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Garvan Award Address of the American Chemical Society: Peptidase Activity and Peptide Metabolism in *Escherichia coli* K-12*,†

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ABSTRACT: The ability of *Escherichia coli* to use simple peptides as sources of amino acids for growth results from the presence of constitutive peptidases which behave like cryptic (partially latent) enzymes in intact bacteria, lysozyme spheroplasts, and osmotically shocked cells. The degree of crypticity observed is related to the physiological state of the bacteria, and is largely determined by environmental factors such as the metal ion content and pH of the growth media. Indeed, certain media will produce bacterial cells that actively concentrate dipeptides *per se;* nevertheless, rupture of these bacteria yields cell extracts characterized by normal levels of dipeptidase activity. Of the total peptidase activity in cell extracts, the major portion is recovered in the soluble fraction, and this fraction contains several peptidases which differ in their action on a variety of di- and tripeptides. Differences in apparent substrate specificity also serve to distinguish the soluble enzymes from those in the ribosomal fraction. Some of the dipeptidase activity of the ribosomal fraction is lost when cells are cultured under conditions leading to the production of excessive amounts of acid as a by-product of glucose metabolism, and a drop in the specific activity of both subcellular fractions occurs when cultures in a glucose-containing medium cease to grow rapidly. Both subcellular fractions attack tripeptides only *via* the initial removal of the N-terminal amino acid, and such aminopeptidase activity usually is higher toward the substrates Met-X-Y and Leu-X-Y than toward Phe-X-Y. These observations form the basis of a more general discussion of bacterial metabolism and protein synthesis *in vivo*.

It is just 25 years since Gray and Tatum (1944) reported that treatment of *Escherichia coli* K-12 with X-rays gives rise to mutant strains characterized by specific growth-factor requirements. Among the first mutants produced by treatment with X-rays or with nitrogen mustard (Lederberg and Tatum, 1946) were a number of amino acid auxotrophs which have proved to be excellent tools for the study of the metabolic relation between amino acids and peptides. It was my good fortune to participate, with E. L. Tatum and J. S. Fruton, in the first experiments concerned with this aspect of metabolism in *E. coli* (Simmonds *et al.*, 1947a). At the start, our aim was to determine whether peptides are intermediates in protein biosynthesis, but the data obtained in the early experiments raised questions that prompted investigations into the bacteriostatic effect of simple peptides, the uptake of peptides into cells, and the role of peptide-cleaving enzymes in making peptide-bound amino acids available for growth. Most recently, the study of bacterial peptidases again focused attention on protein biosynthesis, for some of the peptidase-catalyzed reactions have been implicated in the final steps by which proteins are formed in bacterial systems.

The Leucine Auxotroph

The results from our initial experiments (summarized by Fruton and Simmonds, 1950) were consistent with the view that peptides always undergo hydrolysis before the constituent amino acids become available for growth, and that such hydrolysis occurs rapidly, because cultures of a given auxotroph which are provided with equivalent concentrations of amino acids and peptides usually show identical growth curves. Pep-

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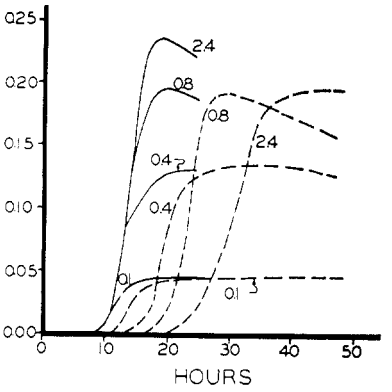

FIGURE 1: Growth curves for leucine–threonine auxotroph (No. 679–680) in presence of excess threonine and either L-leucine (solid lines) or glycyl-L-leucine (dashed lines). Concentration of test compounds, in μmoles per 10 ml of growth medium, indicated for each curve (from Simmonds and Fruton, 1949).

tides of L-leucine form a unique group, and serve not only as growth factors but also as growth inhibitors (Simmonds *et al.*, 1947b, 1951). Growth inhibition results from the bacteriostatic action of the peptide (Figure 1), and hydrolysis of the peptide by the cells both relieves the bacteriostatic effect and provides the leucine required for the growth of a leucine auxotroph.

The extent to which a leucine peptide prolongs the lag time of the growth cycle of the leucine auxotroph is influenced by the "age" of the cells comparising the inoculum for the growth test (Simmonds and Fruton, 1949). If one starts the test with cells taken from the surface of a 24-hr peptone–yeast extract–agar slant, the bacteriostatic effect is large (Table I). When the inoculum consists of cells from a culture growing in a basal medium supplemented with leucine, or with a peptide such as Gly-Leu[1] ("young" cells), the bacteriostatic effect is small. A large effect again is seen when the cells comprising the inoculum have been left in the liquid culture for about 6 days ("aged" cells); such extreme sensitivity of bacteriostatic peptides develops only in static cultures, where the continued metabolism of glucose and the poor aeration permit a drop in the pH of the culture fluid.

These observations (Table I), and other data concerning the effect of culture pH (Meisler and Simmonds, 1963), led to the prediction that only "aged" cells from static cultures would lack the ability to hydrolyze leucine peptides at a rapid rate. This prediction proved only partially correct: compared to whole cells harvested from a 24-hr culture, whole cells from a 168-hr static culture do, indeed, show little hydrolytic activity toward Gly-Leu and Leu-Gly; however, by 48 hr, the whole-cell activity has dropped about 75% from the 24-hr level (Table II). Also in contradiction to our prediction is the observation that a similar (though somewhat slower) loss in whole-cell activity results when cultures are incubated with vigorous aeration which prevents acidification of the culture fluid (Simmonds, 1966).

