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Amino-acid Utilization in Bacterial Growth

1. PEPTIDE UTILIZATION BY A LEUCINE-REQUIRING MUTANT OF *ESCHERICHIA COLI*

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In the course of studies on the quantitative determination of amino-acids with lactic acid bacteria, numerous authors have studied the availability of peptides in place of the corresponding amino-acids. In all cases so far published, it has been found that the peptides either show no activity, or an activity inferior or equal to that of the constituent amino-acids. The magnitude of the response of the organisms depended on the nature of the amino-acids (other than the one missing in the assay) present in the peptide and on their sequence in the peptide molecule.

In two cases only, a growth response greater with peptides than with the sum of equimolar quantities of the constituent amino-acids has been found. This effect has been observed (a) with the strain SF, a member of the genus *Pseudomonas*, studied by

Simmonds & Fruton (1949, 1950, 1951); (b) with the two proline-requiring mutants of *Escherichia coli* (K 12-679-183 and 58-6317), studied by the same authors (Simmonds & Fruton, 1948). In the case of the strain 'SF', the organism can grow on leucine or glycine as the sole source of carbon and nitrogen, but a better growth is obtained on equimolar amounts of L-leucylglycine. However, the organism can also be grown on a variety of amino-acids as nitrogen source, such as L-phenylalanine, L-alanine, L-glutamate, L-isoleucine and L-valine. Thus, this organism exhibits no specificity as to its amino-acid requirement.

On the contrary, the proline-requiring mutants of *Esch. coli* present an absolute requirement for proline. A number of proline dipeptides and tripeptides show growth-promoting activity 1.5-3

times that of proline or of proline plus the corresponding amino-acid (or acids), independently of the nature of the other amino-acids and of their sequence in the molecule.

These two different cases seem to invalidate the view previously expressed by Simmonds, Tatum & Fruton (1947), that peptides must be hydrolysed to the corresponding amino-acids prior to their utilization for growth. This view was based in part on the fact that the leucine-requiring mutant K 12-679-680 does not give a better growth response with leucine peptides than with leucine alone or leucine plus the corresponding amino-acids.

In the present paper, we describe a genotypically leucine-requiring mutant which can be rendered phenotypically leucine-peptide requiring. This was accomplished with a number of natural inhibitors which seem to act in competitively inhibiting the incorporation of leucine into peptides. The results of our experiments suggest that, at least in the presence of these inhibitors, the peptides of leucine are not utilized through mere hydrolysis.

We should like to emphasize the importance of antagonisms between amino-acids in the growth of micro-organisms. These antagonisms may be regarded as regulating the rate of synthesis of various amino-acids and peptides. Such regulations may possibly help to ensure the synthesis of specific protein structures.

EXPERIMENTAL

Strains utilized. Strain ML 328c, a leucine-requiring strain of *Esch. coli*, obtained by the penicillin selection method of Davis (1949, 1950), has been utilized. This strain was obtained by Monod (unpublished) from strain ML3. We wish to thank Dr Monod for supplying us with this mutant and the parent strain. The organism was maintained on meat-broth agar slants by weekly transfers.

Testing methods. The assays were conducted in 16×1.8 cm. tubes. The liquid volume was 5.0 ml. The medium used was that of Monod (1942), containing 5 g. glucose/l.; 3 ml. of this medium were added to each tube and the volume made up to 5 ml. by the addition of the inoculum, other substances and water. All the substances except the α -keto- and α -hydroxy- γ -methyl-*n*-valeric acid and the peptides were added prior to sterilization, which was effected in the autoclave at 115° for 20 min. The α -keto- γ -methyl-*n*-valeric acid, α -hydroxy- γ -methyl-*n*-valeric acid and the peptides were sterilized by filtration and added after autoclaving. The tubes were then cooled and inoculated. The inoculum was prepared in the following manner: a loopful of organisms from an 18 hr. slant was suspended in 10 ml. of sterile water and brought to a standard turbidity. The suspension was then diluted 100-fold and 0.5 ml. of this final suspension was added to each tube. This corresponds roughly to an inoculum of 10^6 cells per tube. The contents of the tubes were then mixed and the tubes incubated at 37° in an upright position without shaking for periods of 17-24 hr., according to the experiment. At the end of this period, growth was estimated turbidimetrically with the Meunier electrophotometer

(green filter); 100 divisions of the drum correspond to 1.3×10^8 cells per ml. Under these conditions, maximal growth of the parent strain ML3 corresponds to 150 divisions (18 hr. period). The mutant was stable and no prototrophic growth was noted in the tubes without added leucine, even after several days.

