*CONTROL OF ISOLEUCINE, VALINE, AND LEUCINE BIOSYNTHESIS, I. MULTI-VALENT REPRESSION**

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Repression of the formation of biosynthetic enzymes by the ultimate end product of their action has been shown to be an important aspect of metabolic regulation in microorganisms.[1] In *Escherichia coli* and *Salmonella typhimurium* each of the last four steps in the synthesis of valine and isoleucine is catalyzed by the same enzymes.[2] Repression of these enzymes presents a unique situation since two ultimate end products are formed by their action. In addition, the initial reaction leading to leucine biosynthesis involves an intermediate in the valine pathway.[3] In this report we would like to describe experiments which indicate that valine, isoleucine, and leucine are required for the repression of the enzymes necessary for isoleucine and valine biosynthesis. In contrast to these results, the enzymes of the leucine pathway appear to be repressed only by leucine.

Materials and Methods.—Organisms: The following derivatives of *S. typhimurium* strain LT-2 were used: *leu* 130 *ilva* 224 which lacks enzymes 3 and 7 (Fig. 1) and therefore requires isoleucine, valine, and leucine; *leu* 124 which lacks the initial enzyme in leucine biosynthesis (Fig. 1, enzyme 6); and *ilva* A-8 which is an isoleucine-valine auxotroph deficient in enzyme 3 (Fig. 1) and which was kindly supplied by Dr. M. Demerec. A mutant derived from *E. coli* strain W, M4862-H5, which has a partial block in enzyme 3 and lacks enzyme 5 (Fig. 1), was employed. It has an absolute requirement for isoleucine and valine and a partial requirement for leucine.

Chemicals: α-Hydroxy-β-carboxyisocaproate was isolated from culture filtrates of *Neurospora crassa* using the method of Gross *et al.*[4] α,β-Dihydroxyisovalerate was prepared according to the procedure of Sjolander *et al.*[5] Other compounds were of reagent grade.

Enzyme methods: For this study the following enzymes were examined: (1) L-threonine deaminase (Fig. 1, enzyme 1) which is involved only in the formation of isoleucine; (2) dihydroxyacid dehydrase (Fig. 1, enzyme 4) which is necessary for the biosynthesis of isoleucine and valine; and (3) α-hydroxy-β-carboxyisocaproate decarboxylase (Fig. 1, enzyme 8) which is required solely for the synthesis of leucine.

The procedure for growth of the organisms, preparation of extracts, and measurement of threonine deaminase activity has been described previously.[6] The chemostat experiments followed the usual method.[7] Dihydroxyacid dehydrase activity was measured according to the procedure of Meyers and Adelberg[8] except that in the assay α,β-dihydroxyisovalerate was increased to 20 μmole per ml, the buffer was pH 8.0, and $MgCl_2$ was employed in place of $MgSO_4$. α-Hydroxy-β-carboxyisocaproate decarboxylase activity was determined by measuring the amount of α-ketoisocaproate formed by the indirect ketoacid procedure of Friedemann and Haugen.[9] The assay mixture contained per 2 ml: Tris-HCl buffer, pH 8.0, 300 μmole; $MnCl_2$, 1.0 μmole; KCl, 100 μmole; DPN, 2 μmole, α-hydroxy-β-carboxyisocaproic acid, 1.0 μmole, and crude bacterial extract. The mixture was incubated at 37°C for 10 min after which 3 ml of phenylhydrazine reagent was added to stop the reaction.

The specific activity for all the enzymes examined is expressed as the μmoles of keto acid formed per mg protein per hr.

Results.—The results in Table 1 reveal that an excess of isoleucine, valine, and leucine was needed to repress L-threonine deaminase and dihydroxyacid dehydrase. When any one of these amino acids was added in limiting amounts, in the presence of an excess of the other two compounds, a 5- to 10-fold increase was noted in enzyme activity. In contrast, the activity of the decarboxylase, which is specific for leucine formation, increased only when the cells were grown in the presence of

TABLE 1

EFFECT OF ISOLEUCINE, VALINE, AND LEUCINE ON REPRESSION OF THE ISOLEUCINE-VALINE AND LEUCINE ENZYMES IN *S. typhimurium leu* 130 *ilva* 224 AND *E. coli* M-4862 H5

Amino acid limiting during growth*	Specific Activity: Threonine deaminase — *S. typhimurium*	Threonine deaminase — *E. coli*	Dihydroxyacid dehydrase — *S. typhimurium*	Dihydroxyacid dehydrase — *E. coli*	α-Hydroxy-β-carboxyisocaproate decarboxylase — *S. typhimurium*	α-Hydroxy-β-carboxyisocaproate decarboxylase — *E. coli*
None[a]	7	4	2.6	5.8	6.6	1.2
Isoleucine[b]	74	74	23	30	3.6	3.6
Valine[c]	74	77	26	37	3	2.4
Leucine[d]	66	58	27	24	35	12

