Effect of threonine on the sensitivity of _Escherichia coli_ K12 to valine under the shift-down condition\(^1\)

—Derepression of acetohydroxy acid synthase and repression of the valine-transport system by threonine—

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ABSTRACT

1. Valine at a concentration of as low as 0.1 \(\mu\)g per ml inhibited growth of strain W3110-215 (\(i^{-}F^{-}\text{thy}^{-}\text{deoC}^{-}\)), a derivative of _Escherichia coli_ K12, after shift-down from nutrient broth to minimal medium.

2. The inhibition by valine at this low concentration was overcome by 0.05 \(\mu\)g or more of isoleucine per ml.

3. On agar medium, valine-inhibition was reversed with unique recovery patterns by alanine, homoserine, leucine, methionine and threonine, suggesting the differences in their mechanisms of action.

4. Cells acquired resistance to 0.1 \(\mu\)g of valine per ml on incubation for more than 40 min in minimal medium containing threonine. The growth of these cells was still sensitive to 1.0 \(\mu\)g or more of valine per ml. Cells incubated in the medium without threonine remained sensitive to 0.1 \(\mu\)g of valine per ml throughout the incubation period of 6 h.

5. The cells acquired resistance because threonine enhanced derepression of synthesis of acetohydroxy acid synthase, which is sensitive to valine, and maintained the repressed condition of the valine-transport system of the cells.

1. INTRODUCTION

Acetohydroxy acid synthase (AHAS) (EC 4.1.3.18, acetolactate synthase) of _Escherichia coli_ (Leavitt and Umbarger 1962) and _Salmonella typhimurium_ (Bauerle et al. 1964) is the first enzyme on the biosynthetic pathway common to isoleucine and valine. The enzyme in _Escherichia coli_ K12 is subject to feedback-inhibition by valine (Leavitt and Umbarger 1962; Bauerle et al. 1964; Umbarger and Brown 1955). As this inhibition of AHAS blocks the syntheses of both isoleucine and valine, the cells do not grow in minimal medium containing valine. This phenomenon is called valine-inhibition (Leavitt and Umbarger 1962). In previous works, more than 0.1 mM (11.7 \(\mu\)g/ml)

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valine was usually added to the culture medium to induce complete growth inhibition (Leavitt and Umbarger 1961, 1962; Umbarger and Brown 1958; O’Neill and Freundlich 1972; Blatt et al. 1972).

We found, however, that when cells of E. coli strain W3110-215 (λ-F-thy-deoC−), a derivative of E. coli K12, were grown in nutrient broth and then shifted down to minimal medium, valine at a concentration of as low as 0.9 μM (0.1 μg/ml) was enough to inhibit their growth for more than 6 h. This valine-inhibition was reversed completely by addition of more than 0.4 μM (0.05 μg/ml) isoleucine. On agar medium, the inhibition was partially reversed by alanine, homoserine, leucine, methionine or threonine as well as by isoleucine. In liquid medium containing one of these compounds in addition to 0.1 μg of valine per ml, cells did not grow for at least 6 h, but grew to a turbid suspension when incubated overnight.

This report describes the effect of threonine on the sensitivity of cells to valine-inhibition and also discusses the mechanism of the phenomena described above.

2. MATERIALS AND METHODS

Organism and media: A derivative of Escherichia coli K12, W3110-215 (λ-F-thy-deoC−) (Okada et al. 1969), was employed in this study. Glucose Simmons medium (Okada et al. 1962) containing 2 μg of sodium citrate (C6H5NaO7·2H2O) per ml instead of sodium glutamate was used as the minimal medium, because some lots of commercial sodium glutamate contain sufficient contaminating valine to inhibit cell growth (Okada et al. 1976). The minimal medium was supplemented with 10 μg of thymine per ml to support growth of the cells. The enriched medium used was nutrient broth containing 5.0 g of NaCl, 10 g of polypeptone and 10 g of meat extract (Wako’s Ehrlich Meat Extract, Osaka) in 1,000 ml of distilled water. Agar media were prepared by adding 1.5% agar to the liquid media. When cells were plated on agar medium by a double agar layer method, soft agar (0.6% agar in saline) was used as the upper layer.