Materials. L-Leucine, L- and D-isoleucine, L- and D-valine, DL-serine, DL-alanine, L-arginine, L-ornithine, DL-lysine, L-tyrosine, L-phenylalanine, L-histidine, L-proline, L-glutamic and aspartic acids, DL-threonine, glycine, synthetic DL-norleucine, and all the peptides were Hoffmann-La Roche products. Synthetic DL-methionine and DL-tryptophan were gifts from L'Alimentation Equilibree.

α -Keto- γ -methyl-*n*-valeric acid was prepared by using ophio-L-amino-acid-oxidase of the venom of *Vipera aspis* acting on L-leucine, according to Singer & Kearney (1950). We thank Dr P. Boquet who supplied us generously with the snake venom. α -Hydroxy- γ -methyl-*n*-valeric acid was a generous gift from Dr D. M. Bonner, to whom our thanks are due.

RESULTS

Leucine requirement of the leucine-requiring mutant

Under the conditions defined above, the concentration of L-leucine required to produce half-maximal growth of strain ML328c is 7×10^{-6} M (incubation time, 18 hr.). Under the same conditions, the concentration of α -keto- γ -methyl-*n*-valeric acid required to produce half-maximal growth is also 7×10^{-6} M.

The mutant shows poor growth even after 40 hr. with α -hydroxy- γ -methyl-*n*-valeric acid at a concentration of 10^{-4} M. The factor limiting its utilization may well be the rate of its conversion to the α -keto derivative (see Holden, Wildman & Snell, 1951). Since identical growth is obtained with L-leucine and identical concentrations of DL-leucine (expressed in L), it is assumed that D-leucine does not promote the growth of the mutant.

Action of amino-acids other than L-leucine in the culture medium

No inhibition of growth of the parent strain ML3 is noted in the presence of added naturally-occurring amino-acids. Cohen, Hirsch & Raynaud (1951) have already found that the growth of a valine-requiring mutant of strain ML3 is inhibited competitively by a number of amino-acids, among which isoleucine is the most potent.

The following amino-acids were tested as inhibitors of the growth of the leucine-requiring mutant ML328c and were found not to suppress the growth at concentrations up to 10^{-2} M: DL-alanine, L-glutamate, L-aspartate, L-arginine, L-ornithine, DL-lysine, L-tyrosine, DL-tryptophan, L-proline, L-phenylalanine and glycine. The L-leucine concentration was 10^{-5} M.

The following amino-acids, at the concentrations given in parentheses, totally suppress the growth of

the mutant (L-leucine concentration as above): DL-norleucine (2.5×10^{-4} M), L-isoleucine (10^{-3} M), L-valine (10^{-2} M) and DL-methionine (10^{-2} M). Irregular results were obtained with DL-serine (10^{-2} M). The concentrations given for the racemic compounds are those of the L-isomer.

Study of the inhibition of the growth of the leucine-requiring mutant by L-isoleucine

Due to the high inhibition index of methionine and valine and to the fact that the concentration of L-leucine could not be lowered below 10^{-5} M without affecting growth in the controls, a thorough study of the inhibition of growth by these two amino-acids could not be made. The case of the inhibition of the growth of the leucine-requiring mutant by norleucine, which inhibits also the growth of the wild type ML3, will be dealt with in a forthcoming publication.

