THE UTILIZATION OF LEUCINE DERIVATIVES BY A
MUTANT STRAIN OF ESCHERICHIA COLI*

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The ability of suitable strains of Escherichia coli to use certain peptides
and other derivatives of phenylalanine and tyrosine as growth factors in
place of the free amino acids has been reported recently from these labora-
tories (1). In the present communication, there are described the results
of a study of the growth-promoting action of derivatives of leucine for a
leucineless mutant strain.

The leucineless strain is a double mutant of strain K-12 of Escherichia
coli and differs from the latter by its requirement for exogenous sources of
threonine and leucine (2). The threonine requirement of the double
mutant was induced by the irradiation of strain K-12 with x-rays (3). A
second treatment of the resultant threonineless mutant (strain 679)
with x-rays produced the double mutant (strain 679-680) requiring leucine
as well as threonine (2). The experiments reported here are concerned
only with the leucine requirement, and, in all the tests, the culture media
contained ample DL-threonine (0.5 mg. per 10 ml.) to meet the require-
ment for that compound. In the following discussion, therefore, strain
679-680 will be considered as a leucineless strain.

Testing Methods—The methods used to study the ability of the mutant
to use leucine and its derivatives were similar to those described pre-
viously (1). Tests were carried out with 10 ml. of leucine-free minimal
medium (3), to which threonine and the test compound were added prior
to sterilization by autoclaving. After inoculation with a drop of an
aqueous suspension of cells, the cultures were incubated at 30° for 24
hours. For test compounds which proved to be active, a complete growth
curvature with graded amounts of compound was obtained, and the molar
concentration of the compound producing half maximal growth was
estimated from this curve. The amount of growth was determined by
density measurements in the Evelyn photoelectric colorimeter with a
No. 540 filter. The relative activity of a test substance as compared to
leucine was calculated from the curves for the test and reference com-
pounds obtained on the same day.

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Research Corporation and from the Rockefeller Foundation.
Compounds which did not replace leucine as a growth factor for the mutant were tested for possible sparing action on the leucine requirement. For these tests, the minimal medium was made up with sufficient leucine to permit approximately half maximal growth of the organism, and the growth obtained on addition of the test compound was measured as usual.

**Table I**

*Effect of Leucine Derivatives on Growth of Leucineless Mutant Strain*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity as growth factor</th>
<th>Sparing action on leucine requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m(M \times 10^4)</td>
<td>m(M \times 10^4)</td>
</tr>
<tr>
<td>L-Leucine*</td>
<td>+</td>
<td>2.2</td>
</tr>
<tr>
<td>d-Leucine*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DL-Leucine†</td>
<td>+</td>
<td>4.4</td>
</tr>
<tr>
<td>L-Leucylglycine‡</td>
<td>+</td>
<td>2.8</td>
</tr>
<tr>
<td>n-Leucylglycine†</td>
<td>+</td>
<td>128 (Ca.)</td>
</tr>
<tr>
<td>N-Methyl-DL-leucylglycine (4)$</td>
<td>-</td>
<td>128 (Ca.)</td>
</tr>
<tr>
<td>L-Leucilyglycylglycine‡</td>
<td>+</td>
<td>2.4</td>
</tr>
<tr>
<td>d-Leucilyglycylglycine‡</td>
<td>+</td>
<td>86 (Ca.)</td>
</tr>
<tr>
<td>L-Leucyl-L-tyrosine‡</td>
<td>+</td>
<td>3.2</td>
</tr>
<tr>
<td>L-Leucinamide acetate (5)</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>d-Leucinamide acetate (5)</td>
<td>-</td>
<td>53</td>
</tr>
<tr>
<td>Glycyl-L-leucine‡</td>
<td>+</td>
<td>2.5</td>
</tr>
<tr>
<td>Glycyl-d-leucine (6)</td>
<td>-</td>
<td>106</td>
</tr>
<tr>
<td>Glycylglycyl-L-leucylglycine (7)</td>
<td>+</td>
<td>3.7</td>
</tr>
<tr>
<td>Triglycyl-L-leucylglycine (7)</td>
<td>+</td>
<td>6.9</td>
</tr>
<tr>
<td>Carbobenzoxyglycyl-L-leucine (8)</td>
<td>+</td>
<td>1100 (Ca.)</td>
</tr>
<tr>
<td>Acetyl-L-leucine (9)</td>
<td>-</td>
<td>173</td>
</tr>
<tr>
<td>Acetyl-DL-leucinamide (10)</td>
<td>-</td>
<td>58</td>
</tr>
<tr>
<td>Acetyldehydroleucine (11)</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>Acetylddehydroleucinamide (11)</td>
<td>-</td>
<td>58</td>
</tr>
<tr>
<td>Acetylddehydroleucylglycine (11)</td>
<td>-</td>
<td>44</td>
</tr>
</tbody>
</table>

* Kindly supplied by Dr. W. H. Stein. The purity of these samples was established by the method of Moore and Stein (12).
† Merck preparation.
‡ Hoffmann-La Roche preparation.
§ The figures in parentheses are bibliographic reference numbers.