Culture conditions and shift-down procedure: Cells in the exponential phase of growth in nutrient broth were always used. They were harvested by centrifugation, washed twice with the same volume of saline as that of the harvested culture, and suspended in a half volume of the minimal medium. Aliquots of 1 ml of the bacterial suspension were diluted with 6 ml of the minimal medium supplemented with various compounds indicated, and shaken at 37°C. Growth of cells was followed by measuring the absorbance at 660 nm in a Shimadzu-Bausch-Lomb Spectrophotometer, Spectronic 88. Other experimental procedures are described in each experimental section.

Preparation of cell-free extracts: Cells were harvested by centrifugation
(10,000 × g, 1 min). Then they were washed twice with the same volume of 0.05 M sodium-potassium phosphate buffer (pH 7.0) as that of the harvested culture, suspended in one-fifth volume of the same buffer containing 30% glycerol, and stored at -20°C overnight. The cells were thawed and disrupted in the cold by sonication for 60 s in a Tomy 20KC Ultrasonicator, Model RP16. The sonicated cell preparations were centrifuged at 8,200 × g for 10 min and the supernatants were used as cell-free extracts.

Assay of acetohydroxy acid synthase: Acetohydroxy acid synthase (AHAS) activity was assayed by the method described previously (Bauerle et al. 1964) with a slight modification. The assay mixture consisted of 0.1 ml of cell-free extract and 0.8 ml of 0.1 M sodium-potassium phosphate buffer (pH 8.0) containing the following supplements: 40 μmoles sodium pyruvate, 5 μmoles MgSO₄, 100 μg thiamine pyrophosphate per ml and 5 μg flavine adenine dinucleotide per ml. The reaction was started by adding the cell-free extract at 37°C, and stopped by adding 0.1 ml of 6 N H₂SO₄ at 30 min. The mixture was further incubated at 37°C for 30 min to convert acetolactate to acetoin and the latter was determined by the method of Westerfeld (1945); i.e., 4 ml of distilled water, 1.0 ml of 0.5% creatine and 1.0 ml of 5% α-naphthol solution were added to the mixture and incubated for 60 min at room temperature. Then color developed was determined at 580 nm in a Hitachi UV-VIS Spectrophotometer, Model 181. Specific activity is expressed as μmoles of acetolactate formed per mg protein per h. Protein was determined by the colorimetric method of Lowry et al. (1951).

Assay of [¹⁴C] valine uptake: Uptake of [¹⁴C] valine by cells was assayed by the method of Piperno and Oxender (1968) as modified by Kiritani (1974). Exponentially growing cells in nutrient broth were collected, washed twice with saline by centrifugation and suspended in the same volume of the minimal medium as that of the harvested culture. Then, eight cultures were set up, each consisting of 1 ml of cell suspension and 4 ml of the minimal medium containing 10 μg of thymine per ml. They were numbered from 1 to 8. Chloramphenicol (100 μg/ml) was added to cultures 1-4 immediately and to cultures 5-8 after incubation at 37°C for 1 h. Cultures 1, 3, 5 and 7 were supplemented with 10 μg of threonine per ml. As controls, 2, 4-dinitrophenol (4 mM final concentration) was added with chloramphenicol to cultures 3, 4, 7 and 8. After addition of chloramphenicol, the cultures were incubated for 6 min at 37°C, and then [U-¹⁴C] valine (final concentration; 0.2 μCi/ml in 1.0 mM solution) was added to each culture. At various times after its addition, 0.2 ml samples were withdrawn and quickly filtered on a millipore filter HA25 (0.45 μm pore size), and rapidly washed with 5 ml of the minimal medium at room temperature. The filter was dried with a hair-drier and then solubilized by incubation in a vial containing 1.0 ml of methyl cellosolve for 1 h. Then
5.0 ml of scintillator (5 g PPO and 0.3 g POPOP in 1 liter of toluene) was added, and radioactivity was measured with an Aloka Liquid Scintillation Spectrophotometer, Model LSC-653. Control values, which were less than 500 dpm per ml of the original culture, were subtracted from the sample values. Uptake is expressed in dpm per ml of the original culture $\times 10^{-3}$.