Table 1. *Competitive inhibition of the growth of the leucine-requiring mutant ML328c of Esch. coli by L-isoleucine*

(Incubation: 18 hr. In this and subsequent tables, the concentrations given are the final molar concentrations, and growth is expressed in units of the Meunier photometer. Inhibition index is defined below.)

L-Leucine (M)	L-Isoleucine (M)	Growth (units)	Inhibition index
5×10^{-6}	0	40	
5×10^{-6}	10^{-4}	16	
5×10^{-6}	2.5×10^{-4}	4	50
10^{-5}	0	97	
10^{-5}	10^{-4}	110	
10^{-5}	2.5×10^{-4}	49	
10^{-5}	5×10^{-4}	15	
10^{-5}	10^{-3}	1	50-100
2.5×10^{-5}	0	133	
2.5×10^{-5}	5×10^{-4}	122	
2.5×10^{-5}	10^{-3}	83	
2.5×10^{-5}	2.5×10^{-3}	11	
2.5×10^{-5}	5×10^{-3}	2	100-200
5×10^{-5}	0	135	
5×10^{-5}	10^{-3}	128	
5×10^{-5}	2.5×10^{-3}	127	
5×10^{-5}	5×10^{-3}	60	
5×10^{-5}	9×10^{-3}	1	180
10^{-4}	0	133	
10^{-4}	5×10^{-3}	143	
10^{-4}	9×10^{-3}	124	
10^{-4}	1.6×10^{-2}	18	approx. 160

The low inhibition index of L-isoleucine (that is the ratio L-isoleucine/L-leucine necessary to abolish totally the growth) rendered it feasible to study the inhibition by this amino-acid in more detail. The inhibition was found to be competitive. The inhibition index varies from 40 to 180 for L-leucine concentrations varying from 2.5×10^{-6} to 10^{-4} M (Table 1). This suggests that L-isoleucine interferes

with L-leucine utilization by the mutant. It will become apparent that the inhibitory action of L-valine concerns also L-leucine utilization, although no evidence was obtained as to the competitive nature of the inhibition. The inhibitory effect on growth is restricted to the L-isomers of isoleucine and valine: D-isoleucine (with an index as high as 2000) and D-valine did not prove inhibitory.

Suppression by leucine peptides of the inhibition of the leucine-requiring mutant caused by L-isoleucine and L-valine

In the case here described, the inhibition by L-isoleucine of the growth of the leucine-requiring mutant ML328c on L-leucine could not be prevented by the addition to the medium of the following amino-acids or related compounds at the concentrations of 10^{-4} and 10^{-3} M: L-valine, DL-methionine,

Table 2. *Non-competitive suppression by L-leucylglycine of the inhibition of growth caused by L-isoleucine*

(Incubation: 20 hr. L-leucine: 10^{-5} M in all cases.)

L-Isoleucine (M)	L-Leucylglycine (M)	Growth (units)
0	0	85
10^{-3}	0	1
10^{-3}	5×10^{-6}	70
10^{-3}	10^{-5}	82
2.5×10^{-3}	0	0
2.5×10^{-3}	5×10^{-6}	55
2.5×10^{-3}	10^{-5}	73
5×10^{-3}	0	0
5×10^{-3}	5×10^{-6}	39
5×10^{-3}	10^{-5}	60
5×10^{-3}	2.5×10^{-5}	88
10^{-2}	0	0
10^{-2}	5×10^{-6}	31
10^{-2}	10^{-5}	50
10^{-2}	2.5×10^{-5}	79
10^{-2}	5×10^{-5}	114
2×10^{-2}	0	0
2×10^{-2}	5×10^{-6}	29
2×10^{-2}	10^{-5}	42
2×10^{-2}	2.5×10^{-5}	69
2×10^{-2}	5×10^{-5}	96
2×10^{-2}	10^{-4}	121

DL-threonine, L-histidine, L-arginine, DL-serine, L-phenylalanine, L-alanine, DL-lysine, α -ketoglutarate. The following combinations also proved ineffective: valine plus methionine, valine plus threonine, methionine plus threonine, valine plus methionine plus threonine. We considered then the possibility that the inhibitory amino-acids acted on the synthesis of leucine peptides presumably normally formed from leucine in the course of protein synthesis by the organism under study. We found that L-leucylglycine non-competitively reversed the inhibition caused by L-isoleucine (Table 2). This result suggested that L-leucylglycine was the product of the inhibited reaction. However,

Table 3. *Non-competitive suppression by glycyl-L-leucine of the inhibition of growth caused by L-isoleucine*

(Incubation: 22 hr. L-Leucine: 10^{-5} M in all cases.)