* Minimal medium was supplemented as follows:
[a] L-isoleucine 50 μg/ml, L-leucine 50 μg/ml, L-valine 100 μg/ml.
[b] L-isoleucine 8 μg/ml, L-leucine 50 μg/ml, L-valine 100 μg/ml.
[c] L-isoleucine 50 μg/ml, L-leucine 50 μg/ml, glycyl-L-valine 25 μg/ml.
[d] L-isoleucine 50 μg/ml, L-valine 100 μg/ml, L-leucine 13 μg/ml for *S. typhimurium leu* 130 *ilva* 224 and 1 μg/ml for *E. coli* M-4862 H5. For other conditions see text.

limiting amounts of leucine. These results were the same in *S. typhimurium* or in *E. coli*.

Since the *S. typhimurium* mutant used in this experiment is known to accumulate β-carboxy-β-hydroxyisocaproate[4] the possibility exists that this accumulation acted as a drain on valine (via α-ketoisovalerate), thus resulting in valine deficiency and subsequent derepression of the valine enzymes. To test this possibility, another leucine auxotroph of *S. typhimurium* was employed. This organism was blocked in the initial step in leucine biosynthesis (Fig. 1, enzyme 6) and, therefore, was unable to convert α-ketoisovalerate to any leucine precursor. The results presented in Table 2 show that growth in limiting amounts of leucine again resulted in derepression of threonine deaminase and dihydroxyacid dehydrase as well as the decarboxylase. It should be noted that the use of leucine auxotrophs makes it possible to derepress maximally all the enzymes of isoleucine-valine biosynthesis in a strain prototrophic with respect to these amino acids.

An analogous derepression of enzymes leading to leucine in a strain in which this pathway is intact may be achieved by taking advantage of the fact that limitation of growth with valine necessarily produces a limitation in the amount of leucine. This limitation, in turn, should cause derepression of the enzymes in the leucine pathway. To test this assumption an isoleucine-valine auxotroph of *S. typhimurium* LT-2 strain *ilva* A-8 was employed. The cells were grown aerobically in minimal medium.[6] The medium used for derepression of the decarboxylase contained limiting valine (glycyl-L-valine, 25 μg/ml) and excess isoleucine (50 μg/ml). A control flask contained glycyl-L-valine (25 μg/ml), isoleucine (50 μg/ml), and leucine (40 μg/ml). Extracts of cells grown in limiting valine had sixteen times as much decarboxylase activity as those of cells grown in limiting valine and excess leucine.

Discussion.—Repression of enzyme synthesis poses a serious problem in those metabolic sequences where a number of end products are derived from a common precursor. In such situations, overproduction of one of the end products could conceivably lead to the curtailment of the formation of the common intermediate, thus blocking the synthesis of the other essential metabolites. One solution to this problem appears to be the production of multiple enzymes which catalyze the formation of the common intermediate. The synthesis of each enzyme is subject to control specifically by its respective end product.[10] However, in the isoleucine-

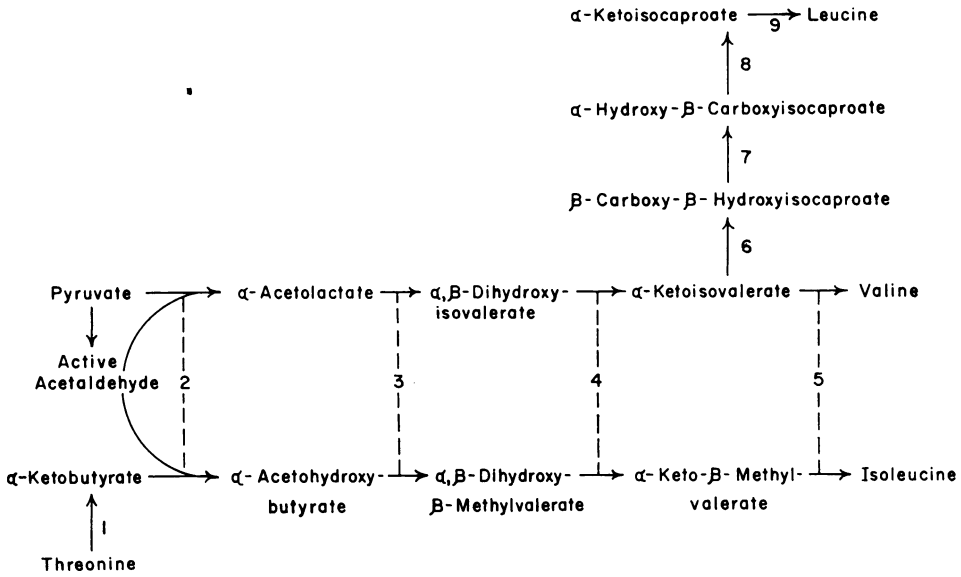

FIG. 1.—Pathway of biosynthesis of isoleucine, valine, and leucine.

