Regulation of the Biosynthesis of the Branched-Chain Amino Acids

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I. Historical Introduction

For a long time the branched-chain amino acids have been considered under a common heading in biochemistry textbooks. The basis for this grouping was solely their structural relationship since, before the advent of isotopic tracer techniques and mutant methodology, so little was known of their catabolism and nothing was known of their biosynthesis. Indeed, on the basis of their catabolism, they might have been considered separately for studies with the diabetic dog revealed that leucine was a "ketogenic" amino acid, valine was "glycogenic" whereas isoleucine was both.

One of the earliest rational reasons for suspecting that they might be metabolically related was an antagonism observed by Gladstone (*21*) in 1939. He noted that the addition of any one of the three branched-chain amino acids to an otherwise sufficient medium prevented the growth of the anthrax bacillus. The three amino acids added together, however, were stimulatory. Gladstone suggested that each of the amino acids might have interfered with either the *utilization* or the *formation* of the other two. Although this case has not been reexamined, examples could be cited today that illustrate both possibilities suggested by Gladstone.

With the elucidation of the biosynthetic pathways leading to the branched-chain amino acids by isotopic, enzymatic, and genetic studies, it became quite clear that the three amino acids were indeed quite closely related to each other with respect to their biosynthesis. Although it is not within the scope of this chapter to review the evidence that led to the demonstration of these pathways, reference will be repeatedly made to the responsible enzymes and the reactions they catalyze, which are schematically represented in Fig. 1. For a fairly complete review

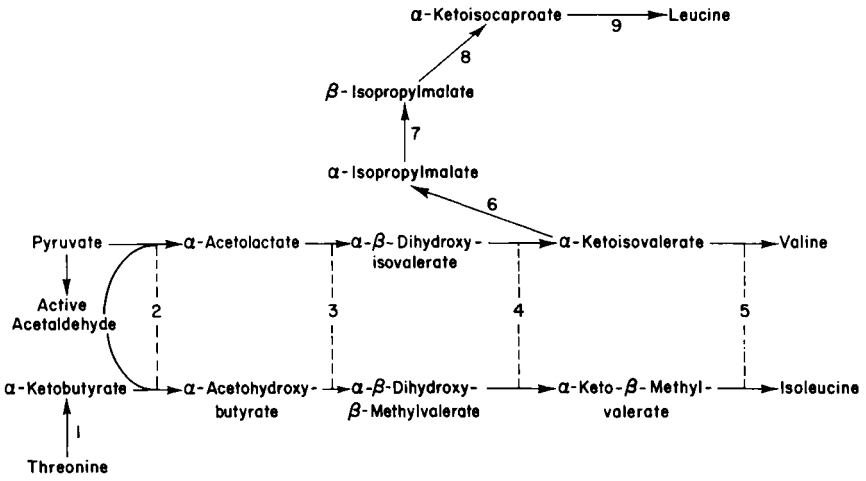

FIG. 1. Biosynthetic pathways leading to the branched-chain amino acids. The enzymes catalyzing the reactions are indicated by arabic numerals; 1, threonine deaminase; 2, acetohydroxy acid synthetase; 3, acetohydroxy acid isomeroreductase; 4, dihydroxy acid dehydrase; 5, transaminase B and, for the valine pathway only, valine-alanine-aminobutyrate transaminase; 6, α-isopropylmalate synthetase; 7, isopropylmalate isomerase; 8, β-isopropylmalate dehydrogenase; 9, transaminase B and a glutamate-leucine transaminase with an undefined specificity.

of that evidence, the reader is referred to an older review (*54*) and, since only isotope data supported the pathway to leucine at the time of its preparation, to a series of three papers documenting that pathway in *Neurospora* and *Salmonella* (*4, 23, 29*).

II. Regulation of Metabolite Flow by End-Product Inhibition

Because the control of the formation of the branched-chain amino acids by end-product inhibition of enzyme action can be considered more simply than the control of their formation by repression of enzyme syn-

thesis, the former will be considered first. Although the two mechanisms can be considered separately, one should not assume *a priori* that the two are completely independent. As shall be shown, the two can be closely interrelated, and even though the examples of their interrelationship to be cited are trivial, one should not yet assume even for these pathways that end-product repression and end-product inhibition are not more fundamentally intertwined in mechanism.

A. Inhibition of Threonine Deaminase

One of the earliest described examples of the almost, but not quite, universal pattern of control of metabolite flow by end-product inhibition is the inhibition of threonine deaminase by isoleucine (*51*). This pattern, inhibition of the first specific enzyme in the pathway by the end product, is also found in the pathways to valine and leucine. Thus far, in all organisms examined the same patterns are found, although in certain details the nature of the interactions appear to vary from one organism to another.

The early studies were made on threonine deaminase of *Escherichia coli* and were characterized by the fact that saturation curves for substrate ([V] *vs.* [S]) and for inhibitor (percent inhibition *vs.* [I]) were sigmoid and that nearly straight lines could be obtained in Lineweaver and Burk or Dixon plots of the kinetic data if the plots were made against $1/[\text{substrate}]^2$ or against $[\text{inhibitor}]^2$, respectively (*51, 52*). In other words, there were cooperative effects in the binding of both substrate and inhibitor implying multiple binding sites on the enzyme with interactions between the sites. Furthermore, the inhibition by isoleucine appeared competitive with substrate and thus enhanced the cooperative interactions (sigmoidicity) seen in the saturation of the enzyme by substrate.