It should be borne in mind that compounds which proved to be inactive by the testing methods employed in these studies may actually be capable, under different experimental conditions, of replacing leucine as a growth factor or of sparing the leucine requirement of the *leucineless* mutant.

**Leucine Requirement**—Under the conditions employed in this study, the
concentration of L-leucine required to produce half maximal growth of strain 679-680 was 0.029 mg., or $2.2 \times 10^{-4}$ mM, per 10 ml. of medium (Table I). Similarly, the concentration of DL-leucine for half maximal growth was $4.4 \times 10^{-4}$ mM. d-Leucine was inactive as a growth factor in concentrations up to $75 \times 10^{-4}$ mM per 10 ml. of medium and, in these concentrations, had neither a sparing action nor an inhibitory effect on the growth obtained in the presence of $2 \times 10^{-4}$ mM of the L form. DL-Isoleucine, in concentrations up to $23 \times 10^{-4}$ mM per 10 ml., was neither active in place of leucine nor inhibitory to growth in the presence of leucine.

**Utilization of Leucine Peptides**—As shown in Table I, all of the peptides of L-leucine which were tested served as growth factors for the mutant. L-Leucylglycine, L-leucylglycylglycine, and glycyl-L-leucine were approximately as active as L-leucine. The activity of diglycyl-L-leucylglycine and triglycyl-L-leucylglycine was somewhat less than that of the di- and tripeptides, but even the pentapeptide had about one-third the activity of L-leucine. L-Leucinamide, however, was relatively inactive.

The suggestion was made previously (1) that peptides of an amino acid which is required for growth by a mutant strain of *Escherichia coli* must be hydrolyzed by the bacterial enzymes before the essential amino acid becomes available as a growth factor. The fact that none of the leucine peptides mentioned above was more active than leucine itself is consistent with this view. The relative activity of the leucine derivatives in promoting the growth of the leucineless strain is similar to that of corresponding derivatives of phenylalanine and of tyrosine for the phenylalanineless and tyrosineless strains (1). Thus, the dipeptides of the essential amino acid and glycine were approximately as active as the essential amino acid itself, while the amino acid amide was considerably less active, and the carbobenzoxyglycyl derivative showed only a slight activity. These differences in the rate of utilization of the leucine derivatives may be taken to indicate comparable differences in the rate of cleavage of these compounds by the bacterial peptidases.

Although the dipeptides of glycine and L-leucine were, on a molar basis, as active as L-leucine with respect to the amounts required to produce half maximal growth, these dipeptides were markedly inhibitory at high concentrations. As can be seen in Fig. 1, L-leucine was not inhibitory in concentrations up to 1 mg. ($76 \times 10^{-4}$ mM) per 10 ml. of medium. Only slight inhibition was noted for L-leucylglycylglycine, diglycyl-L-leucylglycine, and triglycyl-L-leucylglycine at concentrations of 30 to $50 \times 10^{-4}$ mM, but very striking inhibition was observed for similar concentrations of glycyl-L-leucine and L-leucylglycine. On the other hand, L-leucyl-L-tyrosine did not show significant inhibition in concentrations up to $102 \times 10^{-4}$ mM.
In the presence of sufficient L-leucine (2 × 10⁻⁴ mM) to permit half maximal growth, glycyl-L-leucine and L-leucylglycine (1 mg., or 53 × 10⁻⁴ mM, per 10 ml. of medium) did not spare the requirement of the mutant for L-leucine, although a sparing action would be expected on the basis of the growth-promoting action of the dipeptides. The extent of growth actually obtained in the presence of the dipeptides was approximately equal to that obtained with the same amount of L-leucine in the absence of the dipeptides. The dipeptides, therefore, must have exerted an inhibitory effect in this test. In another test in which 23 × 10⁻⁴ mM of L-leucine, i.e., enough leucine for maximal growth, was present in 10 ml. of medium, the addition of 53 × 10⁻⁴ mM of either of the dipeptides resulted in growth equivalent to only 40 per cent of the maximum.

The inhibition of bacterial growth produced by glycyl-L-leucine and L-leucylglycine cannot be ascribed to the glycine liberated on hydrolysis of the dipeptides, since mixtures of equimolar amounts of glycine and L-leucine, in concentrations up to 76 × 10⁻⁴ mM of each amino acid, had exactly the same activity as L-leucine alone. Furthermore, the longer peptides which contained two or more glycine residues per molecule were only slightly inhibitory. It is of interest that glycyl-L-leucine and L-leucylglycine also were inhibitory to the wild type strain (K-12) of Escherichia coli. This organism gave maximal growth in the presence of 5.3 × 10⁻⁴ mM or less of either dipeptide, but was completely inhibited in the presence of 15.9 × 10⁻⁴ mM.