L-Isoleucine (M)	Glycyl-L-leucine (M)	Growth (units)
0	0	104
10^{-3}	0	7
10^{-3}	5×10^{-6}	101
10^{-3}	10^{-6}	114
2.5×10^{-3}	0	0
2.5×10^{-3}	5×10^{-6}	70
2.5×10^{-3}	10^{-6}	99
5×10^{-3}	0	4
5×10^{-3}	5×10^{-6}	57
5×10^{-3}	10^{-6}	81
5×10^{-3}	2.5×10^{-6}	118
10^{-2}	0	3
10^{-2}	5×10^{-6}	34
10^{-2}	10^{-6}	56
10^{-2}	2.5×10^{-6}	103
10^{-2}	5×10^{-6}	116
2×10^{-2}	0	3
2×10^{-2}	5×10^{-6}	31
2×10^{-2}	10^{-6}	41
2×10^{-2}	2.5×10^{-6}	82
2×10^{-2}	5×10^{-6}	106
2×10^{-2}	10^{-4}	127

Utilization of leucine peptides by the leucine-requiring mutant

If leucine peptides are the products of the reaction blocked by L-isoleucine, growth on these peptides instead of L-leucine should be insensitive to the presence of L-isoleucine, or at least the molar ratio of L-isoleucine to the peptide necessary to suppress the growth should be much higher than that of L-isoleucine to L-leucine. It was found: (a) that the growth response of the organism to both peptides is similar to that obtained on equimolar amounts of L-leucine or of L-leucine plus glycine; DL-leucylglycylglycine (calculated as the L-isomer) is as active for growth as L-leucylglycine; (b) that the growth of the organism on optimal concentrations of the peptides is completely insensitive to L-isoleucine and L-valine (Tables 5 and 6). This result seems to substantiate the hypothesis that the peptides are the product of the inhibited reaction.

Table 4. *Suppression by L-leucylglycine and glycyl-L-leucine of the inhibition of growth caused by valine*

(Incubation: 24 hr.)

L-Leucine (M)	L-Valine (M)	L-Leucylglycine (M)	Glycyl-L-leucine (M)	Growth (units)
10^{-5}	0	0	0	92
10^{-5}	10^{-2}	0	0	3
2×10^{-5}	10^{-2}	0	0	4
10^{-5}	10^{-2}	10^{-5}	0	93
10^{-5}	10^{-2}	0	10^{-5}	84

Table 5. *Insensitivity of the growth on leucine peptides to isoleucine*

(Incubation: 20 hr.)

L-Leucine (M)	L-Leucylglycine (M)	Glycyl-L-leucine (M)	L-Isoleucine (M)	Growth (units)
2.5×10^{-5}	0	0	0	80
2.5×10^{-5}	0	0	5×10^{-3}	1
0	2.5×10^{-5}	0	0	97
0	0	2.5×10^{-5}	0	87
0	2.5×10^{-5}	0	10^{-2}	73
0	2.5×10^{-5}	0	2×10^{-2}	63
0	0	2.5×10^{-5}	10^{-2}	71
0	0	2.5×10^{-5}	2×10^{-2}	55

Table 6. *Insensitivity of the growth on leucine peptides to valine*

(Incubation: 24 hr.)

