Isoleucine and Valine Metabolism in Escherichia coli

VII. A NEGATIVE FEEDBACK MECHANISM CONTROLLING ISOLEUCINE BIOSYNTHESIS*

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As a dividend from the study of biosynthetic pathways in microorganisms, the concept has gradually emerged that an exogenous supply of a metabolite, which can readily enter the cell, will generally interfere with the intracellular synthesis of that metabolite. This interference results in the preferential utilization of the exogenous supply by the organism. The first indication of this interference was obtained in studies on the accumulation of precursors by organisms blocked genetically or by an antimetabolite in the biosynthesis of purines (1), pyrimidines (2), and amino acids (3). These studies also showed that, in at least some cases, the formation of the metabolite in question is suppressed at an early step.

Suppression of metabolite synthesis by an exogenous supply of the metabolite accounts as well for some of the results obtained by Roberts et al. (4) using the technique of isotope competition in the study of biosynthetic pathways in Escherichia coli. The extent of such suppression is shown by the finding of Warner (5) that E. coli (with a potential to synthesize all its metabolites) exhibited virtually the same limited biosynthetic activity as did Leuconostoc mesenteroides (with many biosynthetic deficiencies) when each was grown in a richly supplemented medium.

Conceivably, three kinds of mechanism might underlie preferential utilization of exogenous metabolites. One would be a simple mass-law effect on equilibria along the biosynthetic chain. This possibility can be eliminated a priori for those cases in which the effect of the end product is to prevent accumulation of an intermediate preceding a blocked reaction. A second mechanism would be cessation of synthesis of at least one enzyme in the biosynthetic pathway. A third mechanism would involve inhibition of the activity of at least one such enzyme. In either of the last two mechanisms, some simple molecular interaction would be necessary to transmit (via a feedback loop) the information that the end product was available in the medium. This might be done most directly if the end product itself inhibited the formation of the enzyme or its activity. Fig. 1 illustrates diagrammatically these two sites of inhibition.

Several examples of an end product affecting Site I in Fig. 1, loss of a biosynthetic enzyme activity as result of growth in the presence of the end product, have been recorded. In 1953, Cohn et al. (6) and Wijesundera and Woods (7) observed that cells of E. coli grown in the presence of methionine were unable to convert homocysteine to methionine. More recently, Gorini and Maas (8) have shown that exogenous arginine prevents the formation of ornithine transcarbamylase, an essential enzyme for arginine biosynthesis.

Examples of feedback control in which the end product exerts its effect at Site II in Fig. 1 have now been observed in several laboratories. In a preliminary note from this laboratory, it was reported (9) that L-isoleucine is a strong competitive inhibitor of L-threonine deamination, the first step in the sequence of reactions leading to isoleucine. Yates and Pardee (10) have shown that the formation of ureido-succinate is inhibited by uridine triphosphate. Strehler (11) has shown that proline inhibits the conversion of glutamate to Δ1-pyrroline-5-carboxylic acid. More recently the inhibition of acetolactate formation by L-valine has been observed in this laboratory. Moyed1 has observed that histidine inhibits the formation of the precursor of imidazole glycerol phosphate (12). It seems quite significant that in each of these examples, the end product has been shown to inhibit the earliest step leading specifically to its own synthesis.

This paper will describe some characteristics of the inhibition of L-threonine deaminase2 by L-isoleucine and some consequences of this inhibition for the economy of the growing cell.

MATERIALS AND METHODS

The minimal medium of Davis and Mingioli (15), modified by the omission of sodium citrate, was supplemented as indicated. Growth experiments were performed by methods described previously (16). The mutants of E. coli employed were Strain 12B14, a threonine auxotroph, and Strain M4862-G5, which will be described below.

The accumulation of α-keto-β-methylvaleric acid was estimated approximately by the difference in the color obtained when ethyl acetate and when toluene were employed in the indirect method of Friedemann and Haugen (17). As observed by Meister (18), the 2,4-dinitrophenylhydrazones of α-keto-β-methylvalerate and α-keto-isovalerate are extracted by 10 per cent sodium carbonate from toluene but not from ethyl acetate.