Changeux (*10, 11*), investigating further the specific question of the mechanism by which the cooperative interactions and the competitive antagonism between two dissimilar amino acids occurred, made the very important observation that heating the enzyme, or treating it with mercuric salts, resulted in a loss of sensitivity to inhibition by the end product and a loss of the cooperativity observed in saturation by substrate. This observation was interpreted to indicate that the binding sites for substrate and inhibitor were distinct, the competitive interaction notwithstanding.

Another important observation made by Changeux in these studies was that valine overcame the substrate–substrate interaction (i.e., "nor-

malized" the kinetics) and antagonized the inhibition by isoleucine. Thus, under certain conditions (particularly at low substrate levels), valine stimulated the activity of threonine deaminase and was considered to be an "activator." Studies with the enzyme from *S. typhimurium*, which is almost certainly similar to the *Escherichia coli* enzyme, revealed that valine did not activate either desensitized threonine deaminase or a mutant threonine deaminase which was not inhibited by isoleucine and which exhibited "normal" kinetics (hyperbolic substrate saturation curves) (*18*). Thus, valine, the activator, appeared to overcome substrate–substrate interactions, and isoleucine, the inhibitor, appeared to enhance them.

With the results of Changeux' experiments and the somewhat similar, but more detailed, studies of Gerhart and Pardee (*20*) on the aspartate transcarbamylase of *E. coli* as examples, Monod and Jacob (*38*) pointed out the general importance of the kind of regulatory properties exhibited by these enzymes as a basis not only of end-product inhibition, but also of a way to achieve a physiological coordination of reactions that were not biochemically linked. Specifically, it was pointed out that for valine to stimulate the flow of metabolite along the pathway parallel to that by which it was synthesized when isoleucine was limiting (see Fig. 1) might be as important as the inhibition of that flow when isoleucine was in excess. Although it has not yet been possible to determine whether the interaction of valine with threonine deaminase is physiologically significant, the concept of activation by physiologically related but chemically unrelated metabolites is now matter of factly accepted, and numerous examples have been recognized both in catabolism and biosynthesis.

The second generally important feature of Changeux' results highlighted by Monod and Jacob in their essay was that the competitive inhibitor, isoleucine, was not an isosteric one (binding at the same site as did substrate) but was an allosteric one (binding at a different site) and that therefore its effect was transmitted through the tertiary or perhaps through the quaternary structure of the protein. On the basis of the concept of Koshland (*32*) that proteins are flexible and undergo induced changes in structure upon binding small molecules, this idea seemed quite plausible, although quite vague in terms of mechanism.

The concept of an allosteric protein has since become more formalized and restricted with respect to a mechanism that accounts for cooperative ligand binding (which most regulatory proteins do) in terms of a multimeric protein (which most regulatory proteins are). The present concept, introduced by Monod in collaboration with Wyman and Changeux (*39*),

is that allosteric proteins are composed of identical protomers (containing at least one polypeptide) which are symmetrically arranged and that all the subunits can exist in two states which differ in their affinity for the allosteric ligand. Furthermore, the change of state or allosteric transition is a concerted one in which hybrid enzymes do not exist as stable intermediates. The allosteric ligand therefore is considered as a trapping agent which preferentially binds one form of the enzyme. Interestingly, the mathematical equations describing the ligand binding predicted by this model can account for a surprising number of real systems (including, as will be discussed below, the steady-state kinetic data of threonine deaminase). It currently seems unlikely that the allosteric model in its pure form accounts for all the properties of regulatory proteins, but the concept has probably had a greater impact upon the field of enzymology than any single idea since Sumner's crystallization of urease demonstrated the protein nature of an enzyme.

Many of the earlier observations on threonine deaminase can indeed be interpreted in terms of the allosteric model of Monod *et al.* (*39*). Thus, the enzyme from *E. coli* and *S. typhimurium* appeared to be normally in the inactive form and could be stabilized in that form by isoleucine (a negative effector). Activation occurred by adding valine or substrate (positive effectors).

It must be recalled in considering the early results that, owing to difficulties in the purification of the enzyme, it was always examined in crude extracts or in partially purified preparations. Such extracts might have had some variable amount of valine or isoleucine or both retained by the cells during the harvesting of the cells and the preparation of the extract, or even liberated from extract protein by protease activity after dialysis. Furthermore, small amounts of isoleucine were often added which were subinhibitory at high (saturating) levels of substrate but which at low levels of substrate (below $S_{0.5}$) could have been inhibitory.

