THE NUTRITIONAL REQUIREMENTS OF THREONINELESS MUTANTS OF ESCHERICHIA COLI

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In recent years, the study of the growth requirements of biochemically deficient mutants of microorganisms has yielded considerable information concerning the biosynthetic pathways for several amino acids and vitamins. For example, growth studies of a series of Neurospora and bacterial mutants requiring isoleucine and valine demonstrated that the precursors of these amino acids are the corresponding α-keto acids (Umbarger and Adelberg, 1951). Evidence was presented to show that the keto acids, in turn, are formed from the corresponding α,β-dihydroxy acids. At the same time it was suggested, in view of earlier experiments using labeled acetate (Tatum and Adelberg, 1951), that valine and isoleucine had a common four-carbon precursor and that the biosynthetic pathway was closely associated with the biosynthesis of threonine.

This relationship was demonstrated by the fact that one class of isoleucineless mutant had an alternative requirement for d-threonine or several other four-carbon compounds. Another class responded to the same compounds and, in addition, to L-threonine. A third class of threonineless mutant indicated that L-threonine itself is not a precursor of isoleucine since mutants of this class responded only to L-threonine. In a condensed form, the scheme proposed earlier is reproduced below:

\[ \text{Valine} \rightarrow \text{d-threonine} \rightarrow \alpha\text{-aminoacetic acid} \rightarrow \alpha\text{-keto-} \beta\text{-hydroxybutyric acid} \rightarrow \text{Isoleucine} \]

No mutants were available to determine the sequence of the compounds enclosed in brackets

\[ \text{X} \]

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In the above scheme or whether any were only indirectly related to the biosynthetic pathway. Later it was observed that X in the proposed scheme is probably homoserine. Thus the biosynthesis of isoleucine seemed to be closely related not only to threonine but also to methionine (Teas et al., 1948).

In order to examine more critically the role of the four-carbon compounds in isoleucine and valine synthesis, quantitative growth studies have been performed using a representative strain from each of the three classes of threonineless mutants of Escherichia coli. The present paper will report the results of these studies and will discuss their implications.

MATERIALS AND METHODS

Organisms. Three threonine requiring mutants were employed. All were derived from strain K-12 of E. coli by ultraviolet irradiation. Mutant selection was aided by the use of penicillin (Davis, 1949). Strain 12B14 grew on minimal agar plates only when supplemented with L-threonine. Strain JHM-544, kindly supplied by Miss Pauline Miller, grew on minimal agar supplemented with d-threonine; L-threonine was inactive. The third strain, growing on either L- or d-threonine, RSS-60, was isolated from strain RSS-5, originally identified as an organism inhibited by threonine. The apparent inhibition later was attributed to the fact that strain RSS-5 was a mixed culture containing a few threonineless and many prototrophic cells. Growth of the former in the presence of threonine caused the accumulation of an inhibitor which suppressed the growth of the prototrophs. Young cultures, therefore, showed less growth in the presence of threonine than in
its absence. Strain RSS-5 was obtained and kindly donated by Miss R. S. Savat.

Screening of nutriles. The preliminary screening of various enrichments was performed by the auxanographic method (Pontecorvo, 1949).

Quantitative growth measurement. The basal medium of Davis and Mingioli (1950) was employed with the addition of 0.0045 per cent phenol red. The amino acids or other growth factors were added before autoclaving. For each enrichment tested, a series of Wassermann tubes each containing 3.0 ml of the basal medium supplemented with increasing amounts of the growth factor and a corresponding series of 125 ml Erlenmeyer flasks also containing 3.0 ml of medium were inoculated with one capillary drop of a suspension of the mutant under examination. The suspension was prepared from an 18 hour broth culture washed once with distilled water. After 24 hours' incubation at 35°C in an incubator saturated with water vapor to minimize evaporation of the medium, the cultures were acidified with one drop of 4 N HCl to convert the indicator to the yellow (acid) hue. The optical density was determined at 590 mμ using a Coleman model 11 universal spectrophotometer which had been adapted for 2.5 ml volumes. The optical densities reported here have been corrected for the inoculum by subtracting the optical density of inoculated tubes containing no enrichment.

* "Tryp broth"—a pancreatic digest of beef heart containing about 10 per cent meat prepared in this laboratory.

Results and Discussion

Preliminary observations. On the basis of the different supplements which permitted growth of the three mutants in auxanographic agar plates, it was concluded that each of the three strains differed from the wild strain by a unique mutation. In addition, it was observed that the type of growth zone in the seeded agar plate varied with the compound under test as well as the mutant used as the inoculum. For example, when minimal agar plates were seeded with E. coli, strain JHM-544, the growth zone which resulted at the site of application of a loopful of 1 per cent L-isoleucine was relatively small and very dense. On the other hand, L-isoleucine applied in the same way to the surface of minimal agar plates seeded with strain RSS-60 induced a very broad, diffuse growth zone. While no significance was attached to this observation initially, it later became quite apparent that these differences made possible a prediction as to the rapidity of growth response of a given mutant to the various supplements. In table 1 are given compounds which the three mutants would utilize for growth and the type of growth zone observed.