The preparation of extracts and the assay of L-threonine de-

1 H. S. Moyed, personal communication.
2 It should be emphasized here that E. coli has two L-threonine deaminases, each of which also attacks L-serine. One is an adaptive (degradative) enzyme described by Wood and Gunsalus (13). The second is the biosynthetic L-threonine deaminase (14) which is constitutive and is essential for the synthesis of L-isoleucine. In this paper the term L-threonine deaminase refers only to the biosynthetic enzyme.
FIG. 1. Mechanisms for end product control of biosynthesis. Glucose is metabolized to an intermediate, A, which is directed toward synthesis of an end product, P, by the action of enzyme a. The intracellular pool of product, P<sub>int</sub>, is maintained by synthesis from A or by entrance into the cell of exogenous product, P<sub>ex</sub>. Oversynthesis of P is prevented by its control (at Site I) over the system forming enzyme a or by its control (at Site II) over the action of enzyme a. Removal of P<sub>int</sub> by conversion to cell constituents lifts the inhibition thus allowing either formation of additional enzyme a or conversion of A to P.

Enzyme a

Forming System

Amino Acids

Glucose

Cell

Cell Constituents

A

II

P<sub>ex</sub>

P<sub>int</sub>

Enzyme a

Fig. 2. Effect of L-isoleucine on growth and α-keto-β-methylvalerate formation by E. coli strain M 4862-G5. O—O, minimal medium; ▲—▲, same supplemented with 10 μg. each of L-valine and L-leucine and 4 μg. of L-isoleucine per ml.; ○—○, and Δ—Δ, samples receiving 100 μg. of L-isoleucine per ml. at time indicated by arrow. Growth determined turbidimetrically using a Klett-Summerson colorimeter with a K-42 filter. α-Keto-β-methylvalerate determined as described in "Materials and Methods" using 1 ml. of culture fluid partially freed of cells by centrifugation.

Accumulation of α-Keto-β-Methylvalerate—The relationship between cell growth and precursor accumulation in this double mutant was studied in the following way. In the experiment represented in Fig. 2, cells were grown in medium supplemented with 200 μg. each of L-valine and L-leucine and 20 of L-isoleucine per ml. In this medium growth had ceased, owing to isoleucine exhaustion, several hours before the cells were harvested, and α-keto-β-methylvalerate was being actively accumulated. The cells were centrifuged, washed twice with M/5000 phosphate buffer, pH 7.0, and inoculated to a moderate optical density in two flasks, one containing only minimal medium and the second supplemented with a limiting amount of L-isoleucine and a slight excess of L-leucine and L-valine. The cells were shaken in a water bath at 37°, and at intervals samples were removed for determination of cell density and of keto acid production. After 2.5 hours the contents of the flasks were subdivided, and to one sample of each an excess of L-isoleucine (100 μg./ml.) was added. Measurements were then continued as before.

As shown at the top of Fig. 2, the medium containing amino acids permitted growth until the limiting amino acid, L-isoleucine, was exhausted. Upon addition of excess L-isoleucine, as indi-
GROWTH

FIG. 4. Sparing effect of L-isoleucine on L-threonine utilization by E. coli strain 12B14. Each point is the maximal increase in turbidity of a flask culture supplemented as shown.

In the medium that permitted growth, the accumulation of the isoleucine precursor was suppressed for about 30 minutes. It is evident that keto acid accumulation began shortly before growth stopped owing to exhaustion of isoleucine. A more detailed study of the relationship between cessation of growth and onset of keto acid formation by this mutant is represented in Fig. 3. Initially there was very little accumulation of keto acid, the rate roughly paralleling the increase in cell density. However, shortly before the final increase in cell density, there was a rapid increase in the rate of accumulation, after which the rate remained unchanged. It would thus appear that the last portion of exogenous L-isoleucine to be utilized for growth was ineffective in suppressing the pathway to endogenous isoleucine.

The inverse relation between the presence of L-isoleucine and the accumulation of the keto acid indicated by the effect of isoleucine exhaustion is further emphasized by the observation that accumulation ceases abruptly upon the addition of L-isoleucine (indicated by the arrow in Fig. 2). This effect of isoleucine clearly does not depend upon resumption of growth since it is observed even in the suspension (open circles) where growth was prevented by some other deficiency. The conclusion seems warranted that an excess of L-isoleucine inhibits without delay, and presumably by some rather direct mechanism, the synthesis of α-keto-β-methylvalerate and isoleucine.

Sparing Action of L-Isoleucine on L-Threonine Utilization—Some indication of the site of action of L-isoleucine on its own synthesis came from a consideration of its effect on the threonine auxotroph, Strain 12B14. Although no compounds were found to substitute for this amino acid, L-isoleucine exerted a sparing action on the utilization of L-threonine. As is shown in Fig. 4, the yield of cells from a given amount of L-threonine was about doubled by the presence of excess L-isoleucine.

