS I and AHAS III. Spontaneous regulatory mutations in two loci adjacent to *ilvG* promoting expression of this gene occur and are selected in valine-containing minimal medium. Since the structural gene for a Val^r AHAS isoenzyme appears to be normally expressed in other *Enterobacteriaceae* (11, 36, 74, 117), *E. coli* could be envisioned as deriving from a different strain (or vice versa) after some alteration in organization and expression of the *ilv* genes. If we assume that having *ilvG* unexpressed is not a fortuitous regulatory failure for *E. coli* K-12 and that having it expressed is not a useless function for *S. typhimurium* (and presumably for *E. coli* 9723 and W), then we should conclude that the *ilvG* problem points to a major aspect of evolutionary divergence among these *Enterobacteriaceae.*

The problem of *ilvG* expression is interesting also in the framework of the structure and function of the *ilvREJGDAO* gene cluster. Regulation of these genes cannot be explained in terms of the current ideas on gene expression. We think that this is very useful at a time in which the operon model (56) is being extended and put in a new context. We believe that the studies on the regulation of the *ilvREJGDAO* cluster, through fine genetic analysis and in vitro work with purified deoxyribonucleic acid, which are in progress in several laboratories will allow the establishment of new paradigms for biological regulatory mechanisms.

The isolation of Val^r mutants has been essential in revealing the existence, in *E. coli* K-12, of two normally expressed AHAS isoenzymes catalyzing the first biosynthetic step common to the branched-chain amino acids. As we have discussed above, these isoenzymes appear to play an essential role in the control of carbon flow through the pathway and to be involved in metabolic interactions between branched-chain amino acid biosynthesis and lysine biosynthesis. Biochemical studies with purified preparations and analysis of regulatory mutants, presently in progress, should add more insight into the properties of these isoenzymes and will probably lead to a more general understanding of the role of isoenzymes in diverging metabolic pathways.

Work on the phenomenon of valine inhibition has been very useful in the study of the process of peptide uptake. Furthermore, it has been essential for the understanding of the genetics of multiplicity of active transport for branched-chain amino acids: this has proven to be an interesting model system for energy-mediated cell permeability and is now being extensively studied.

Valine inhibition has also been successfully used for investigating the utilization of peptides as a source of amino acids: Miller and Schwartz (69) have recently described *E. coli* K-12 mutants resistant to valine containing peptides which are deficient in several peptidases.

ADDENDUM

The *E. coli* K-12 linkage map of Bachmann et al. (7) reported *ilvB* and *ilvG* as genes for subunits of acetolactate synthase I and *ilvH* and *ilvI* as genes for subunits of acetolactate synthase II. In the present paper we use a different terminology (see Fig. 1 and Table 6), in agreement with later experimental acquisitions and with a consolidated use in the most recent literature.

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