Quantitative growth response. Considering first the behavior of the strain responding to D-threonine, but not L-threonine, strain JHM-544, figure 1 shows that L-isoleucine permitted good growth in both tube and flask cultures. In contrast, D-threonine could be utilized only at higher concentrations under the highly aerobic conditions afforded by the shallow layer of medium in

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tbody>
<tr>
<td><strong>Growth zones in auxanographic plates using Escherichia coli mutants</strong></td>
</tr>
<tr>
<td><strong>COMPOUND APPLIED</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>L-Isoleucine</td>
</tr>
<tr>
<td>Na dl-a-keto-beta-methylvalerate</td>
</tr>
<tr>
<td>DL-a-aminobutyric acid</td>
</tr>
<tr>
<td>Na a-ketobutyrate</td>
</tr>
<tr>
<td>D-Threonine</td>
</tr>
<tr>
<td>DL-Homoserine</td>
</tr>
<tr>
<td>L-Threonine</td>
</tr>
<tr>
<td>a-Keto-beta-hydroxybutyric acid†</td>
</tr>
</tbody>
</table>

* 1 loopful 1 per cent solution.
† Earlier observations (Umbarger and Mueller, 1951). Compound not available for retesting.

Key: D = dense; H = diffuse border; F = faint; S = sharp border.
the Erlenmeyer flasks. This suggests, perhaps, that D-threonine does not lie directly in the biosynthetic pathway but may be converted slowly by an oxidative process to a normal metabolite. On the other hand, L-isoleucine and D-threonine permitted only a low level of growth in the 24 hour incubation period. The low level type of growth utilized very efficiently by strain RSS-60. On the other hand, L-isoleucine and D-threonine permitted only a low level of growth in the 24 hour incubation period. The low level type of growth

**Figure 1.** Growth response of *Escherichia coli*, mutant JHM-544, to increasing concentrations of growth factor.

**Figure 2.** Growth response of *Escherichia coli*, mutant RSS-60

In addition this organism grew very well in both tube and flask cultures supplemented with DL-α-aminobutyric and α-ketobutyric acids. The curves obtained were similar to those obtained with increasing concentrations of L-isoleucine and hence are not shown.

The growth response curves obtained when strain RSS-60 (utilizing either D- or L-threonine) was employed were strikingly different from those obtained using strain JHM-544 as shown in figure 2. L-Threonine and DL-homoserine were response also was noted when α-ketobutyric and DL-α-aminobutyric acids were employed as enrichment (not shown).

The shape of the growth curves obtained when *E. coli*, strain RSS-60, was grown in the presence of L-isoleucine and D-threonine suggested that the growth on these compounds was slower than on L-threonine and DL-homoserine and that with a longer incubation period a higher level of growth might have been expected. That increasing the incubation period did result in increased growth
in the case of L-isoleucine is shown in figure 3. In the same experiment, readings made at 60 hours were somewhat erratic due, apparently, to prototrophic growth. Nevertheless, the continued growth during the additional 20 hours as shown in the graph suggests that when a low level growth response was observed the results indicated a slow utilization of the compound under test.

On the basis of these observations it became possible to correlate the growth response in liquid medium with that observed in auxanographic agar plates. In the auxanographic procedure of determining the spectrum of compounds on which an organism will grow there are two competing rate factors controlling the size and density of the growth zone. One is the rate of diffusion of the enrichment outward from the point of application, and the other is the growth rate of the organism itself. For example, the slow growth of mutant RSS-60 on L-isoleucine permitted that compound to diffuse a considerable distance from the point of application before it had all been assimilated. In contrast, L-threonine permitted a more rapid growth of the cells, and the supply thus was exhausted before it had diffused appreciably, resulting in a compact, dense halo of growth as indicated in table 1.

This variation in density and size of the growth zones brought about by variation in growth rate also can be simulated by varying the number of cells used to seed the agar. For example, when one-tenth the usual number of cells was used as the inoculum, the zones were much more diffuse even on compounds permitting rapid growth. This is understood readily if one considers that, in the presence of 10 cells per unit volume, a doubling of each of the cells will extract from the medium only one-tenth as much nutrient as will the doubling of 100 cells per unit volume in the same period. Since the compound being tested has the same rate of diffusion regardless of cell number, the compound will be permitted to diffuse farther in less heavily seeded agar plates. These factors should be considered when attempts are made to use the auxanographic procedure as a quantitative assay method.