Chemicals: L-Valine, 2, 4-dinitrophenol, PPO (2, 5-diphenyloxazole) and dimethyl POPOP (p-bis[2-(4-methyl-5-phenyloxazole)] benzene) were purchased from Nakarai Chemical Co. Threonine, cocarboxylase (thiamine pyrophosphate), sodium pyruvate, creatine and $\alpha$-naphthol were from Wako Pure Chemical Industries Co. Flavine adenine dinucleotide was from Sigma Chemical Co. and chloramphenicol was from Sankyo Co. [U-14C] Valine (specific activity, 250 mCi/mmole) was obtained from the Radiochemical Centre (Amersham).

3. RESULTS

Hypersensitivity to valine under the shift-down condition

When cells of strain W3110-215 harvested from cultures in minimal medium or nutrient broth were suspended in minimal medium supplemented with or without valine, growth of cells transferred from nutrient broth was inhibited by addition of valine (0.1 $\mu$g/ml), but no such inhibition was observed for cells pre- grown in minimal medium (Fig. 1). The inhibitory concentration of
Valine was examined by transferring cells grown in minimal medium and in nutrient broth to fresh minimal medium containing valine at various concentrations (Fig. 2). Cells from cultures in minimal medium grew normally in the presence of 0.1 μg of valine per ml, but their growth was completely inhibited by valine at a concentration higher than 1.0 μg per ml (Fig. 2A). On the other hand, under the shift-down condition, growth of cells was inhibited completely for a period longer than 6 h by as little as 0.1 μg of valine per ml (Fig. 2B). Various derivatives of E. coli K12 were examined for their sensitivity to the valine inhibition. The results showed that they were all sensitive to valine; indicating that the phenomenon is common to all the derivatives of E. coli K12 tested (data are not shown). Next, the effect of isoleucine on the valine-inhibition was examined to know whether the mechanism of the inhibition is the same as that reported by Leavitt and Umbarger (1962). Under the shift-down condition, cells were grown in minimal medium containing either 0.1 μg (Fig. 3A) or 10 μg (Fig. 3B) of valine per ml with isoleucine at various concentrations. The results indicated that the inhibition by 0.1 μg of valine per ml was reversed completely by addition of 0.06 μg or more of isoleucine per ml (Fig. 3A) and that the inhibition by 10 μg of valine per ml was reversed by 3.0 μg or more of isoleucine per ml (Fig. 3B).
Fig. 3. Effect of isoleucine concentration on growth of the strain W3110-215 in the presence of valine. Cells grown in nutrient broth were shifted down to minimal medium containing either 0.1 μg (Fig. 3A) or 10 μg (Fig. 3B) of valine per ml with isoleucine at various concentrations. The absorbances at 660 nm after incubation for 7 h are plotted against the concentration of isoleucine. The broken line indicates the absorbance of the culture without valine after incubation for 7 h.

Fig. 4. Effects of isoleucine, alanine, methionine and threonine on valine-inhibition on agar medium. Cells grown in nutrient broth were washed and plated on minimal agar medium containing thymine. A filter paper disc soaked in 10 mM valine was placed in the center of each plate and another paper disc soaked in isoleucine (a), alanine (b), methionine (c) or threonine (d), all at the concentration of 10 mM, was placed 1 cm apart from the disc soaked in valine. The plates were incubated at 37°C overnight. The pattern with leucine, which is not shown, was similar to that with threonine (d). Bright area, growth; shadowed area, growth inhibition.
Effects of various amino acids on valine-inhibition

Cells grown in nutrient broth were transferred to 2.0 ml of minimal liquid medium containing 0.1 μg of valine per ml and 10 μg per ml of either one of 18 amino acids, the constituents of protein excluding isoleucine and valine, or homoserine or hydroxyproline. Then the cells were incubated at 37°C overnight. Results indicated that, among the amino acids examined, alanine, homoserine, leucine, methionine and threonine could overcome the inhibition by valine at low concentrations (data are not shown).