It was therefore of interest that in this author's laboratory, different preparations of *E. coli* and *S. typhimurium* extracts yielded substrate saturation curves with varying degrees of sigmoidicity. The first report which was based upon results with a fairly pure preparation of threonine deaminase from *S. typhimurium* was that of Maeba and Sanwal (*35*). These workers did, in fact, observe a noncooperative binding of threonine in the absence of isoleucine, but the usual cooperative binding when isoleucine was present. In retrospect, it may be that these experiments were conducted with enzyme that was free of isoleucine. However, Burns and Zarlengo (*5*) have shown that the effect of very low levels of iso-

leucine (sufficient to render the substrate saturation curves sigmoid) can be overcome in buffer of the ionic strength of that used by Maeba and Sanwal (*35*). It is now clear, however, that the purified threonine deaminase of *S. typhimurium* exhibits cooperative binding of substrate only in the presence of isoleucine. Thus, it now appears that, in terms of the allosteric model of Monod *et al.* (*39*), the equilibrium between the active state and inactive state of threonine deaminase strongly favors the active form so that normal, Michaelis-Menten kinetics are observed except in the presence of the negative effector (isoleucine).

There have been several examples of threonine deaminase which, in crude systems, appeared to be different from those of *S. typhimurium* and *E. coli* in that the enzyme in crude systems exhibited "normal" kinetics except when isoleucine was added to the assay system. An example is the activity found in extracts of *Rhodospirillum rubrum* (*41*). In this laboratory the same was found to be true of the activity in *Micrococcus denitrificans* and *Bacillus subtilis*. The enzyme from the latter was purified and studied in some detail on the assumption that it was different from the *S. typhimurium* enzyme then being purified at Duke. It now appears, however, that the differences between the two enzymes are rather trivial and, although different types of experiments have been done with each enzyme, many of the findings are interpretable by a common model.

Considering first the findings already published on the *S. typhimurium* enzyme, the enzyme appears to be composed of four identical subunits, with a molecular weight of about 200,000 (*60*). In view of the fact that it was possible to demonstrate only two pyridoxal phosphate sites on the enzyme, it would appear that the enzyme is actually a dimer of subunits which contain two identical peptides arranged so that only one substrate site on each subunit is functional. Compatible with this view is the observation that the value of n in an empirical "Hill plot" (*12*) never exceeds 2 for either substrate or inhibitor.

The *B. subtilis* enzyme is very nearly the same size and has been observed in the electron microscope to be composed of two, nearly spherical, subunits (*27*). Half-molecules, but not quarter molecules, have been obtained. It has not yet been possible to demonstrate a reassociation of the half-molecules into active enzyme.

Kinetic studies have been more extensive with the enzyme from *B. subtilis*. However, enough comparative studies have been done with the enzyme from *S. typhimurium* to suggest that the differences will be found to be minor. Using the purified *B. subtilis* enzyme, Hatfield (*27*) found that the steady-state kinetic data (i.e., steady-state velocities

in the presence of varying amounts of substrate and inhibitor) could be readily interpreted, with the proper choice of constants, by the equations describing the allosteric model of Monod *et al.* (*39*). To apply these equations, which involve binding data, to kinetic data, it was necessary to employ the convention introduced by Frieden (*19*), which assumed that isoleucine was purely a "K-system" inhibitor (i.e., it affected substrate binding but not V_{max}), an assumption which seemed justified at least in the ranges of substrate and inhibitor employed. Essentially, the conversion consists of equating the binding function, Y_F, of the original equation with the observed v/V_{max} values. Presumably, too, constants could be chosen so that other models could be used to account for the data. For example, the linear model of Koshland *et al.* (*33*), which can readily be applied to a two-subunit protein and which involves a sequential, rather than a concerted, change in conformation of the subunits, would probably also account for the kinetic data obtained with the *B. subtilis* enzyme.

It is very important to emphasize that it was the steady-state kinetic data that could be explained by the allosteric model of Monod *et al.* (*39*). This limitation was necessary because it was observed that the transition from the inhibited form of the enzyme to the active form was a slow process requiring time periods that were readily demonstrable on an ordinary recording spectrophotometer when a coupled assay (in which the product was reduced by lactic dehydrogenase) was employed. While the transition could be faster (less than 10 seconds) than could be conveniently observed with such an instrument, it could require 15 or 20 minutes to reach a steady-state velocity.

The transition was examined in detail under the rather special conditions in which all the enzyme was initially in the inhibited state, i.e., enzyme was preincubated with isoleucine at a concentration of about 30 times the K_i. A 5-minute preincubation period was sufficient to convert the enzyme, which was in the active state in its isolated form, to the inactive state. The rate of increase in activity was then determined after adding an amount of substrate that would reverse completely the inhibition. It was observed that the rate constant describing the conversion of inactive enzyme to active enzyme was directly proportional to substrate concentration (*27*). Although experiments of this kind were not useful in deciding between a concerted or a sequential transition of the individual subunits, they were useful in deciding between two other alternatives distinguishing the models of Monod *et al.* (*39*) and of Koshland *et al.* (*33*), namely, whether the transition was induced or spontaneous. Specifically, two reaction sequences, which for simplicity

concerned only one of the two presumed subunits, were considered:

$$FI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} P \quad (1)$$

$$FI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FIS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ESI \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} P \quad (2)$$

It was further assumed that during the transition period the intermediates in either sequence would be close to zero concentrations. The integrated differential equation describing the appearance of active enzyme in sequence (1) revealed that the proportionality of rate of activation and substrate concentration was compatible with the concept of a spontaneous reaction. However, the terms in the equation which contained 1/S also contained I. Thus, the rate constant describing the activation should also have been related (inversely) to inhibitor concentration. When this prediction was tested, the rate constant was shown to be *independent of inhibitor concentrations* over the ranges of substrate and inhibitor concentration chosen for study. Variants of sequence (1), such as *introducing an activator* site for substrate or assuming the $F \rightleftharpoons E$ transition to be immeasurably fast, did not alter the general form of the equation.