The nature of the very small amount of keto acid (note the difference in scale between Figs. 2 and 3) which appeared during the first minutes of the experiment shown in Fig. 3 is not known. In some experiments, even larger amounts of this material appeared only to disappear before the L-isoleucine was exhausted. It seems unlikely that the color is the result of α-keto-β-methylvalerate.

Fig. 3. Relationship between exhaustion of L-isoleucine and accumulation of α-keto-β-methylvalerate by E. coli strain M 4862-G5. △—△, minimal medium supplemented with 15 µg. of each L-valine and L-leucine per ml. and 4 µg. of L-isoleucine per ml. ■—■, minimal medium supplemented with 15 µg. of each L-valine and L-leucine per ml. and 7 µg. of L-isoleucine per ml. Determinations were as in Fig. 2 except that culture fluids were used without centrifugation.

Fig. 4. Sparing effect of L-isoleucine on L-threonine utilization by E. coli strain 12B14. Each point is the maximal increase in turbidity of a flask culture supplemented as shown.

The diminished growth rate is probably the result of the excessive isoleucine to valine ratio which would tend to exclude valine from the cells (30). Under this condition of limiting L-valine, acetolactate accumulates (12).

In the unsupplemented medium, there is also a moderate increase in optical density. The nature of this increase is not clear but it is doubtful whether in the absence of the required amino acids it represents normal cell growth. The addition of L-isoleucine has no effect on the subsequent density in this medium.

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Inhibition of L-Threonine Deamination—The first step in the conversion of L-threonine to L-isoleucine is its deamination to yield α-ketobutyrate. Since this reaction is irreversible, it is the only one of the several reactions that isoleucine might inhibit in suppressing its own synthesis that could also account for the sparing action on threonine utilization.

Examination of extracts has shown that indeed L-threonine deamination is inhibited more strongly by L-isoleucine than by any other amino acid tested (9). At that time, evidence was presented that the inhibition by L-isoleucine was competitive. In Fig. 5 data plotted by the method of Lineweaver and Burk (24) are presented as further evidence for a competitive antagonism. It is to be noted that the abscissa is $1/S^2$ rather than the usual $1/S$ since this reaction appears to be bimolecular with respect to both substrate and inhibitor. Attempts to alter the conditions of assaying enzyme activity so as to obtain the usual monomolecular kinetic behavior have been unsuccessful.

It has been emphasized that there is a high degree of specificity in the interaction between L-threonine deaminase and L-isoleucine. For example, L-valine, which so often competes with L-isoleucine in biological systems, has no effect on the deamination of L-threonine. However, L-leucine, another amino acid often antagonistic to isoleucine, is an inhibitor, though a much weaker one. Further study has shown that this inhibition, like that by L-isoleucine, is competitive and has the kinetics of a bimolecular reaction. But, unlike L-isoleucine for which the enzyme has a very great affinity, L-leucine appears to combine with the enzyme no better than does the substrate, L-threonine. Some indication of the relative affinities of L-threonine deaminase for these amino acids can be seen in Table I. (Because of the second order kinetics followed by L-threonine and L-serine deamination the apparent dissociation constants have the dimensions of a concentration squared.) The data shown are obtained with crude extracts which vary somewhat in the $K_a$ obtained, owing perhaps to varying content of free L-isoleucine. The precise values are therefore not particularly significant. Nevertheless, the data emphasize the particular effectiveness of L-isoleucine in inhibiting L-threonine deamination, and hence its potential efficiency in controlling this reaction.

### Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$K_{\text{Substrate}}$ (mole/l.)$^2$</th>
<th>$K_{\text{Inhibitor}}$ (mole/l.)</th>
<th>Concentration for half maximal activity</th>
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<tr>
<td>L-Threonine</td>
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<td>$9 \times 10^{-9}$</td>
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<tr>
<td>L-Serine</td>
<td>$18 \times 10^{-9}$</td>
<td>$13 \times 10^{-9}$</td>
<td>$9 \times 10^{-9}$</td>
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<tr>
<td>L-Isoleucine</td>
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<td>$9$ to $11 \times 10^{-9}$</td>
<td>$3 \times 10^{-5}$</td>
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<tr>
<td>L-Leucine</td>
<td>$10^{-5}$</td>
<td></td>
<td>$10^{-5}$</td>
</tr>
</tbody>
</table>

Fig. 5. Competitive inhibition of L-threonine deamination by L-isoleucine. $S$ = Molar concentration of L threonine. $V$ = Klett reading obtained with 0.25 ml. of the 1 ml. reaction mixture containing 0.2 ml. of extract equivalent to 25 mg. wet cells and incubated at 37° for 30 minutes.