The fact that whole cells show equal hydrolytic activity toward the two leucine peptides (in an assay system closely approximating the composition of the growth medium) accords with the fact that the two peptides show equal effectiveness both as growth factors and as growth inhibitors. But these facts are difficult to reconcile with the observation that broken-cell preparations show much more activity toward Leu-Gly than toward Gly-Leu, and that the levels of activity seen in the preparations made from 24-hr cultures is maintained throughout the aging process (Table II). In addition, the results of assays on the broken-cell preparations indicate that Gly-Leu and, especially, Leu-Gly are acted upon by enzymes which are partially latent (cryptic) in whole cells, and also tell us that assays on broken cells do not provide a reliable guide to the responses which are to be expected *in vivo*, and especially in growth tests.

We know that the prolonged lag times seen for cultures provided with leucine peptides are not the result of a failure of the cells to take up the peptides. In addition to the improbability of the idea that a highly bacteriostatic compound fails to enter cells readily, tests made on both "young" and "aged" cells of the leucine auxotroph show that Gly-Leu is taken up as rapidly as is leucine (Meisler and Simmonds, 1963). More importantly, and in complete agreement with the results of peptidase assays on whole cells, "young" cells exposed to glycyl-L-[^{14}C]-leucine contain labeled leucine but no labeled Gly-Leu,

TABLE I: Production of Sensitivity to Glycyl-L-leucine in Leucine–Threonine Auxotroph No. 679–680.[a,b]

Preparation of Inoculum				Growth Test Data[c]	
Medium and Conditions	Time (hr)	Culture Optical Density	Culture pH	Leucine $T_{0.5}$ (hr)	Gly-Leu $\Delta T_{0.5}$ (hr)
Slant	24[d]			34	+29
A; stationary incubation	32[e]	0.07	6.4	18	+9
	47	0.16	5.6	17	+8
	102	0.14	5.3	18	+6
	127	0.13	5.4	26	+11
	153[f]	0.15	5.5	47	+31
Slant	24[d]			22	+25
A; shaken incubation	24[e]	0.29	6.2	15	+6
	40	0.56	5.8	16	+5
	119	0.50	6.1	21	+2
	146[f]	0.56	6.5	15	+5

[a] From Meisler and Simmonds (1963). [b] Cells from a peptone–yeast extract–agar slant were inoculated into a series of tubes of basal growth medium A (containing threonine; initially at pH 6.5) and supplemented with 0.24 mM L-leucine or with 0.24 mM glycyl-L-leucine. At each time shown, a sample was removed from a culture growing on leucine to provide the inoculum for a new growth test in the same types of media. [c] $T_{0.5}$ indicates time required for leucine-supplemented culture to reach one-half maximal growth, and $\Delta T_{0.5}$ is prolongation of the $T_{0.5}$ that results when medium contains glycylleucine. [d] "Slant" cells. [e] "Young" cells. [f] "Aged" cells.

[1] The abbreviated designations of amino acid residues are those listed in *Biochemistry* 5, 1445 (1966), and denote the L forms.

TABLE II: Hydrolysis of L-Leucine Peptides by the Leucine–Threonine Auxotroph No. 679–680.[a,b]

Culture Age at Harvesting (hours)	Activity (μmoles hydrolyzed/hr per mg of protein)			
	Whole Cells[c]		Broken Cells[d]	
	Gly-Leu	Leu-Gly	Gly-Leu	Leu-Gly
24[e]	1.29	1.35	0.89	4.1
48	0.26	0.37	1.07	5.1
72	0.12	0.27	0.83	4.1
168[f]	0.05	0.13	0.83	5.0

[a] From Simmonds (1966). [b] Assay system (pH 7.1): potassium phosphates, Na_2SO_4, $MgSO_4$, and trace elements at concentrations present in basal growth medium plus 2 mM dipeptide. [c] Cells from basal growth medium containing 0.24 mM L-leucine harvested by centrifugation and washed with 0.9% NaCl. [d] Washed whole cells frozen and crushed in a Hughes press, and crushed material suspended in 0.9% NaCl. [e] "Young" cells. [f] "Aged" cells.

TABLE III: Effect of Amino Acids and Peptides on Growth of *E. coli* K-12.

Supplement[a]	$T_{0.1}$ (hr)[b]	Supplement[b]	$T_{0.1}$ (hr)[b]
None	18		
L-Phenylalanine	18	L-Leucine	22
L-Phenylalanine + glycine	19	L-Leucine + glycine	23
Glycine	18		
Glycyl-L-phenylalanine	19	Glycyl-L-leucine	48
L-Phenylalanylglycine	19	L-Leucylglycine	50

[a] Cells from peptone–yeast extract–agar slant inoculated into basal medium S (Simmonds and Toye, 1967) containing 0.2% glucose and supplemented, as shown, with 0.24 mM concentrations of each amino acid or dipeptide. [b] Time required for culture absorbancy to attain a value of 0.10 as measured by periodic readings (Evelyn colorimeter) during growth cycle.

whereas only labeled Gly-Leu is detected in "aged" cells (Meisler, 1963).