In contrast, the equation describing the appearance of active enzyme via reaction sequence (2) was compatible with the dependence of the rate of activation upon substrate concentration and its independence of inhibitor concentration. Thus, the experiments favored the concept of an *induced* transition. An apparently noncatalyzed activation could also be observed upon removal of inhibitor by gel filtration. However, even at room temperature, the transition was extremely slow, requiring about 75 minutes under the conditions employed. The nature of the transition is unknown. It does not appear to involve an association or dissociation reaction, however.

Finally, it might be mentioned that threonine deamination can also serve a degradative role in many microorganisms. Obviously, an enzyme with regulatory properties as rigidly related to isoleucine biosynthesis as the one described here could not catalyze a catabolic deamination. An enzyme in *E. coli* that is catabolic is described elsewhere in this volume (*59*). It, too, is a regulatory protein with properties that make it uniquely suited to its presumed catabolic role.

B. Inhibition of Acetohydroxy Acid Synthetase

The enzyme catalyzing the first step in the biosynthesis of valine from the key intermediate, pyruvate, and the one that is inhibited by

valine is acetohydroxy acid synthetase (*53*). The effectiveness of this inhibitory effect in most strains of bacteria would be expected to be very low, since the enzyme catalyzes not only the synthesis of acetolactate, the valine precursor, but also that of acetohydroxybutyrate, the isoleucine precursor. Thus, quenching of the valine pathway by exogenous valine would be expected to be accompanied by a quenching of the isoleucine pathway as well. As will be discussed below, in most strains there is probably a compensatory process (multivalent repression) that would prevent the quenching of isoleucine biosynthesis but that compensatory response would lead to increased valine production as well. Furthermore, there is no evidence that the failure of valine to prevent isoleucine formation in these strains is related to that compensatory process. Paradoxically, although *in vitro* experiments pinpoint a control point for regulation of valine biosynthesis, there is little evidence obtained with growing cells that the sensitivity of the enzyme to valine is physiologically significant. Indeed, one of the few experiments that bears directly on the question was one performed by the Biophysics Group at the Carnegie Institution of Washington's Department of Terrestrial Magnetism during their isotope competition studies on strain B of *E. coli* (*44*). These workers found that the amount of carbon flow over the pathway to valine was virtually the same whether exogenous valine was supplied or not. Thus, isotope competition in this case was probably due to a "swamping" of the endogenously formed pool of valine with exogenous valine. In contrast, flow over the isoleucine pathway was quenched very efficiently by exogenous isoleucine. Nevertheless, the acetohydroxy acid synthetase of *E. coli* strain B is inhibited by valine *in vitro*.

The exception to the ineffectiveness of valine in interfering with isoleucine biosynthesis is found in the K12 strain of *E. coli*, a strain in which valine inhibits growth unless isoleucine or one of its six-carbon precursors is added to the medium (*2*, *34*). Although the sensitivity of the enzyme to inhibition by valine is not the only factor involved in the inhibition of growth (see below), one mechanism of valine resistance in mutants of the K12 strain is the formation of an enzyme less sensitive to valine than that in the wild type (*34*, *42*).

Before acetolactate was proposed by Strassman *et al.* (*49*) as an intermediate in the biosynthesis of valine, it was clearly implicated as an intermediate in the formation of acetoin, a catabolic product of glucose dissimilation by *Aerobacter aerogenes* (*30*). The enzymes responsible for the two roles for acetolactate formation, however, are different (*26*). The valine-inhibited enzyme with a pH optimum of about 8.0 is repressed

in rich medium, whereas the other is formed in rich medium containing sugars, but only after the pH drops to near its optimal pH (6.0). This feature is compatible with its role in acetoin formation by *A. aerogenes* when the fermentation mixture becomes acidic.

Halpern and Even-Shoshan (*25*) have described mutants of *A. aerogenes* that lack the pH 6.0 enzyme as well as mutants that lack the pH 8.0 enzyme. Only the absence of the pH 8.0 enzyme could be correlated with auxotrophy for isoleucine and valine.

Studies by Störmer (*46*) have clearly demonstrated the fact that the two physiologically distinct enzymes in *A. aerogenes* are also biochemically different. The pH 6.0 enzyme was crystallized and shown not to be a flavoprotein (*47*). In contrast, the valine-sensitive enzyme exhibits a requirement for FAD and even in crude extracts, it is almost completely resolved for this compound (*48*). In passing, it might be stated that, thus far, in the author's laboratory attempts to purify the valine-sensitive enzyme from *E. coli* or *S. typhimurium* have been unsuccessful. Stabilizing the enzyme has been a problem, the enzyme appearing to be most stable under conditions that are optimal for catalytic activity (M. Kisumi, personal communication).