Fig. 6. Relationship of growth and L-threonine deaminase activity to the appearance of α-keto-β-methylvalerate in cultures of E. coli M 4862-G5. •, ○, minimal medium; ▲, △, minimal medium supplemented with 10 μg. of each L-valine and L-leucine per ml and 5 μg. of L-isoleucine per ml.; ■, □, minimal medium supplemented with 15 μg. of each L-valine and L-leucine per ml. and 10 μg. of L-isoleucine per ml. Open symbols, growth; closed symbols, broken lines, L-threonine deaminase activity; closed symbols, solid lines, α-keto-β-methylvalerate.
Effect of L-Isoleucine on Appearance of L-Threonine Deaminase Activity—Returning to Fig. 2, it can also be seen that during the brief period of growth, there was an increase in capacity to form keto acid. This increase was greater than would be expected on the basis of the increase in cell density, implying that there was a preferential formation of one or more enzymes required for the synthesis of α-keto-β-methylvalerate.

The data in Fig. 6 suggest such preferential formation of L-threonine deaminase which is the only enzyme in this sequence that could be tested. In this experiment, samples were removed for turbidity and keto acid determination as before. In addition, L-threonine deaminase activity of the intact cells was measured in samples that had been chilled in ice and centrifuged. It can be seen that L-threonine deaminase activity was quite low in the cells at the beginning of the experiment, but in the suspensions given L-isoleucine there was an increase in activity just before the break in the growth curve. In this experiment, unlike those shown in Figs. 2 and 3, the final increase in turbidity was gradual as though the last portion of L-isoleucine were being utilized only slowly. It is striking, however, that L-threonine deaminase activity was preferentially increased during this period. It would thus appear that isoleucine exerted an inhibitory effect on the formation of L-threonine deaminase as well as on its action.

**DISCUSSION**

In the two mechanisms shown in Fig. 1 for blocking of biosynthesis by an end product, the most obviously important difference for the physiology of the cell would be the rapidity with which the biosynthesis is affected by changes in environment. According to Mechanism I, addition of P to the medium would prevent further formation of enzyme a. Synthesis of A would continue though at a linear rate rather than an exponential one. Without a means for destruction of enzyme a, this kind of control could not account for strong suppression of synthesis of certain amino acids observed by Roberts et al. (4) when E. coli was transferred from minimal medium to media containing those amino acids. In the reverse situation, i.e. resumption of synthesis of P upon exhaustion of P, synthesis of enzyme a would be necessary. Whether or not this shift to intracellular formation of the product would be accompanied by a temporary cessation of growth (dianuxie) would depend on how efficiently the last portion of P was directed toward synthesis of enzyme a.

Further evidence that Mechanism I could not account for control of metabolite synthesis is found in the fact that when an auxotroph stops growing because of exhaustion of its growth factor it fails to excrete the whole array of other metabolites (e.g. amino acids and nucleotides) which it is capable of forming. It must be inferred that the formation of these other metabolites is prevented by feedback loops similar to Mechanism II in Fig. 1. This mechanism, involving inhibition by P of the action of enzyme a would respond much more rapidly to environmental changes. Enzyme a would cease functioning as soon as the exogenous product (P_e) entered the cell in sufficient amount. Conversely, synthesis of P would resume as soon as the level of F_int was sufficiently lowered to permit enzyme a to function. This level of F_int would then be maintained as a steady state pool. As P would be removed through synthesis of cellular constituents the pool would be continuously replenished from A.

In the examples of Mechanism II that have been cited, the reaction inhibited by end product is the first in the sequence leading solely toward the synthesis of the end product in question. This common feature is important. For example if isoleucine blocked its own pathway by preventing the utilization of α-ketobutyrate, threonine deamination might have proceeded unchecked and α-ketobutyrate would have been excreted as an end product of glucose metabolism. On the other hand isoleucine could also have blocked its own pathway by inhibiting an earlier step in the pathway, for example, the formation of threonine. In such a case, isoleucine would inhibit the growth of the wild strain and threonine would reverse the inhibition noncompetitively.

Thus, control of biosynthesis by means of a feedback loop involving the initial step in a pathway that is inhibited by the end product would appear to be the only control mechanism which is simple, efficient and without the above drawbacks. It might be anticipated that this pattern will be found to underlie many additional examples of the effect of end product on metabolite synthesis.