One other case of the concentration of a dipeptide *per se* by *E. coli* is known to me: that, reported by Kessel and Lubin (1963), of a mutant of strain W which concentrates Gly-Gly. In the case of the strain W mutant, however, a cell-free extract failed to hydrolyze Gly-Gly, whereas an extract of the strain K-12 leucine auxotroph readily hydrolyzed Gly-Leu.

The Phototroph, Strain K-12

Leucine peptides also are bacteriostatic for the prototrophic strain K-12 (Table III), and the prototroph has been used in our more recent work, in part to simplify large-scale preparation of cells, but mainly to avoid complications which might arise from an imbalance in amino acid metabolism peculiar to the leucine auxotroph (Meisler, 1963).

The action of preparations of strain K-12 on leucine peptides resembles that of the auxotroph. Furthermore, whole-cell activity toward the analogous (nonbacteriostatic) phenylalanine dipeptides parallels the activity toward the leucine peptides (Table IV), which accords with the fact that the dipeptides support equal rates of exponential growth by a glycine auxotroph although, for this mutant, as for strain K-12, only the leucine compounds are bacteriostatic (Simmonds and Miller, 1957).

With strain K-12, the relation between the activity values of whole cells and broken cells (Table IV) shows the cleavage of all the dipeptides to be catalyzed by enzymes which are partially cryptic even in bacteria from a 24-hr culture. As part of an attempt to explain crypticity, consideration was given to the possibility that the cell contains one group of peptidases which are readily detectable in assays on whole cells because they are located, like alkaline phosphatase (Malamy and Horecker, 1964), close to the cell wall, while a second group of peptidases are readily detected only after complete disruption of a cell because they are located, like β-galactosidase (Neu and Heppel, 1964), within the bacterial spheroplast. This idea was discarded after we found that the peptidases resemble β-galactoside (Neu and Heppel, 1964, 1965) in being completely retained within EDTA-lysozyme spheroplasts and osmotically shocked cells (Simmonds and Toye, 1966; Van Lenten and Simmonds, 1967). Moreover, intact spheroplasts and shocked cells exhibit levels of peptidase activity comparable to the levels found for intact bacteria, so the factors controlling the crypticity of peptidases *in vivo* remain operative in the intact spheroplast and shocked cell.

The search for these controlling factors was more successful once attention was directed to the role of the H^+ and metal ion content of culture media. For example, when cells are cultured in the usual synthetic medium (medium S), whole-cell activity toward Leu-Gly and Phe-Gly begins to fall when exponential growth ceases and the pH of the culture fluid drops below pH 6 (Table V, *cf.* line 3 *vs.* lines 1 and 2); this is a true increase in crypticity, for the activity of the cell extract remains high. If the culture is at pH 5 for 3 to 4 hr after the cessation of growth (line 4), the cell extract also shows decreased activity, which indicates that production of acid *in vivo* first enhances crypticity and later causes enzyme inactivation.

During growth in the metal-deficient medium S-M (which lacks the $MgCl_2$ and trace elements of medium S), the culture pH remains above pH 6 even on prolonged incubation (Table V, last line), and the whole-cell activity is constant throughout the growth cycle. The peptidase activity of whole cells from medium S-M is higher than that of cells growing rapidly in medium S, although extracts made from the two types of cells show the same levels of activity. Consequently, metal ions must play a special role in the regulation of peptidase activity *in vivo*.

To learn more about the effects of H^+ and metals, cultures growing in medium S were acidified to pH 5 by the addition of HCl. This stops culture growth but causes only an increase in crypticity (Table VI). In a similar experiment with cultures

TABLE IV: Peptidase Activity of Various Preparations of Strain K-12.[a,b]

Culture at Harvesting (hours)	Preparation Tested	μmoles Hydrolyzed/hr per mg of Cell Protein			
		Gly-Leu	Gly-Phe	Leu-Gly	Phe-Gly
24	Whole cells	0.50	0.55	0.68	0.74
48	Whole cells	0.15	0.29	0.46	0.52
144	Whole cells	0.08	0.16	0.15	0.12
24	Broken cells	1.6	5.4	7.7	23
48	Broken cells	1.3	3.5	9.5	29
24	Broken cells	0.9	4.4	9.0	28
	Cell-free extract[c]	1.4	4.3	8.4	27
		[2.1][d]	[6.4]	[12.6]	[41]

[a] From Simmonds (1966). [b] Cell preparations made and assayed as in Table II. [c] Supernatant fluid obtained by centrifugation of broken-cell suspension. [d] Values in brackets represent activity/mg of protein in extract.