The third organism used in this study, E. coli, strain 12B14, showed a good growth response when the minimal medium was supplemented with its more specific growth requirement, L-threonine. For purposes of comparison, the growth response to increasing concentrations of L-threonine is shown in figure 4.

**Sparring of the L-threonine requirement.** According to the scheme proposed earlier, mutant RSS-60 utilized L-threonine or D,L-homoserine not only as a source of L-threonine for its proteins but also as a source of isoleucine and valine. The slow growth on D-threonine and L-isoleucine thus might indicate that the conversion of these compounds to L-threonine is more sluggish than is the formation of L-valine and L-isoleucine from either homoserine of L-threonine. It was to be expected, however, that a sparing action on the homoserine and the L-threonine requirement would be exerted by L-isoleucine as well as its supposed precursor D-threonine. That such a sparing action did occur is shown in table 2. The reduction in the requirement of mutant RSS-60 for L-threonine and homoserine when L-isoleucine is added is compatible with the idea that a portion of these four-carbon amino acids is utilized for isoleucine synthesis.

While valine alone is inhibitory to strain K-12 and mutants derived from it, this amino acid could be tested also for a sparing action on the four-carbon requirement of strain RSS-60 when it was tested in the presence of L-isoleucine, the antagonist of valine inhibition. In such experiments it was observed that the addition of valine permitted no enhancement of the sparing action brought about by isoleucine alone. This observation suggests that contrary to the scheme proposed earlier, valine is not formed from the same four-carbon intermediate as isoleucine. In agreement with this suggestion is the observation
that mutant JHM-544, believed to be blocked in the conversion of homoserine to the active four-carbon fragment, grew less well on valine plus isoleucine than it did on isoleucine alone.

On the other hand, the results obtained using valine could be due to an artifact inherent in the experimental material since there is a mutual antagonism between isoleucine and valine. Thus, amino acids chosen though even the mutants that were noninhibitory, the efficiency of utilization of each may be decreased in the presence of each other. It would be difficult to design an experiment to test the sparing effect of valine in which this possibility was circumvented. Nevertheless, the evidence obtained thus far makes it appear that in both mutants there is no impairment of valine synthesis.

Further evidence that the scheme as originally proposed is incorrect was obtained when it was observed that strain 12B14, which grew only on L-threonine when various supplements were tested singly, also showed an enhanced growth on L-threonine if D-threonine or L-isoleucine was present in the medium. The sparing effect of isoleucine is decreased if valine is also present. This observation suggests that L-threonine is utilized by strain RSS-60 and strain 12B14 for the same purpose and that perhaps L-threonine also lies directly in the pathway of isoleucine synthesis. The cause underlying the inability of strain 12B14 to respond to isoleucine and D-threonine when added singly is unexplained at present. It is possible that this behavior results from some sort of internal inhibition preventing other compounds from being utilized unless small amounts of suboptimal amounts of an amino acid mixture (Davis, 1950a), the growth of strains JHM-544 and RSS-60 was enhanced in the vicinity of strain 12B14. By means of chromatographic procedures with ion exchange resins and filter paper, it was shown that mutant 12B14 excreted small amounts of valine and α-aminobutyric acid when grown on limiting amounts of L-threonine. Since mutants JHM-544 and RSS-60 respond to α-aminobutyric acid, the observed

![Figure 4. Growth response of Escherichia coli, mutant 12B14](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Enrichment</th>
<th>µg/ml</th>
<th>Optical Density of Flask Cultures</th>
<th>Other Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>8 µg/ml D-threonine</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>4</td>
<td>0.030</td>
<td>0.485</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.249</td>
<td>0.536</td>
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<tr>
<td></td>
<td>16</td>
<td>0.463</td>
<td>0.561</td>
</tr>
<tr>
<td>DL-Homoserine</td>
<td>4</td>
<td>0.151</td>
<td>0.675</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.316</td>
<td>0.659</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.451</td>
<td>0.759</td>
</tr>
</tbody>
</table>

* These optical density values represent percent transmittance readings of 14, 13, and 15. The differences are thus within the limit of error of the spectrophotometer used.
feeding could be accounted for by the presence of this compound. In addition, several other materials were excreted by strain 12B14 which yielded ninhydrin reacting spots on filter paper chromatograms. The quantities were so small that identification has not been possible.

From the results obtained in the experiments described here, it would seem that the scheme originally presented by Umbarger and Adelberg (1951) should be modified with respect to the origin of the carbon chain of valine. In addition, the peculiar behavior of strain 12B14 in responding only to L-threonine yet showing an increased growth with this amino acid when isoleucine is also present would suggest either that L-threonine is a more direct precursor of isoleucine than the scheme suggests or that the genetic change in strain 12B14 has not resulted in the mere absence of an enzyme converting homoserine to L-threonine. Similarly, the blocks in strains JHM-544 and RSS-60 may also be the result of some metabolic error other than the absence of enzyme systems catalyzing the reactions indicated by the broken lines in the scheme.