The following derivatives of D-leucine were also investigated with the
leucineless mutant: D-leucylglycine, D-leucylglycylglycine, D-leucinamide acetate, and glycyld-Leucine (Table I). Only the first two compounds exhibited any growth-promoting activity, which corresponded to 2 to 3 per cent of that of the L isomers. The activity of D-leucylglycine and D-leucylglycylglycine, both of which are commercial preparations, may well be due to traces of the L isomers present in the samples.

Utilization of Acetyl-L-leucine—Although acetyl-L-leucine did not replace L-leucine as a growth factor for the leucineless mutant, it did exert a sparing action on the leucine requirement. The addition of increasing amounts of acetylleucine to a culture medium containing $2 \times 10^{-4}$ M of L-leucine per 10 ml. gave significantly more growth than was expected from the quantity of L-leucine used. This stimulation of growth is not due to traces of L-leucine admixed with the acetyl compound, since no growth-promoting action could be detected in the absence of L-leucine with concentrations of acetyl-L-leucine as high as $173 \times 10^{-4}$ M. The presence of even 0.3 per cent of L-leucine in such a sample of acetyl-L-leucine would have been sufficient to produce detectable growth.

As shown in Fig. 2, the degree to which the requirement for L-leucine could be spared by acetyl-L-leucine was dependent upon the amount of L-leucine present in the medium. The conversion of the acetyl derivative to leucine, or to some compound with leucine activity, apparently is the result of a metabolic process associated with growth. In agreement with this view is the fact that growing cells of the wild type strain (K-12) were found to be capable of converting acetylleucine to a compound which is a growth factor for the leucineless mutant. When K-12 was grown for 24 hours at 30° in 10 ml. of medium containing 2 mg. of acetyl-L-leucine, a
microbiological assay of the resultant medium indicated the presence of L-leucine, or of a substance with L-leucine activity, in an amount equivalent to about 12 per cent of the starting material. Resting cells of the wild type strain did not bring about this conversion. The presence of some unchanged acetyl-L-leucine in the medium in which K-12 cells were grown was also demonstrated by sparing action tests with the leucineless mutant.

The ability of both the wild type strain and the mutant to convert acetylleucine to leucine, or a leucine-like compound, is in accord with the view that the metabolism of the parent strain and that of a mutant arising from it are identical, except for the metabolic reaction governed by the gene which has undergone mutation (13). In the present case, it is apparent that the mutation responsible for the leucine requirement is not connected with the metabolism of acetylleucine by Escherichia coli.

The conversion of acetyl-L-leucine to the corresponding amide appears to suppress the sparing action. As has been noted above, the conversion of an amino acid to its amide was found to reduce markedly its growth-promoting action. In the case of acetyl-L-leucine, such a conversion decreased more than 100-fold the sparing action characteristic of the parent compound.

The response of the leucineless mutant to acetylleucine is quite distinct from that of the phenylalanineless and tyrosineless mutants. For these latter mutants, acetylphenylalanine and acetyltyrosine neither filled nor spared the amino acid requirement (1). It is of interest in this connection that the original K-12 strain was found to be incapable of hydrolyzing acetyltyrosine (14).

**Effect of Derivatives of Dehydroleucine**—In order to examine further the possible metabolic role of dehydroamino acids (cf. (1, 14)), several derivatives of α, β-dehydroleucine were assayed as potential growth factors for the leucineless mutant. Acetyldehydroleucine, acetyldehydroleucinamide, and acetyldehydroleucylglycine were inactive in the growth tests; nor did they exert a sparing action on the leucine requirement of the mutant (Table I). In the case of acetyldehydroleucine, at least, a sparing action should have been demonstrable if the mutant was able to reduce the α,β-double bond to yield acetyl-L-leucine. Experiments in which the disappearance of the α,β-double bond in derivatives of dehydroamino acids was followed spectrophotometrically had indicated that growing cultures of Escherichia coli strain K-12 did not readily metabolize acetyldehydroleucine (14). The inactivity of that compound in the tests with the leucineless mutant strain, therefore, is not unexpected.

**SUMMARY**

The utilization of derivatives of leucine has been studied with an x-ray-induced mutant strain of Escherichia coli which requires for growth an
exogenous source of L-leucine. All of the leucine peptides which were tested were found to serve as growth factors for the leucineless mutant. Acetylleucine, however, was not an active growth factor, although it did spare the requirement of the mutant for L-leucine. Derivatives of dehydroleucine were neither active growth factors nor did they spare the leucine requirement.

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