TABLE V: Comparison of Whole Cells and Cell-Free Extracts.[a,b]

Medium	Culture Protein (mg/ml)	Culture pH	Extract Protein (%)[d]	Whole Cells (units/mg of protein)[c]			Cell Extract (units/mg of protein)[c]		
				Gly-Phe	Leu-Gly	Phe-Gly	Gly-Leu	Leu-Gly	Phe-Gly
S	0.10	6.7	69	0.9	1.6	5.2	12	20	62
S	0.32	6.5	72	0.4	1.2	3.1	7.4	14	38
S	0.60[e]	5.7[f]	43	0.7	0.5	1.1	19	23	53
S	0.50[e]	5.0	44	0.1	0.2	0	2.2	5.8	11
S-M	0.04	6.7	55	3.8	8.2	16	9.6	20	67
S-M	0.18	6.5	33	5.4	9.7	23	9.2	26	72
S-M	0.33[g]	6.5	30	5.0	7.7	19	18	26	76

[a] From Simmonds and Toye (1967). [b] Strain K-12 grown in basal medium containing 0.2% glycerol (S) or in that medium devoid of $MgSO_4$ and trace elements (S-M). Cells harvested and washed with Tris–KCl–$MgCl_2$ buffer (pH 8); cell-free extracts prepared by centrifugation following sonic disruption of whole-cell suspensions. [c] Assay system (pH 8.0); 12 mM Tris, 40 μM EDTA, 100 μM Mn^{2+}, 400 μM Mg^{2+}, 1 mM K^+, and 2 mM substrate; 1 unit is the amount causing the hydrolysis of 1 μmole of substrate/hr. [d] Protein in extract as percentage of protein in whole cells. [e] Culture in stationary phase at harvesting. [f] Culture incubated with vigorous aeration to prevent marked drop in pH. [g] Cells harvested about 20 hr after maximal culture growth was attained.

growing in medium S-M, acidification is followed by a fall in the activity of whole cells and of cell extracts. Thus, exposure of cells to H^+ in the absence of metal ions (as in medium S-M) leads to an inactivation of peptidases which can be prevented by the presence of metal ions (as in medium S).

From an experiment in which cultures were allowed to grow in metal-deficient medium S-M and then were supplemented with metallic salts, we learned that a mixture of Mg^{2+} plus the trace elements enhances crypticity at physiological pH (Table VII, expt 1). The fall in whole-cell activity is mainly due to the presence of Zn^{2+} (expt 2 and 3), and if the Zn^{2+} concentration is raised tenfold above the level used in medium S, a fall in the activity of the cell extract also is seen (expt 3).

The effect on cells *in vivo* of a very high concentration of Zn^{2+} is consistent with the effect observed when Zn^{2+} is added to a cell extract or to the soluble fraction prepared from a cell extract (Simmonds and Toye, 1967). In such *in vitro* systems, Zn^{2+} (100 μM) markedly inhibits hydrolysis of leucine dipeptides and phenylalanine dipeptides, and Zn^{2+} inhibition is only slightly reversed by EDTA (40 μM). Mn^{2+} and Co^{2+} also influence the peptidase activity of extracts, and a study of their effects provided data indicative of the presence of three soluble peptidases: one peptidase, which is insensitive to EDTA, is activated by Co^{2+} and catalyzes the hydrolysis of Gly-Leu and Gly-Phe; the other two peptidases, both of which are EDTA-sensitive, differ in their response to Co^{2+} and Mn^{2+} and in their action on Leu-Gly and Phe-Gly.

We can explain crypticity, therefore, in the following way. Growth of cells in the presence of metallic salts (as in medium S of Table V) permits the intracellular formation of metal–protein complexes that lack full enzymic activity, and breaking open the cells leads to dissociation of the inactive complex to

TABLE VI: Effect of Acidification of Culture Media on Peptidase Activity.[a]

Growth Medium[b]	pH before Treatment	Treatment[c]	Interval after Treatment (hours)	Substrate in Assay[d]	Whole Cells (units/mg of protein)	Cell-Free Extract (units/mg of protein)
S[e]	6.2	None	1.0	Phe-Gly	4.4	27
		Acid	0.5	Phe-Gly	2.6	27
		Acid	1.0	Phe-Gly	1.9	28
S-M[f]	6.7	None	0.5	Phe-Gly	21	73
		Acid	0	Phe-Gly	9.2	33
		Acid	0.5	Phe-Gly	4.5	
S-M[g]	6.7	None	24	Phe-Gly	19	76
		Acid	24	Phe-Gly	3.9	19
		None	24	Leu-Gly	7.7	26
		Acid	24	Leu-Gly	2.9	6.2
		None	24	Gly-Phe	5.0	18
		Acid	24	Gly-Phe	1.4	4.4

[a] From Simmonds and Toye (1967). [b] Strain K-12 grown in presence (S) or absence (S-M) of $MgSO_4$ and trace elements as described in Table V. [c] Each culture divided in several portions: one portion used as unacidified control and others brought to pH 4.9 by addition of 1 N HCl; incubation then continued for indicated time. [d] Assay system as in Table V. [e] Extract protein equivalent to *ca.* 63% of whole-cell protein in all cultures. [f] Extract protein *ca.* 34% of whole-cell protein in all cultures. [g] Extract protein *ca.* 30% of whole-cell protein in unacidified control and about 18% of whole-cell protein in acidified culture.