Next, cells grown in nutrient broth were washed and plated on minimal agar medium containing thymine by the double agar layer method, and two filter papers (6 mm in diameter), one saturated with 10 mM solutions of valine and the other with one of the effective amino acids (see above), were placed on each plate 1 or 2 cm apart from each other. Each amino acid showed a different recovery pattern on the plate after incubation overnight at 37°C as seen in Fig. 4.

Time course experiments showed that growth inhibition by 0.1 μg of valine per ml was not reversed during incubation for 6 h by addition of 10 μg of alanine or leucine per ml to the liquid minimal medium. The valine-inhibition was overcome almost completely by isoleucine, but the onset of growth was delayed about 3 h when methionine was added instead of isoleucine (Fig. 5).
Valine-inhibition was not overcome by threonine in liquid medium during the first 6 h of incubation at 37°C (Fig. 6A). However, the results in Fig. 4 suggest that previous contact of cells with threonine was effective in making them resistant to valine-inhibition. An experiment was performed to examine this possibility.

Cells were shifted down from nutrient broth to minimal medium with or without 10 μg per ml, and valine (0.1 μg/ml) was added after incubation for 0, 60 or 120 min. As in the previous experiments, when valine was added at zero time to the culture medium containing threonine, cells did not start growing until 6 h. However, when valine was added after 60 or 120 min of the contact of cells with threonine, they grew equally well as in the medium without valine (Fig. 6A). When valine was added to cultures without threonine, no growth was observed, even when valine was added after incubation for 60 or 120 min at 37°C (Fig. 6B).

To determine more precisely the incubation time with threonine necessary for cells to become refractory to valine-inhibition, we added valine to cultures incubated in medium containing threonine at 0, 10, 20, 30, 40 and 60 min after the shift-down and measured the turbidities of them 7 h after the addition of valine. As shown in Fig. 7, when cells were incubated in medium containing threonine, levels of their resistance to inhibition by valine (1.0 μg/ml) began

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**Fig. 6. Effects of threonine and valine on growth of the strain W3110-215.** Cells grown in nutrient broth were shifted down to minimal medium with (A) or without (B) threonine (10 μg per ml). Valine (final concentration; 0.1 μg per ml) was added to cultures at 0 h (●), 1 h (△) or 2 h (▲) after the shift-down. Valine was not added to the control culture (○).

**Effect of threonine on valine-inhibition**

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Fig. 7. Effect of threonine on sensitivity of the strain W3110-215 to valine. Cells grown in nutrient broth were transferred to minimal medium with (●) or without (○) threonine at 10 µg per ml, and valine (0.1 µg per ml) was added at 0, 10, 20, 30, 40 or 60 min after the transfer. The absorbance of cultures at 660 nm after incubation for 7 h from the addition of valine is plotted against the time of valine addition. The following broken lines indicate absorbances at 660 nm after incubation for 7 h without valine: ————, with threonine; ————, without threonine.

Fig. 8. Sensitivity of the strain W3110-215 to valine. Cells grown in nutrient broth were transferred to minimal medium containing 10 µg of threonine per ml. After incubation for 0 (○) or 60 (●) min at 37°C, valine at various concentrations was added to the cultures. The absorbance at 660 nm after incubation for 7 h from the addition of valine is plotted against the concentration of valine. The following broken lines indicate the absorbance of cultures after incubation for 7 h without valine: ————, with threonine; ————, without threonine.
to elevate at 20 min and attained the maximum level at 40 min after the shift-down. Cells incubated without threonine remained sensitive to valine throughout the incubation period.

Next, we compared the levels of resistance to the valine-inhibition between cells incubated in minimal medium containing threonine for 0 and 60 min after the shift-down. The results in Fig. 8 indicate that 0.1 µg of valine per ml was sufficient for maximal growth inhibition of the former cells, whereas at least 1.0 µg of valine per ml was necessary to inhibit growth of the latter. These results also indicate that cells acquired resistance to 0.1 µg of valine per ml by treatment with threonine were still sensitive to 1.0 µg of valine per ml.