For all practical purposes, *E. coli* and *S. typhimurium* do not have the pH 6.0 enzyme found in *A. aerogenes*. However, in extracts in which the valine-sensitive acetohydroxy acid synthetase is strongly repressed, another activity with an optimum at about pH 6.0 is readily apparent. Evidence that this activity *can* function in valine biosynthesis in *E. coli* was provided by a series of experiments by Ramakrishnan and Adelberg (*43*), who obtained the only acetohydroxy acid synthetase-negative mutants yet reported in *E. coli*, an observation that allowed them to locate the position of the structural gene and an adjacent element, which was presumably the operator region.

The procedure these workers employed to obtain acetohydroxy acid synthetase-negative mutants was first to select mutants that were resistant to inhibition by α-aminobutyrate (*43*). Some of the resistant mutants had a derepressed level of acetohydroxy acid synthetase, a derepression attributed to an O^c mutation of an "operator" region specific for the acetohydroxy acid synthetase structural gene. Selection of α-aminobutyrate-sensitive mutants from one of these strains led to the isolation of mutants with lesions in the acetohydroxy acid synthetase structural gene.

Although acetohydroxy acid synthetase belongs to the generic group of proteins that can clearly be termed "regulatory," the enzymes from *E. coli* and *S. typhimurium* do not exhibit cooperative binding of either

substrate or inhibitor. Thus, neither enzyme falls into the generic group of allosteric proteins as rigidly defined by Monod *et al.* (*39*). The enzyme from *E. coli* exhibits a competitive antagonism between valine and pyruvate (*53*). On the other hand, valine inhibits the enzyme from *S. typhimurium* noncompetitively with respect to substrate (*34*).

C. Inhibition of α-Isopropylmalate Synthetase

The third end-product-sensitive enzyme in the pathways to the branched-chain amino acids is the leucine-inhibited enzyme α-isopropylmalate synthetase. This enzyme was highly purified from *Neurospora* by Webster and Gross (*57*), who demonstrated that the saturation of the enzyme by the two substrates, acetyl coenzyme A and α-ketoisovalerate was noncooperative, i.e., normal Michaelis-Menten saturation curves were seen. At pH 7.5, it was observed that leucine inhibited the enzyme competitively with respect to acetyl coenzyme A. At lower pH, the antagonism was of the "mixed" type (i.e., both K_m and V_{max} were affected) with respect to acetyl coenzyme A, as it was with respect to α-ketoisovalerate at all pH values examined. The interaction of inhibitor with enzyme, however, was cooperative, with n values in "Hill plots" of about 1.5.

Physical studies on the enzyme pointed to a structure with a molecular weight of 143,000 which upon treatment with guanidine yielded subunits of about 48,000 (*58*). Analysis of tryptic peptides pointed to a trimeric structure of the enzyme.

Gross and Webster (*24*) described some mutants that were resistant to 5′,5′,5′-trifluoroleucine and which contained α-isopropylmalate synthetases that were resistant to inhibition by leucine. These mutants were of interest because, as will be discussed below, the subsequent two enzymes in the pathway, α-isopropylmalate isomerase and β-isopropylmalate dehydrogenase, were derepressed in these mutants. In yeast, similar mutants containing α-isopropylmalate synthetases resistant to inhibition by leucine do not have derepressed levels of the subsequent two enzymes (*45*). The yeast enzyme, however, has been studied only in crude extracts. In each organism, such mutants overproduce and excrete leucine.

The enzyme from *S. typhimurium* has recently been purified by Kohlhaw *et al.* (*31*). The saturation curves for both substrates reveal normal (noncooperative) kinetics. However, in the presence of leucine, the apparent binding of acetyl coenzyme A, which was competitive with leucine, became increasingly cooperative. As was found with the *Neurospora* enzyme, the enzyme was more strongly inhibited by leucine at pH 6.5 than at pH 8.5, the optimal pH for the *Salmonella* enzyme.

The inhibition by leucine is not complete, particularly at higher acetyl coenzyme A concentrations. The binding of leucine is cooperative, with n values in "Hill plots" of close to 2.

The binding of leucine to the enzyme leads to a reversible retardation of the enzyme on a Sephadex G-100 column that is indicative of a reduction in molecular size, presumably to "half molecules." Disc gel electrophoresis of the purified enzyme yielded two bands of active enzyme, but in the presence of leucine only one (the faster migrating) band was found. The nature of the leucine effect is not understood but is undoubtedly related to binding at the inhibitor site since a leucine-resistant mutant enzyme does not exhibit the leucine-dependent shifts.

The α-isopropylmalate synthetase of yeast is also inhibited by leucine (*45*). Trifluoroleucine-resistant mutants with leucine-insensitive enzymes have been isolated. The enzyme has not been purified, however.

III. Control of Enzyme Level in the Pathways to the Branched-Chain Amino Acids

The second mode of control of biosynthetic function, regulation of enzyme amount, can now be rather clearly defined on the physiological level for the biosynthesis of the branched-chain amino acids in *E. coli* and *S. typhimurium*. For other forms of bacteria, and for *N. crassa* and yeast, less is known although some comparisons are possible. It would appear that, in bacteria, the differences are rather trivial whereas there are probably basic differences between the physiological patterns of control of enzyme amount in prokaryotic and in eukaryotic cells. In neither system, however, can much be stated definitively regarding the molecular mechanism of regulation of the levels of these enzymes (i.e., at the level of gene expression). Because of its greater simplicity from a comparative point of view, the leucine pathway will be considered first.