TABLE VII: Effect of Addition of Metal Ions to Cultures Grown in Metal-Deficient Medium.[a,b]

Expt	Supplement Added[c]	Culture Protein at Harvesting (mg/ml)	Substrate in Assay[d]	Whole Cells (units/mg of protein)	Cell-Free Extract (units/mg of protein)
1	None	0.16	Gly-Phe	2.4	10
	Mg^{2+} + TES	0.23	Gly-Phe	1.0	7.3
	None		Leu-Gly	4.6	18
	Mg^{2+} + TES		Leu-Gly	0.9	15
	None		Phe-Gly	14	61
	Mg^{2+} + TES		Phe-Gly	3.6	42
2	None	0.18	Phe-Gly	12	53
	$Mg^{2+} \pm Zn^{2+} \pm [Fe^{2+} + Cu^{2+}]$	0.22	Phe-Gly	7.4	46
	$Zn^{2+} \pm [Fe^{2+} + Cu^{2+}]$	0.18	Phe-Gly	8.0	49
3	None or Mg^{2+}	0.26	Phe-Gly	27	60
	Mg^{2+} + TES	0.33	Phe-Gly	7.7	63
	Zn^{2+}	0.27	Phe-Gly	17	81
	Zn^{2+}, 151 μM	0.28	Phe-Gly	4.6	55
	Zn^{2+}, 310 μM	0.30	Phe-Gly	1.9	37

[a] From Simmonds and Toye (1967). [b] Each strain K-12 culture, grown in medium S-M (Table VI), divided into several portions: one used as control and others supplemented as shown; incubation then continued for 3 hr. [c] Final concentrations: 400 μM Mg_2SO_4, and (supplied singly or as trace element solution, TES), 31 μM $ZnSO_4$, 3.6 μM $FeSO_4$, 1.6 μM $CuSO_4$, 0.92 μM H_3BO_3, 0.36 μM $MnCl_2$, 0.20 μM Na_2MoO_4. [d] Assay system as in Table V.

yield an active enzyme. The metal–protein complexes within the cell render the protein resistant to denaturation by H^+ of exogenous origin; but the protein is not fully resistant to H^+ produced within the cell, probably because excessive acid production overwhelms the homeostatic mechanisms. The fact that cells grown in the absence of metallic salts (as in medium S-M) cannot form inactive metal–protein complexes in large amounts explains why such cells appear to have more pep-

TABLE VIII: Effect of Supplementary Carbon Source on Peptidase Activity.

Preparation of Culture[a]		Soluble Fraction (sp act.)[c]		Ribosomal Fraction (sp act)[c]		R/[R + S][d] (%)	
Carbon Source	Absorbance[b]	Phe-Gly	Leu-Gly	Phe-Gly	Leu-Gly	Phe-Gly	Leu-Gly
Glycerol	0.22	130	33	4.1	9.3	1.5	12
Glycerol	0.37	50	13	5.1	11	5.4	34
Glycerol	0.52[e]	78	25	4.2	15	1.5	14
Glycerol	0.63[f]	49	13	2.3	9.5	1.2	15
Glucose	0.36	137	27	6.5	9.8	1.2	8.4
Glucose	0.49	140	49	17	21	2.4	7.9
Glucose	0.50	156	24	17	7.6	2.4	6.7
Glucose	0.73[e]	43	16	3.3	3.1	3.2	7.7
Glucose	0.75[f]	66	17	2.3	3.2	1.1	5.7

[a] Cultures grown in peptone-containing medium A-M of Simmonds and Toye (1967) supplemented with 0.6% glycerol or glucose; initially at pH 7.5. Cell-free extract (prepared as in Table V) fractionated by high-speed centrifugation (Simmonds and Toye, 1967). [b] Culture absorbance at harvesting (Bausch and Lomb Spectronic 20; 580 mμ). [c] Units per milligram of protein in fraction; assay system as in Table V. [d] Total activity in ribosomal fraction divided by sum of total activity in each subcellular fraction. [e] Culture just entering stationary phase; glycerol culture at pH 7.4 and glucose culture at pH 7.2 at harvesting. [f] Culture at maximal absorbance *ca.* 5 hr before harvesting; glycerol culture at pH 7.2 and glucose culture at pH 7.0 at harvesting.

tidase activity (in whole-cell assays) and why their intracellular peptidases are inactivated by exogenous H^+.

Soluble and Ribosomal Fractions of Strain K-12

Separation of cell-free extracts into soluble and ribosomal fractions shows that almost all the dipeptidase activity is localized in the soluble portion of the cytoplasm (Simmonds and Toye, 1967). Until recently, the available evidence indicated that the activity of each subcellular fraction (Simmonds and Toye, 1967), as well as that of the unfractionated extract (Simmonds, 1966; Simmonds and Toye, 1966; Van Lenten and Simmonds, 1967), is ascribable to peptidases having the properties of constitutive enzymes. We now know that the addition of glucose in place of glycerol to a peptone-containing growth medium alters the peptidase content of strain K-12.

Among the results of the more recent work is the observation that a medium containing peptone as the only carbon source and a medium containing peptone plus glycerol produce cells with the same dipeptidase activity (S. Simmonds and N. O. Toye, unpublished data). As found earlier (Simmonds and Toye, 1967), the soluble fractions from cells grown with glycerol are more active toward Phe-Gly than toward Leu-Gly and Gly-Phe; the ribosomal fractions are most active toward Leu-Gly, and contain from 12 to 34% of the total extractable activity toward this substrate (Table VIII).

When glycerol is replaced by glucose, the most striking change is in the peptidase activity of the ribosomal fractions. The ribosomal fractions made from glucose-grown cells account for only 6–8% of the total activity toward Leu-Gly; moreover, they show the same specific activity toward Leu-Gly and toward Phe-Gly.