**Effect of threonine on derepression of AHAS**

There are many examples that cells are more resistant to an inhibitor of an enzyme when the enzyme activities are higher. This is because of the increase in the number of targets of the inhibitor. Therefore, there is a possibility that addition of threonine rendered cells resistant to valine (0.1 µg/ml) by inducing synthesis of AHAS.

As seen in Table 1, in the absence of threonine, the enzyme activity increased 2.5~4.4 folds during incubation for 60 min in minimal medium, and in the presence of threonine, the activity further increased to the levels about 1.5~4.0 times higher than those obtained in its absence. These results indicate that threonine accelerates derepression of the synthesis of AHAS.

**Effect of threonine on the valine-transport system**

Another possibility is that cells acquired resistance to valine, because synthesis of the valine-transport system could not be derepressed in the presence

### Table 1. Effect of threonine on the synthesis of AHAS

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Nutrient broth</th>
<th>Minimal medium</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−Thr</td>
<td>+Thr</td>
</tr>
<tr>
<td>1</td>
<td>0.19</td>
<td>0.83</td>
<td>1.46</td>
</tr>
<tr>
<td>2</td>
<td>0.22</td>
<td>0.56</td>
<td>1.74</td>
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<tr>
<td>3</td>
<td>0.12</td>
<td>0.35</td>
<td>1.45</td>
</tr>
<tr>
<td>4</td>
<td>0.23</td>
<td>0.72</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Abbreviations: −Thr, without threonine; +Thr, with threonine.
of threonine and remained at a restricted level for the entrance of valine into cells. To examine this possibility, the efficiency of transport of [14C] valine by cells was assayed in the presence or the absence of threonine. Uptake of [14C] valine by cells as a function of time is illustrated in Fig. 9. The intracellular pool of [14C] valine was saturated after incubation for 1.5-2.0 min at 37°C. The saturation level in the cells with no incubation after the shift-down was not influenced by the presence of threonine in the minimal medium (Fig. 9A). However, on incubation for 60 min in the absence of threonine, the saturation level increased about 2-fold, while, when cells were incubated for 60 min in the presence of threonine, it did not (Fig. 9B). Thus, threonine at the concentration of 10 μg per ml inhibits derepression of the valine-transport system.

4. DISCUSSION

When cells of the strain W3110-215, a derivative of E. coli K12, were shifted down to minimal medium, their growth was inhibited by valine at as low a concentration as 0.1 μg per ml (Figs. 1 and 2). This inhibition was reversed completely by isoleucine (Fig. 8), indicating that the inhibition is caused by the same mechanism as that reported previously for the inhibition by valine at higher concentrations (Umbarger and Brown 1955; Leavitt and Umbarger 1962). Since the syntheses of biosynthetic enzymes in general are assumed to be repressed in nutrient broth (Anraku 1968), the synthesis of AHAS was
also assumed to be repressed in cells grown in nutrient broth. As expected, AHAS activity in these cells was low, as shown in Table 1. The low activity of AHAS might be responsible for the hypersensitivity of cells to valine after the shift-down. The sensitivity of the cells to inhibition by 0.1 μg of valine per ml was minimized when they were kept in contact with threonine in minimal medium for 40 min in the absence of valine (Figs. 6 and 7). During this period, threonine derepressed the synthesis of AHAS (Table 1) and maintained the valine-transport system at a repressed level (Fig. 9). The former phenomenon results in increase in the number of targets of valine, the feedback inhibitor, while the latter restricts the entrance of valine into the cells. Thus, these two phenomena together result in increased resistance of the cells to valine.