A. Repression of the Leucine Biosynthetic Enzymes

The regulation of the level of leucine biosynthetic enzymes conforms to the "classical" pattern that would be predicted on the basis of the Jacob-Monod model (*28*) with a few modifications here and there. However, alternative models would be equally apt, and it should be emphasized that the facts presently known do not warrant the assumption that regulation does indeed occur by a simple, repressor-operator model.

There is, however, in *Salmonella* a functional unit of four genes that define the structure of the three specific enzymes required for leucine biosynthesis (*36*). That the cluster does function as a unit is indicated

by the amounts of enzyme found under conditions of repression and derepression. The expression of the cluster does seem to be coordinate, although, owing to a differential stability of the three enzymes, coordination is somewhat difficult to demonstrate (*3*). Analysis of the regulation of this (*leu*) gene cluster has been possible because of the isolation of several different kinds of mutants in which regulation of the cluster was altered. One of these is a mutant in which there is repression of function of the entire cluster by some as yet unidentified component of normal cytoplasm. The mutant, strain *leu 500,* is a leucine auxotroph only in cells containing an intact *supX* gene (*40*). (*SupX* was so named because mutations affecting it suppress the "0^x" or "xenesthetic" mutation in strain *leu 500*). Many of the mutations which suppress the *leu 500* mutation are deletion mutations affecting the adjacent *cysB* region and the tryptophan operon. [The latter are of considerable interest since they provide a mechanism for the direct selection of deletion mutations which extend into or even through the tryptophan operon from the operator end (*37*)].

A set of mutants affecting expression of the *leu* operon were obtained by Calvo (*8*), who isolated a large collection of trifluoroleucine-resistant mutants of *S. typhimurium.* One group of these was shown to have elevated levels of the leucine biosynthetic enzymes and, as a result, was found to overproduce and excrete leucine. The lesion in these mutants was found to be linked to the *leu* operon and was shown to lie in what must be the operator region of the *leu* operon (*7*). The lesions that have been studied in detail appear to lie between the position of the *leu 500* site and what has been recognized as the most operator-proximal mutational site in the *leuA* cistron.

A second group of trifluoroleucine-resistant mutants studied by Calvo and his co-workers (*6*) comprised those in which the lesions were unlinked to the *leu* operon. These were characterized phenotypically by being excretors not only of leucine, but of isoleucine and valine as well. These mutants exhibit derepressed levels of both the leucine biosynthetic enzymes and the isoleucine and valine biosynthetic enzymes. While these mutants might be compared superficially to mutants in which the repressor of the Jacob-Monod model (*28*) has been modified, analysis of their function does not support so simple an interpretation. Actually, two classes of such mutants have been identified. One, which exhibits some repressibility when the level of exogenous leucine is very high, has a leucyl-tRNA synthetase with a reduced affinity for leucine (Calvo, personal communication). The second class exhibits very little repressibility even with high exogenous leucine levels. The biochemical lesion in this class has not yet been elucidated.

The present picture of repression of the *leu* operon is thus unclear. For leucine to trigger repression, its activation does appear necessary. Whether leucine tRNA is itself involved in repression or is necessary for one step leading to the formation of the repressor cannot be stated with certainty. Until now, no mutants have been described which appear to lack the postulated repressor of the Jacob-Monod model. Thus, it is possible neither to support nor to supplant the operator-repressor model for the regulation of the *leu* operon in *Salmonella*.

The relative effectiveness of end-product inhibition and repression in leucine biosynthesis in *Salmonella* was demonstrated in some novel experiments by Calvo and Calvo (*9*). Trifluoroleucine-resistant mutants which contain normal isopropylmalate synthetase, but have derepressed levels of the enzyme, excrete leucine. The same is true for the mutant that contains a leucine-insensitive enzyme but has lower enzyme levels because of repression by endogenously formed leucine. For both the growth rate is normal. Thus, repressibility of the leucine operon is sufficient to prevent overproduction of leucine only if the first enzyme is end-product sensitive. When both lesions were combined in the same organism, excretion of leucine was extremely heavy, and the growth rate was actually reduced unless valine was added.

In *Neurospora,* the studies of Webster and Gross (*57*) on factors regulating the level of enzymes in the pathway to leucine have revealed a physiological pattern that is indeed different from that found in *Salmonella*. As mentioned earlier, the first enzyme in the pathway is inhibited by leucine (*57*). It is also repressed by leucine. On the basis of his analysis using mutants lacking the first enzyme and mutants in which end-product sensitivity of the first enzyme was lost, Gross (*22*) has postulated that α-isopropylmalate, the product of the first enzyme, is the *inducer* of the second and the third enzymes in the pathway.

The physiological pattern in yeast is still different in that incorporation of excess leucine into the medium represses the second and third enzymes, but actually increases the amount of the first enzyme (*45*). The first enzyme, however, is repressed when threonine as well as leucine are present in excess. At the present time, no satisfactory molecular models for the regulation of the leucine biosynthetic enzymes in either yeast or *Neurospora* have been postulated.