As an alternative interpretation of the genetic blocks in these organisms, it seems plausible that there is an oversynthesis of some normal metabolite in the mutant cells. The excessive amount of such a metabolite might disrupt the ordinarily well controlled biosynthetic processes of the cell. If such were the case, then the compounds serving as growth factors for these organisms would do so because of their capacity in overcoming to greater or lesser degrees the imbalance brought about by the overproduction of that metabolite. Such interplay between biosynthetic systems has been discussed by Davis (1950b) and demonstrated in the transamination reactions of isoleucine and valine (Umbarger and Magasanik, 1952).

Attempts have been made to demonstrate the accumulation of inhibitors in the culture fluids of these three organisms. As reported above, valine, which is a growth inhibitor of the wild strain, is accumulated in cultures of strain 12B14. Similarly, culture filtrates of E. coli, strain RSS-60, are inhibitory to the wild strain, and the property is overcome by isoleucine as would be expected if valine had been accumulated. In the parent strain the four-carbon compounds employed in this study (in contrast to isoleucine) are unable to reverse the inhibitory action of valine so that the overproduction of valine alone does not explain the growth factor requirements of these organisms.

In this connection it is of considerable interest when auxanographic plates seeded with the parent strain (K-12) are tested with all the readily available amino acids, zones of increased growth have been observed only in the vicinity of those compounds permitting growth of mutant RSS-60. Thus it is tempting to suggest that in strain K-12 there is a partial metabolic block which can be overcome by isoleucine and by compounds thought to serve as precursors of isoleucine. In mutant RSS-60, the block may have become so accentuated that an absolute requirement for any one of these compounds has resulted.

The aim of these studies has been to demonstrate the pathway of biosynthesis of isoleucine and its relation to threonine synthesis. It was supposed that the mutants chosen would serve as ideal tools to investigate this problem just as the experience of others (Gale, 1951) with other biosynthetic systems. That this has not held true in this particular instance suggests that other tools must be sought to discover the complete biosynthesis of threonine and isoleucine.

This somewhat pessimistic view is strengthened further by the recent report by Garner and Teas (1952) who showed that a methionineless Neurospora crassa mutant which accumulated threonine continued to do so even after crossing with a threonineless mutant. In a somewhat similar case, Maas (1952) has obtained a valineless mutant which accumulates valine. The occurrence of organisms which require a given nutrient for growth and also accumulate it indicates that the growth requirement is not due to a deficiency in synthesizing enzymes. It would be interesting to know whether some of the amino acid requirements established for other microorganisms with more complex growth requirements such as the lactobacilli and streptococci are not due to other than the lack of appropriate biosynthetic systems.

At present, no unified scheme for the biosynthesis of threonine and isoleucine in biological systems can be devised from growth studies employing bacterial mutants. It is quite possible that the studies such as are reported here and the similar studies with N. crassa mutants (Garner...
and Teas, 1952) have led away from rather than toward the elucidation of the biosynthetic pathway. Certainly, in view of the differences in the alternative requirements of several of the two groups of mutants, no biosynthetic scheme can be suggested which would not require the invocation of internal metabolic inhibitions in order to explain the genetic blocks.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the technical assistance of Mrs. Martha Wallace.

SUMMARY

Quantitative growth experiments with three threonineless mutants of *Escherichia coli*, strain K-12, were performed. Strain RSS-60 grew fast on L-threonine or DL-homoserine but grew slowly on D-threonine, α-ketobutyrate, α-aminobutyrate, or L-isoleucine. Strain JHM-544 grew fast on α-keto- or α-aminobutyrate or L-isoleucine but utilized D-threonine only at high concentrations under aerobic conditions. Strain 12B14 grew only when L-threonine was supplied.

L-Isoleucine and D-threonine exerted a sparing action on the L-threonine requirement of strain 12B14 as well as strain RSS-60. A similar sparing effect was observed on the DL-homoserine requirement of strain RSS-60. The inhibitory amino acid, L-valine (in the presence of the antagonist, L-isoleucine), exerted no such sparing action.

These observations are not compatible with the scheme proposed by Umbarger and Adelberg (1951). It was concluded that the growth studies performed with the three mutants described here and those reported using *Neurospora crassa* mutants do not permit the construction of a scheme relating the biosynthesis of isoleucine, valine, and threonine.

REFERENCES


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Umbarger, H. E., and Magasanik, B. 1952 Competitive interactions between isoleucine and valine transamination in *Escherichia coli*. Federation Proc., 11, 301.