L-Leucine (M)	L-Leucylglycine (M)	Glycyl-L-leucine (M)	L-Valine (M)	Growth (units)
10^{-5}	0	0	0	92
10^{-5}	0	0	10^{-2}	3
0	10^{-5}	0	0	100
0	0	10^{-5}	0	95
0	10^{-5}	0	2×10^{-2}	81
0	0	10^{-5}	2×10^{-2}	90

Hydrolysis of leucine peptides by washed suspensions of the leucine-requiring mutant

To interpret our results, it was important to ascertain whether or not the peptides were hydrolysed by the leucine mutant. We used the technique of paper chromatography to test this possibility.

Esch. coli ML328c (1 l.) was grown for 18 hr. in a synthetic medium containing L-leucine (2×10^{-5} M). The culture was then centrifuged, the cells washed twice with saline and resuspended in 3 ml. of sterile water. Four tubes were run for each peptide, and contained 5 mg. of peptide, 0.12 ml. water and 0.13 ml. bacterial suspension (containing 2 mg. N/ml.). The total volume was 0.75 ml. Tubes were also run that contained bacteria plus leucine, bacteria plus glycine, bacteria plus both amino-acids and bacteria alone. The tubes were incubated at 37° for periods of 30 min., 1, 4 and 24 hr. After centrifugation, a sample of the supernatant was spotted on Whatman no. 1 filter paper and ascending chromatography was run with butanol-acetic acid as solvent. The chromatogram was developed with ninhydrin (0.1%) in CHCl_3 containing 0.1% collidine.

In the case of glycyl-L-leucine, no hydrolysis was evident after 0.5, 1 and 4 hr. After 24 hr. slight spots of glycine and leucine were present, which indicated a slight hydrolysis. In the case of L-leucylglycine, very slight hydrolysis was noticeable as early as 0.5 hr., and increased up to 24 hr.

Peptide specificity

DL-Alanylglycine, glycylglycine, glycylglycylglycine, glycyl-L-tyrosine, glycyl-L-tryptophan did not reverse the inhibition caused by L-isoleucine of the growth of the leucine-requiring mutant. Other peptides containing or not containing leucine were not available.

DISCUSSION

An explanation of the observed facts might be based on the now classical criteria concerning the interpretation of competitive inhibition. According to these concepts, the fact that supplying the organism with L-leucylglycine or glycyl-L-leucine suppresses the inhibition in a non-competitive manner, suggests that it is the synthesis of leucine peptides from L-leucine which is inhibited; moreover, the fact that these compounds meet the needs of the organism and allow growth independently of the amount of L-isoleucine (or L-valine) points to the same conclusion.

It is difficult to visualize the activity of leucine peptides in suppressing the inhibition caused by isoleucine and valine as resulting from a mere hydrolysis of the peptides to free leucine. In this case, there would still be a competitive inhibition by the inhibitors of the growth on the peptides.

Another alternative would be the incorporation, as such, of the peptides in the bacterial protein. This is rendered improbable by the fact that both L-leucylglycine and glycyl-L-leucine are equally active for growth. It would therefore be difficult to account for the fact that either of the two types of preformed linkage is sufficient to overcome the inhibition caused by isoleucine and valine and to ensure alone the growth of the organism.

Hydrolysis of the peptides could give rise to a form of L-leucine more active for growth, i.e. more readily converted to other peptides. For example, transpeptidation reactions could occur, the supplied preformed peptide being capable of transpeptidation with other amino-acids or other peptides to give all the leucine peptide configurations necessary for the synthesis of bacterial protein. Such reactions have been shown to occur in kidney and pancreas extracts between glutathione on the one hand and leucine, phenylalanine or valine on the other (Hanes, Hird & Isherwood, 1950). The products of the reaction were cysteinylglycine and three new substances. In the case of phenylalanine, the substance has been identified on reasonably good grounds with γ -glutamylphenylalanine. There is good reason to support the suggestion that the proteolytic enzymes themselves may catalyse transpeptidation (Lipmann, 1949; Frantz & Lotfield, 1950; Brenner, Müller & Pfister, 1950; Fruton, 1950). The existence in the leucine-requiring mutant ML328c of peptidases that hydrolyse the peptides used in this study is thus not incompatible with the hypothesis of a utilization of the peptides other than through mere hydrolysis. Hanes *et al.* (1950) announce that they recently obtained with plant enzymes more direct evidence of transpeptidation reactions involving, this time, α -peptides.