We do not know of any previous report on the effect of threonine on the production of AHAS. There are strong evidences that three separate genetic loci, ilvB, ilvG and ilvII (Guardiola et al. 1974b), code for three AHAS isozymes, AHAS I, AHAS II and AHAS III, respectively, in E. coli K12 (Guardiola et al. 1977). AHAS I and AHAS III are sensitive to feedback inhibition by valine (Val⁵) (Guardiola et al. 1977; Davis et al. 1977; De Felice and Levinthal 1977; De Felice et al. 1978), while AHAS II has been believed to be insensitive to valine-inhibition and expressed only in an ilvO⁻ mutant (Favre et al. 1976; Smith et al. 1979). However, a recent paper (Berg et al. 1980) reported that ilvO⁻ mutations reside within ilvG, which alter the valine binding site of AHAS II making it insensitive to valine-inhibition. According to this finding, all three AHAS-isozymes in E. coli K12 are concluded to be sensitive to the inhibition by valine. Therefore, we can predict that AHAS synthesized in the presence of threonine is valine-sensitive. The results shown in Fig. 8 indicate that AHASsynthesized in the presence of threonine are sensitive to valine, because the growth of cells procedingly grown for 60 min in medium containing threonine was inhibited by 1.0 μg or more of valine per ml.

Since E. coli strain W3110-215 is not an auxotroph for isoleucine and/or valine, threonine can be converted to isoleucine via a common biosynthetic pathway for isoleucine and valine in this strain. Therefore, the observed effect of threonine could be due to isoleucine derived from threonine. Although the inhibition-recovery patterns on minimal agar plates showed a great difference between the effects of threonine and isoleucine (Fig. 4), this difference does not necessarily indicate a difference in their mechanisms of action, because valine inhibits the biosynthesis of isoleucine from threonine, making the cells deficient in isoleucine. In spite of the facts described above, the possibility that threonine or one of its derivatives other than isoleucine, such as threonyl-tRNA, participates in regulation of the synthesis of the enzyme cannot be excluded. The effect of isoleucine on the production of Val⁵AHAS has been reported by several workers (Dwyer and Umbarger 1968; Jackson...
AHAS synthesis and valine transport system

and Henderson 1975; Wiginton and Shive 1978). Among these reports, only Wiginton and Shive (1978) indicated a positive effect of isoleucine on the synthesis of Val\(^{\text{A}}\)AHAS. But we do not know of any article reporting a positive effect of threonine on the AHAS synthesis.

In our experiments, valine transport activity was kept under a repressed condition in a medium containing threonine during the incubation period of 60 min. From this observation, it can be assumed that threonine in the medium inhibits valine-transport into the cells. The results in Figs. 6, 7 and 8, however, indicate that 84 µM (10 µg per ml) threonine did not prevent the entry of 0.84 µM (0.1 µg per ml) valine. Therefore, it is unlikely that threonine in the medium inhibited the entry of valine into the cells.

Quay et al. (1975) demonstrated with a temperature-sensitive mutant of leucyl-tRNA synthetase that repression of branched-chain amino acid transport involves the interaction of leucine with its aminoacyl-tRNA synthetase and its cognate leucyl-tRNA species. Leucyl-tRNA is also involved in regulation of the synthesis of isoleucine-valine biosynthetic enzymes (Hatfield and Burns 1970; Calhoun and Hatfield 1973; Cortese et al. 1974; Coleman et al. 1975; Lawther and Hatfield 1978). Since threonine is not converted to leucine, the repression by threonine of the valine-transport system is independent of the repression by leucine. Therefore, it might be possible that the effects of threonine are exerted on valine-transport system and the synthesis of AHAS through cognate tRNA and aminoacyl-tRNA synthetase (Quay et al. 1975).

In addition to isoleucine and threonine, we found that alanine, homoserine, leucine and methionine also overcome valine-inhibition in specific ways (Fig. 4). Guardiola et al. (1974a) assumed that the very high affinity transport process is common for the uptakes of isoleucine, leucine, valine, threonine, methionine and alanine. Methionine is also known to repress the very high affinity transport system (Guardiola et al. 1974a). However, the recovery patterns on agar plates observed in this work (Fig. 4) suggest that the mechanism by which these compounds exert their effects on valine-inhibition is complex.

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**AHAS synthesis and valine transport system**