A second change produced by use of glucose is the appearance of a correlation between peptidase activity and culture age: after cultures cease to grow exponentially (Table VIII, last two lines), the specific activity values of each subcellular fraction drop sharply. This may represent only one aspect of a more general alteration in cell morphology (*cf.* Herbert, 1961), for we find the progression from exponential to stationary phase produces cells containing only about 50% of the expected amount of protein and having a high resistance to ultrasonic oscillation. These changes can be related to the fact that growth in the presence of glucose leads to a greater production of H^+ than is seen in the presence of glycerol (Table VIII, footnotes *e* and *f*) although growth on the two carbon sources is characterized by the same generation time. We infer that the "acid effect" described above for cultures in synthetic media (Tables V and VI) also explains the drop in peptidase activity observed when cultures in the peptone–glucose medium cease to grow. Another possible explanation is that peptidases belong to the group of proteins undergoing rapid degradation in nongrowing cells (Mandelstam and Halvorson, 1960; Pine, 1965; Willetts, 1967). If they do, then we still have to explain why the rate of protein turnover is specifically altered by glucose metabolism, for the constancy of protein content and peptidase activity in cells metabolizing glycerol implies a balance between protein degradation and synthesis in glycerol-grown cells.

The ribosomal fraction of strain K-12 obviously contains at least one peptidase that usually shows highest activity toward Leu-Gly, whereas the soluble fraction appears to contain two enzymes acting on this dipeptide. The presence of Leu-Gly-cleaving enzymes in both subcellular fractions of *E. coli* B was shown by Matheson (Matheson, 1963; Matheson and Tsai, 1965), who subsequently reported that the ribosomal enzyme acts also on methionyl peptides and suggested that a ribosomal peptidase may function in protein biosynthesis to catalyze the removal of amino-terminal methionine residues from polypeptide chains (Matheson and Murayama, 1966).

Ribosomal fractions made from strain K-12 also have activity toward dipeptides of structure Met-X, and it is comparable to their activity toward dipeptides of structure Leu-X (Table IX). In these dipeptides, the nature of the carboxyl-terminal residue may be important, for replacement of a gly-

TABLE IX: Depeptidase Activity of Soluble and Ribosomal Fractions.[a]

Substrate	Soluble Fraction (units/mg of protein)[b]	Ribosomal Fraction (units/mg of protein)[b]	R/[R + S][c] (%)
Phe-Gly	231	1.8	0.3
Met-Gly	211	4.7	0.5
Met-Ser	139	2.7	0.6
Met-Leu	183	16	2.7
Leu-Gly	57	7.9	4.1
Leu-Ser	30	7.5	7.1
Leu-Leu	32	16	13
Leu-Tyr	36	16	12
Gly-Gly	17	<0.7	<1.2
Gly-Met	51	1.0	0.6
Gly-Phe	37	0.8	0.6

[a] Strain K-12 grown to late exponential phase in high-phosphate, peptone- and glycerol-containing medium B-M (Simmonds and Toye, 1967). Cell-free extract (prepared as in Table V) separated by high-speed centrifugation into soluble fraction and ribosomal pellet; pellet washed once in Tris–KCl–$MgCl_2$ buffer (pH 8) before resuspension in buffer for use as ribosomal fraction. [b] Assay system as in Table V; activity measured by procedure of Simmonds and Toye (1966). [c] Total activity in ribosomal fraction divided by sum of total activity in each subcellular fraction.

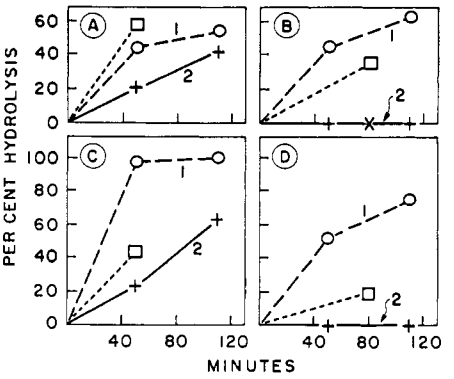

FIGURE 2: Peptidase activity of washed ribosomes prepared (as in Table IX) from strain K-12. Assay system as in Table V; amounts of unreacted substrate and of each product measured, after separation by descending paper chromatography (Simmonds and Griffith), by ninhydrin procedure (Matheson and Tattrie, 1964). Aminopeptidase activity toward tripeptides (○) shown by curves 1, and hydrolysis of resultant carboxyl-terminal dipeptides (+) by curves 2; hydrolysis of dipeptide substrates (□) shown by unnumbered curves. Substrates: A, Met-Leu-Gly and Met-Leu; B, Met-Gly-Gly, Met-Gly, and Gly-Gly (X); C, Leu-Leu-Gly and Leu-Leu; D, Leu-Gly-Gly and Leu-Gly.

cine or a serine in X position by a leucine or tyrosine facilitates hydrolysis.

With the soluble fraction, only the amino-terminal residue appears to be of importance. Here, Phe-Gly and substrates in the Met-X series are hydrolyzed most rapidly (Table IX). Dipeptides of structure Gly-X are poor substrates and each depeptide with an amino-terminal glycine is hydrolyzed much more slowly than is its X-Gly isomer.