B. Repression of the Isoleucine and Valine Biosynthetic Enzymes

The trifluoroleucine-resistant mutants that excrete leucine as well as valine and isoleucine are of special interest when the regulation of the isoleucine–valine-forming enzymes is under consideration, since the

repression of these enzymes in *Salmonella* is multivalent; i.e., for repression to occur, leucine, valine, and isoleucine must be present in excess (*16*). Thus, the trifluoroleucine-resistant mutants in which the enzymes of both pathways are derepressed appear to be altered in a step that is essential for leucine to exert repression on the leucine operon and is essential also for leucine to participate in multivalent repression of the isoleucine–valine (*ilv*) gene cluster.

As implied above in the discussion of an operator-like region specific for the structural gene (*ilv B*) for acetohydroxy acid synthetase, the *ilv* gene cluster does not constitute a single operon. A second distinct operator in *E. coli* was identified by Ramakrishnan and Adelberg (*43*), which upon mutation to the O^c (operator constitutive) state resulted in the derepressed formation of the *ilv A, D* and *E* gene products (enzymes 1, 4 and 5 in Fig. 1). Mutants containing a derepressed *ilv C* gene (which lies between the *ilv B* and *ilv A* genes) have not yet been described, so there is no direct evidence for a third operator region. However, because the isomeroreductase is formed at repressed or derepressed levels in a pattern that is independent of (i.e., not coordinate with) the other four isoleucine- and valine-forming enzymes, there must be some controlling element that provides for the recognition of repressing levels of the branched-chain amino acids or their active derivatives.

Because the mutations in *Salmonella* leading to a leucyl tRNA synthetase with reduced affinity for leucine resulted in faulty recognition of leucine in the multivalent repression of the isoleucine- and valine-forming enzymes, it might be expected that the activating enzymes corresponding to valine and isoleucine might also be involved in the formation of the hypothetical "multivalent repressor." Indeed, the involvement of valyl tRNA synthetase in multivalent repression had been shown even earlier in the demonstration by Eidlic and Neidhardt (*14*) that under conditions of a limited capacity to charge $tRNA^{val}$ with valine, there was a derepression of the *ilv* genes. Further augmenting the idea that multivalent repression might involve activated derivatives of the three branched-chain amino acids was the observation of Szentirmai *et al.* (*50*) on a thiaisoleucine-resistant mutant of *E. coli*. Extracts of this mutant exhibited low levels of isoleucyl tRNA synthetase activity which had a reduced affinity for isoleucine and an even more reduced affinity for the analog, thiaisoleucine. Thus, the altered synthetase resulted in an increased discrimination against the analog. However, it is not clear whether this property or the concomitant derepression of the *ilv A-D-E* operon was the basis for resistance of the mutant.

It should be pointed out that one feature attributed to an operon, viz., the coordinate expression of component genes, was not observed in the experiments with *E. coli* (*13*). Whether this failure was due to a technical difficulty or was an indication of the existence of internal sites of initiation (of either transcription or translation) in the *ilv A-D-E* operon is not clear.

That only three of the five enzymes were found to be derepressed in the mutants was in keeping with the fact that in *E. coli* strain K12, enzymes 2 and 3 in the sequence (see Fig. 1) are not derepressed when isoleucine is limiting (*13*). This difference from the behavior of the same enzymes in *S. typhimurium* will be discussed below.

Whereas the occurrence of the *his S, U,* and *W* mutants in *S. typhimurium* points to direct involvement of histidyl tRNA in the regulation of the *his* operon, analogous evidence is not strong for the *ilv* operon in either *E. coli* or *S. typhimurium*. An interesting correlation between the effects of two analogs of valine differing in their reaction with valyl tRNA synthetase and their effects upon the level of the isoleucine–valine-forming enzymes was reported by Freundlich (*15*). One analog, α-aminobutyrate, competed with valine for activation but was not itself transferred to $tRNA^{val}$. This analog led to a derepression of the *ilv* operon. On the other hand, α-amino-β-chlorobutyrate, which was itself transferred to $tRNA^{val}$, led to repression of the isoleucine–valine-forming enzymes.

These two observations are indeed compatible with the idea that valyl tRNA formation is a necessary reaction in the formation of the "multivalent repressor." However, the observations do not allow such an unequivocal interpretation. Thus, as shown by Ramakrishnan and Adelberg (*43*) and by Freundlich and Clarke (*17*), α-aminobutyrate mimics the effect of valine in inhibiting acetohydroxy acid synthetase *in vitro*. If aminobutyrate were an effective inhibitor of this enzyme *in vivo*, there might be sufficient quenching of valine biosynthesis to lead to a derepression of the *ilv* genes because of limiting valine. Again, the question of the significance of reduced levels of the isoleucine-valine biosynthetic enzymes in the presence of aminochlorobutyrate is difficult to interpret. The difficulty lies in deciding whether the reduced activity of the isoleucine-valine biosynthetic enzymes is due to repression or is a result of formation of inactive protein. Freundlich and his co-workers, however, have taken some pains to demonstrate the continued formation of other enzymes when the isoleucine-valine enzymes were apparently repressed by the substitution of aminochlorobutyrate for valine. It is not clear in any of these experiments whether aminochlorobutyrate has "replaced" valine in the formation of the "multivalent repressor."