valine pathway, each of the last four enzymes (Fig. 1, enzymes 2–5) forms intermediates leading to both isoleucine and valine. Furthermore, three of these enzymes (Fig. 1, enzymes 2–4) are required to produce an intermediate common to valine and leucine. Therefore, each of these enzymes supplies two different intermediates for three ultimate end products, namely, isoleucine, valine, and leucine. Kinetic data from enzyme studies and the occurrence of "single-step" mutants lacking enzymatic activity support the contention that single enzymes catalyze these reactions.[2]

The requirement of both isoleucine and valine for the repression of the enzymes involved in isoleucine and valine biosynthesis prevents the repressive effect of an excess of one of these amino acids from blocking the formation of the other. Since the synthesis of leucine proceeds from an intermediate (α-ketoisovalerate) in the valine pathway, the additional requirement for excess leucine to repress the enzymes in the sequence prevents an excess of valine from impeding leucine formation. This is particularly so because transaminase B (Fig. 1, enzyme 5), which interconverts valine and α-ketoisovalerate, is also repressed along with the rest of the enzymes in the sequence.[11] Therefore, if excess valine and isoleucine were sufficient for repression there would be no mechanism for providing the α-ketoisovalerate for leucine synthesis.[12] The present findings that excess isoleucine, valine, and leu-

TABLE 2

EFFECT OF GROWTH IN LIMITING LEUCINE ON REPRESSION OF THE ISOLEUCINE-VALINE AND LEUCINE ENZYMES IN *S. typhimurium leu* 124

Leucine* (μg/ml)	Specific Activity: Threonine deaminase	Dihydroxyacid dehydrase	α-Hydroxy-β-carboxy-isocaproate decarboxylase
50	8	6	3.6
7.5	87	72	102

* Repressed cells were grown in the presence of 50 μg/ml L-leucine under the usual conditions. The chemostat was used to grow cells under conditions of leucine limitation (7.5 μg/ml). Enzyme activity was measured after 3 generations. For other conditions see text.

cine are all needed to repress the enzymes of isoleucine and valine biosynthesis offer a solution other than multiplicity of enzymes for the general problem of repression in pathways where several metabolites are derived from a common precursor. We suggest that the term multi-valent repression be used to describe this phenomenon.

An additional point of interest is that although the biosynthetic threonine deaminase serves only to provide α-ketobutyrate for isoleucine synthesis, its formation is similarly controlled by the level of all three amino acids. This would not be surprising in the case of *S. typhimurium*, in which the threonine deaminase structural gene lies in a cluster of genes which are concerned with isoleucine and valine biosynthesis.[13] This entire cluster, therefore, like the cluster concerned with histidine biosynthesis,[14] should constitute a single functional unit or "operon."[15] Consistent with this finding was the observation that the activities of threonine deaminase and dihydroxyacid dehydrase in this strain were each about 10-fold greater under conditions of derepression (Table 1). In other words, they appeared to be coordinately repressed.[16] • In contrast, these same enzyme activities in *E. coli* were increased about 17- and 5-fold respectively when derepressed, thus indicating noncoordinate repression. These results would be expected if the corresponding genes were in separate operons. In this regard one map of the chromosome of a different strain of *E. coli* (K-12) does place these genes some distance apart[17] whereas another map of *E. coli* K-12 shows them in the same cluster.[18]

Whether or not the entire sequence of enzymes leading to the biosynthesis of isoleucine and valine is under coordinate control is being further studied in this laboratory.

Summary.—Evidence has been presented that valine, isoleucine, and leucine are required for the repression of the enzymes leading to isoleucine and valine biosynthesis. This phenomenon has been termed multi-valent repression. In contrast, the enzymes of the leucine pathway appear to be repressed only by leucine.

The possible significance of multi-valent repression in the control of isoleucine, valine, and leucine biosynthesis is discussed. In addition, a method is presented whereby derepression of the enzymes in a pathway may be obtained in a strain prototrophic for the pathway.

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[12] This discussion would be only of academic interest if end product inhibition by valine was sufficient to block the initial reaction leading to valine biosynthesis (Fig. 1, enzyme 2). However, in general, end product inhibition by valine is not severe. Indeed, where this mechanism is very effective, as in *E. coli* K-12, excess valine inhibits growth. (Leavitt, R. I., and H. E. Umbarger, *J. Bacteriol.*, **83**, 624 (1962).

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