Although for each substrate tested, the amount of activity in the soluble fraction far exceeds the amount in the ribosomal fraction (Table IX, last column), the ribosomal fraction has high specific activity toward some of the dipeptides, and further tests show that it contains high levels of aminopeptidase activity toward some tripeptides. The tripeptides used are of the type X-Y-Gly, and their degradation initially produces only X and Y-Gly; no X-Y can be detected among the hydrolysis products (S. Simmonds, unpublished data). This is similar to the mode of degradation (by a phenylalanine auxotroph) of the dipeptide amides Gly-Phe-NH_2 and Phe-Gly-NH_2, which are first cleaved to yield the amino-terminal amino acid plus an amino amide (Simmonds and Griffith, 1962).

Comparison of the activity of the ribosomal fraction toward Leu-Leu-Gly (Figure 2C), Leu-Gly-Gly (Figure 2D), and Met-Gly-Gly (Figure 2B) shows similar results: the reaction X-Y-Gly → X + Y-Gly (curves numbered 1) is faster than the analogous reaction X-Y → X + Y (dotted curves). The data suggest that all enzymic activity may reside in an aminopeptidase which also possesses dipeptidase activity toward Leu-Leu (Figure 2C), Leu-Gly (Figure 2D), and Met-Gly (Figure 2B). This suggestion fails to explain the data for Met-Leu-Gly (Figure 2A) because the first step in its degradation is less rapid than the hydrolysis of Met-Leu; the suggestion also fails to explain why Phe-Gly is hydrolyzed (*ca.* 12% in 80 min) and Phe-Gly-Gly is not (0% hydrolysis in 110 min). Consequently, at least two peptidases must be present in the ribosomal preparation, and further study will be necessary before it will be possible to define their substrate specificity.

When the same group of substrates is acted upon by the soluble fraction (Figure 3), the initial step in tripeptide degradation (to yield X + Y-Gly) is never faster than hydrolysis of the analogous dipeptide (X-Y). Indeed, the initial step in the hydrolysis of Met-Leu-Gly (Figure 3A), Leu-Gly-Gly (Figure 3D), and, especially, Leu-Leu-Gly (Figure 3C) appears to be accelerated as a consequence of the hydrolysis of the newly formed depeptide (curves numbered 2). In tripeptides as well as in dipeptides, Met-Y bonds are cleaved much faster than Leu-Y bonds, which is consistent with the presence in the soluble fraction of an enzyme (or enzymes) catalyzing the removal of amino-terminal methionine and leucine from both tri- and dipeptides. However, the soluble fraction lacks activity toward Phe-Gly-Gly (0% hydrolysis on 60 min) in spite of its high activity toward Phe-Gly (*ca.* 43% hydrolysis in 20 min), so we must infer the presence of another enzyme which acts rapidly on a depeptide and slowly, if at all, on the analogous tripeptide.

As mentioned earlier, we had reason to believe the soluble fraction contains two "peptidases" that differ in their action on Phe-Gly and Leu-Gly, and it was tempting to predict that the enzyme acting preferentially on Phe-Gly is a "dipeptidase" whereas the enzyme acting preferentially on Leu-Gly is an "aminopeptidase." This prediction could be tested without delay, because we already had separated from the soluble portion of strain K-12 cells two enzyme preparations which differ in their dipeptide–substrate specificity and which we term soluble fractions L and P (Table X).

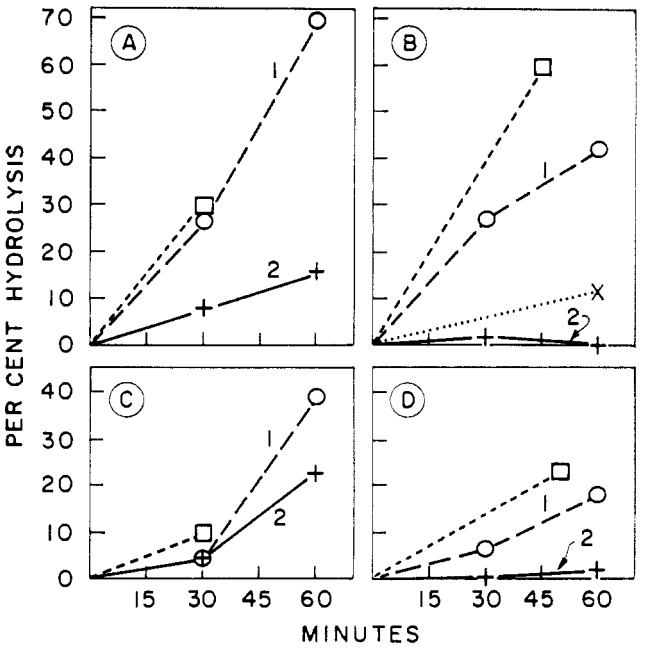

FIGURE 3: Peptidase activity of the soluble fraction prepared from the culture of strain K-12 used in Figure 2. Assay procedure, symbols, curve numbers, and substrates as given for Figure 2.

Soluble fraction L acts rapidly not only on leucyl dipeptides but also on methionyl dipeptides, and shows a clear correlation between its dipeptidase and aminopeptidase activity. Both types of activity are stable at *ca.* 10° for several weeks and are quickly lost when the preparation (column-effluent fractions) is frozen, which leads us to ascribe the activity of fraction L to a single aminopeptidase catalyzing rapid removal of leucine and methionine residues and showing a preference for substrates in which the NH group of the sensitive CO–NH is provided by a leucine rather than a glycine residue.