On the other hand, until some evidence is obtained for or against the existence of such an entity, discussions of the role played by amino-chlorobutyrate may be premature.

Like the pathway to leucine, the question of mechanism of regulation of the pathway to isoleucine and valine is an open one. In each case, it seems likely that the structural genes are adjacent to receptor elements comparable to the operator of the Jacob-Monod (*28*) model that would provide for recognition of the repressor. The idea that the repressors are not the three branched-chain amino acids themselves is evident from the need for activation (*13, 14*). A cheap hypothesis would be that the repressor of the Jacob-Monod model is "activated" not by the amino acids, but by some derivative of them, such as amino acyl tRNA's. Until some genetic or biochemical evidence is obtained for the existence of an intermediary element comparable to the repressor, the cheap hypothesis is also a worthless one since it does not lead to any experimental tests. It is clear from the discussion that the writer is prejudiced in favor of some sort of negative control mechanism. It should be pointed out that the question of a "positive" control system (i.e., a mechanism that promotes a "turn on" of the transcription of the *ilv* gene cluster) is not completely ruled out.

IV. The Inhibition of Growth of *Escherichia coli* Strain K12 by Valine

In this final section it might be appropriate to discuss and reiterate some of the observations pertaining to the phenomenon that originally stimulated interest in the biosynthesis of the branched-chain amino acids. That phenomenon was, of course, the inhibitory effect of valine on the growth of *E. coli* strain K12 and the reversal of that inhibition by isoleucine (*2*).

Until it was realized that the repression of the isoleucine and valine enzymes was multivalent, it appeared that the greater valine sensitivity of the acetohydroxy acid synthetase of the K12 strain was a sufficient explanation of the quenching of isoleucine biosynthesis and the resulting inhibition of growth (*34*). Compatible with this view is the fact that the six-carbon precursors of isoleucine reverse valine inhibition whereas the four-carbon precursors do not. However, if the enzymes of the isoleucine and valine biosynthetic pathway were multivalently repressed, the quenching of isoleucine biosynthesis should lead to a derepression of the very enzyme that is inhibited by valine (*16*). Indeed, derivatives of the K12 strain in which this enzyme is genetically derepressed are valine-resistant (*43*). However, in this respect, the K12 strain of *E. coli* is unique in that only three of the five enzymes needed for isoleucine and valine biosynthesis are derepressed on limiting isoleucine (*13, 55*).

Thus, the addition of valine to a minimal medium not only inhibits the second step in the sequence of reactions leading to valine, but also causes a repression of the sensitive enzyme and the one following it in the sequence. The molecular basis of the repression signal is, of course, unknown.

On the metabolic level, there yet remains one paradoxical observation to be explained. The apparent mutation of the operator locus controlling the *ilv ADE* cluster to the operator-constitutive state leads to a marked (about 25-fold) derepression of the three corresponding enzymes and to a very high level of resistance to valine (*43*). Furthermore, the valine resistance accompanied the postulated $O^+ \rightarrow O^c$ mutation only if the threonine deaminase structural gene was intact. From this fact it might be inferred that a high rate of threonine deamination results in a reversal of the inhibitory effect of valine. However, the addition of α-ketobutyrate or α-aminobutyrate (which can enter the cell sufficiently well to support the growth of threonine deaminase-deficient mutants) to the medium does not reverse the inhibition. (α-Aminobutyrate in higher concentrations is itself inhibitory, presumably by mimicking the effect of valine itself.) In addition, cells in which the same three enzymes are derepressed by a mechanism not involving the operator locus are still valine sensitive (*50*). Although the level of derepression of threonine deaminase in these cells is only 6-fold, there is not even a low level of resistance to valine.

A possible, though rather unlikely, metabolic mechanism by which a high rate of threonine deamination might allow resistance to valine would be the "removal" of the entering valine via a transamination reaction with the α-ketobutyrate produced by the derepressed enzyme. Examination of the internal amino acid pool should readily reveal whether this possibility is tenable. Perhaps the basis of the resistance that accompanies the $O^+ \rightarrow O^c$ mutation lies in the expression of the gene cluster itself. There may be, for example, an isoleucine–valine-synthetizing particle ("ilvasome") such as postulated for *Neurospora* by Wagner *et al.* (*56*). It may be that the O^c mutation allows the formation of an ilvasome in which isoleucine and valine biosynthesis is more efficiently integrated with the needs of the cell and which functions without interruption by exogenous valine. If such were the case, it may then be that the answer to this 23-year-old question must await the technical breakthrough that will permit the study of gene expression in subcellular systems.

NOTE ADDED IN PROOF

In view of the earlier failures to obtain evidence of an operator region that specifically controlled the structural gene for acetohydroxy

acid isomeroreductase (*ilv C*), the recent observations of Dr. Stuart Arfin and Mr. Barry Ratzkin in this laboratory are of interest. They have found that the isomeroreductase is induced by either of the two substrates even in the presence of repressing levels of isoleucine, valine, and leucine. It would therefore appear that earlier observations on the repression and derepression of this enzyme by the three branched-chain amino acids may have been a secondary consequence of the effect of these amino acids on repression and inhibition of acetohydroxy acid synthetase.

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