Transpeptidation reactions would certainly account for the equal activity of the two different peptides of leucine tested in the present study, as well as for the results of Simmonds & Fruton (1948) with their proline-requiring mutants. It appears reasonable to us to consider this possibility as a likely one.

Further work is being done in this laboratory to obtain more information on possible transpeptidation reactions and on their possible significance for the synthesis of bacterial protein.

SUMMARY

1. The growth of a leucine-requiring mutant of *Escherichia coli* is inhibited by the presence in the culture medium of L-isoleucine, L-valine, DL-methionine and DL-norleucine. D-Valine and D-isoleucine are not inhibitory.

2. The inhibition by L-isoleucine is competitive.

3. The inhibition of the growth of the mutant caused by L-isoleucine and L-valine is suppressed in a non-competitive way by the addition of either L-leucylglycine or glycyl-L-leucine.

4. Different explanations for the results obtained

are considered, taking into account that the presence in the mutant of the peptidases that hydrolyse the peptides studied is not incompatible with a utilization of the peptides prior to their mere hydrolysis, for example, through transpeptidation reactions.

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Acetylcholine

1. HYDROLYSIS BY HYDROGEN AND HYDROXYL ION

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This paper presents a study of the acid- and alkali-catalysed hydrolyses of acetylcholine bromide, which may be written

- (1) $\text{CH}_3\text{COOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3 + \text{H}_2\text{O} + \text{H}_3\text{O}^+ \rightleftharpoons \text{CH}_3\text{COOH} + \text{HOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3 + \text{H}_3\text{O}^+$
- (2) $\text{CH}_3\text{COOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3 + \text{OH}^- \rightleftharpoons \text{CH}_3\text{COO}^- + \text{HOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$

Our object has been to obtain kinetic data leading to frequency factors and activation energies, for comparison with similar data on the neutral hydrolysis catalysed by true and pseudo cholinesterases. A comparison of the data obtained here with published data on non-ionic esters, such as methyl acetate, has an intrinsic theoretical interest for reaction mechanisms, because of the positive charge on the acetylcholine ion. To date, the study of the non-enzymic hydrolysis of acetylcholine has been limited to obtaining the percentage non-enzymic hydrolysis in 20 min. at pH values near to 7 (Easson & Steadman, 1936). We shall discuss the equilibrium point of the reaction in a later paper, but in both cases it is far enough to the right for us to neglect the back reaction in our kinetic analysis.

EXPERIMENTAL AND RESULTS

The hydrogen-ion catalysis

Method. Commercial acetylcholine bromide was recrystallized from absolute ethanol until it reached a constant melting point of 143°. Conventional methods were used to follow the reaction. Hydrochloric acid catalyst solution (100 ml.) was brought to the thermostat temperature in a stoppered conical flask. Four concentrations of acid were used, 0.1, 0.075, 0.05 and 0.025 N-HCl. At zero time, a weighed amount of acetylcholine bromide was added and the flask shaken, so that the bromide rapidly dissolved. After varying time intervals (decided after preliminary experiments), 5 ml. portions of the reaction mixture were withdrawn and run into a known amount of standardized NaOH solution plus phenolphthalein, in a conical flask at 0°. The NaOH solution was calculated to neutralize the catalyst present. The solution was then rapidly titrated to the end point with further NaOH. If the titre at infinite time is r_∞ and at time t is r_t , the quantity of ester remaining unhydrolysed at time t is equivalent to $r_\infty - r_t$. The difficulties associated with the determination of r_∞ were avoided by the use of Guggenheim's (1926) method. If r_t be the titration value at time t and $r_{t+\tau}$ the titration value at time $t + \tau$, where τ is a suitable standard time interval, chosen to be at least twice the half-life of the reaction, then Guggenheim's method gives the first-order constant k as the slope of the