A more complicated situation obtains in the case of soluble fraction P, the preparation having more activity toward Phe-Gly than toward Leu-Gly. Compared to fraction L, fraction P shows less variation in its specific activity values for di- and tripeptides, and lacks any obvious correlation between dipeptidase and aminopeptidase activity. To explain its activity, we postulate the presence of at least two enzymes: possibly an aminopeptidase that also acts as a dipeptidase and a dipeptidase that is inactive toward tripeptides. Some support for this idea is provided by the fact that aminopeptidase activity appears to be stable when the preparation is frozen, and dipeptidase activity toward Met-Leu and Leu-Leu also is cold stable; in contrast, dipeptidase activity toward substrates of structure X-Gly is rapidly lost during storage of the preparation at 10° or in the freezer.

TABLE X: Relative Activity of Fractions Separated on DEAE-Sephadex A-50.[a]

Substrate	Soluble Fraction L		Soluble Fraction P	
	Dipeptidase Act.	Aminopeptidase Act.[b]	Dipeptidase Act.	Aminopeptidase Act.[b]
Leu-Gly	100[c]		100[d]	
Leu-Gly-Gly		200		70
Phe-Gly	20		290	
Phe-Gly-Gly		10		55
Met-Gly	85		450	
Met-Gly-Gly		90		140
Leu-Leu	750		160	
Leu-Leu-Gly		750		300
Met-Leu	650		200	
Met-Leu-Gly		650		175

[a] Soluble preparation (similar to that used in Table IX) fractionated on DEAE-Sephadex A-50 in 40 mM Veronal buffer (pH 8) containing 0.1 mM $MnCl_2$ and 5 mM mercaptoethanol and a KCl gradient of 0–0.5 M. About 200 mg of protein applied to 44 × 4 cm column; 15-ml fractions collected at flow rate of 175 ml/hr. Column effluent first scanned for activity toward Leu-Gly and Phe-Gly; peak fractions in each of two areas which showed activity then pooled to provided fraction L (displaced from column at *ca.* 0.25 M KCl) and fraction P (displaced at *ca.* 0.4 M KCl). Assay of each fraction (for substrates listed in table) with a system containing 9 mM Tris (pH 8.0), 40 mM EDTA, 110 μM Mn^{2+}, 4 mM Veronal, 0.5 mM mercaptoethanol, and 2 mM substrate. [b] Activity determined (Simmonds and Toye, 1966) with mixtures of tripeptide, amino-terminal amino acid, and carboxy-terminal dipeptide (in proportions present when extent of hydrolysis (X-Y-G → X + Y-G) is 0, 20, and 40%) used to prepare standard curves for ninhydrin determinations (Matheson and Tattrie, 1964). [c] Specific activity, 80 units/mg of protein, set equal to 100. [d] Specific activity, 70 units/mg of protein, set equal to 100.

Conclusions

That *E. coli* K-12 must be able to hydrolyze a variety of peptides had been evident since amino acid auxotrophs first were found to grow readily on these compounds. Our study of the hydrolytic reactions *in vitro* indicates the existence of soluble enzymes and of enzymes associated with the ribosomes, all of which attack CO–NH bonds linking protein amino acids and have activity generally ascribed to dipeptidases or aminopeptidases. The physiological role of these intracellular peptidases remains an intriguing question. They appear to be constitutive enzymes, for their production is not enhanced when cells are supplied with exogenous peptides that serve as sources of amino acids for growth. They probably play no direct role in the metabolism of cell-wall peptidoglycans, for they clearly differ in activity from the intracellular enzymes described in studies on the biosynthesis and degradation of those substances (Strominger, 1967; Strominger and Ghuysen, 1967; Ghuysen, 1968). It is my belief that the peptidases function in protein turnover (*i.e.*, the degradation of cellular proteins which provides amino acids for the formation of new protein molecules), and also in the synthesis *de novo* of at least some cellular proteins.

The assumption that all polypeptide chains synthesized in bacterial systems contain an amino-terminal methionine prompted a search for methionine aminopeptidase activity in

bacterial extracts. Following the first report by Matheson and Murayama (1966) that ribosomes from *E. coli* B hydrolyze methionyl peptides (structure unspecified) came other reports that extracts hydrolyze Met-Ala and Met-Leu (Fry and Lamborg, 1967; Adams, 1968), Met-Phe (Livingston and Leder, 1969), and methionylpuromycin (Livingston and Leder, 1969; Takeda and Webster, 1968) and aminopeptidase activity toward Met-Ala-Ser (Adams, 1968) and Met-Ala-Ser-Asn-Phe-Thr (Takeda and Webster, 1968) also was described. Although each report is accompanied by the statement that the observed activity can explain the production *in vivo* of the large amount of *E. coli* protein which lacks an amino-terminal methionine, we still must determine how further, and extensive, degradation of the newly formed polypeptides is prevented, for aminopeptidase activity is by no means limited to substrates containing a terminal methionine. In this regard, it seems of interest to recall that the dipeptidase activity of intact cells of strain K-12 is subject to control by environmental factors, in particular the H^+ and metal-ion content of the culture medium. It is not unlikely that similar factors may prove of importance in the control of aminopeptidase activity within cells, and it is to be hoped that the isolation and characterization of the aminopeptidases will provide helpful information on this point.

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