There is another potentially important feature of this pattern of feedback control. It is quite possible for an inhibitor to owe its effect on growth to its resemblance to some metabolite in inhibiting the initial step leading to that metabolite. Although the metabolite would be expected to reverse the action of the inhibitor noncompetitively, there may be competition between the inhibitor and metabolite for entry into the cell. This secondary effect of the inhibitor would, of course, be by-passed by employing a suitable derivative or precursor of the metabolite. Thus, examination of the data of Dunn et al. (25) would lead to the prediction that the growth inhibitor, β-thienylalanine, prevents phenylalanine synthesis in E. coli by inhibiting the same reaction that is sensitive to phenylalanine. In this example, entrance of phenylpyruvate and phenylalanine peptides into the cell is unaffected by the inhibitor so that these derivatives are noncompetitive antagonists.

A second kind of interference with feedback control was presaged by Gladstone (26) in 1939 when he proposed that one of a pair of structurally similar amino acids might prevent synthesis of the second. Thus, it has been suggested that valine is inhibitory to Strain K-12 of E. coli because the enzyme over which valine exerts feedback control is also an essential enzyme in isoleucine biosynthesis (16). Perhaps re-examination of some of the early examples of antimetabolite and drug action would reveal more examples of interference with feedback control as mechanism of action.

While it seems clear that L-isoleucine controls its own synthesis by its effect on the action of L-threonine deaminase, isoleucine also affects the formation of this enzyme. Although cells harvested from media containing excess L-isoleucine contain levels of L-threonine deaminase that are more than account for the role of this enzyme in isoleucine synthesis, the level rises in an isoleucine auxotroph as L-isoleucine approaches exhaustion (Fig. 6). It would appear that under these conditions a "brake" is removed and the last portion of isoleucine is preferentially converted to L-threonine deaminase and perhaps to other enzymes leading to isoleucine. Thus, isoleucine seems to prevent the cell from exceeding the "normal" level of L-threonine deaminase. The "normal" level would be empirically defined as that amount of activity which is found in the wild strain of E. coli grown in minimal medium.

Thus, maximal L-threonine deaminase should be obtained in cells growing at a reduced rate owing to slow permeation of
L-isoleucine. Under these conditions there would be a continuous
source of isoleucine for protein synthesis without a pool of iso-
leucine acting as a "brake" on the formation of L-threonine
deaminase. These conditions were achieved with E. coli
mutant M4862-G5 by using high concentrations (100 μg./
ml.) of L-valine and L-leucine and a low concentration (5 μg./
ml.) of L-isoleucine. In this experiment not only L-threonine
deaminase, but also the rate of α-keto-β-methylvalerate ac-
cumulation increased continuously while growth occurred. This
effect of limiting the growth rate by growth factor on the forma-
tion of n-threonine deaminase has been more elegantly demon-
strated by Gorini and Maas (8) by means of a chemostat.

It has been stressed here that a feedback mechanism in which
an end product blocks the action of an early enzyme essential
to its own synthesis can account for the orderly synthesis of
small molecules exhibited by the growing cell. However, it is
obvious that the growing cell is also provided with a mechanism
which prevents excessive synthesis of enzymes and other macro-
molecules. The suppression of enzyme formation by end prod-
uct such as that observed here or that reported in detail by
Gorini and Maas (8), although failing to account for the con-
trolled synthesis of small molecules, is nevertheless an important
process to the economy of the cell. Elucidation of this control
mechanism should eventually be a part of any theory of protein
synthesis.

SUMMARY

1. L-Isoleucine decreases by half the requirement of an
Escherichia coli mutant for L-threonine, a precursor of isoleucine.

2. An excess of L-isoleucine inhibits the accumulation of its
own precursor, α-keto-β-methylvalerate, by a mutant of E. coli
blocked between the two compounds. By use of a doubly
blocked mutant, it was possible to show that this effect of
L-isoleucine is independent of its effect on growth.

3. Both of these observations, as well as the reported pref-
ential utilization of exogenous isoleucine, can be explained
by the further observation that L-isoleucine completely inhibits
the action of L-threonine deaminase, the initial enzyme in the
sequence leading from L-threonine to L-isoleucine.

4. The apparent affinity of L-threonine deaminase for L-isoe-
leucine is about 100 times its affinity for L-threonine. L-Leucine
is a much less effective inhibitor of the same enzyme.

5. An increase in the amount of L-threonine deaminase per
cell was shown to occur at about the time when L-isoleucine
was disappearing from the medium. It is tentatively concluded
that, whereas L-isoleucine does not completely prevent the
formation of L-threonine deaminase, it does prevent its over-
production.

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