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Soluble Tri- and Dipeptidases in *Escherichia coli* K-12†

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ABSTRACT: As part of a study of the metabolic role of peptidases in *Escherichia coli* K-12, cell extracts were examined for the presence of three enzymes originally identified [Sussman, A. J., and Gilvarg, C. (1970), *J. Biol. Chem. 245,* 6518] in extracts of the lysine auxotroph AS013 by virtue of their activity toward lysine homopolymers. It has now been shown that the activity ascribed to a Co^{2+}-dependent dilysine-specific enzyme is a function of the strain K-12 dipeptidase DP, a metal-dependent enzyme active toward a variety of dipeptides, and that the activity ascribed to a trilysine-specific enzyme is a function of the strain K-12 tripeptidase TP, an aminopeptidase capable of hydrolyzing substrates in the series X-Gly-Gly, X-Gly-X, and X-Leu-Gly (where X is Leu or Met) but devoid of activity toward dipeptides. The third enzyme, an oligopeptidase not previously observed in strain K-12, was found to include among its substrates not only di- and trilysine but other di- and tripeptides that are hydrolyzed by the di-and tripeptidase as well as by aminopeptidases L and AP; the aminopeptidases, however, lack activity toward di- and trilysine. The absence of oligopeptidase activity from extracts of strain AJ005, a "peptidase-deficient mutant" derived from strain AS013 by Sussman and Gilvarg, has been confirmed, and strain AJ005 has been shown to contain all the other peptidases known to be present in strain K-12. Possible functions of the oligopeptidase are proposed on the basis of its observed activity in vitro and of the differences between the growth responses of strains AJ005 and AS013 in various media. Some general aspects of peptide metabolism are discussed with emphasis on the use of peptidase-deficient mutants in the study of this problem, and methods that may prove helpful in the isolation of such mutants are suggested.

Evidence that bacterial enzymes catalyzing the hydrolysis of small peptides participate in a variety of metabolic processes has been accumulating since the role of peptidases in the utilization of exogenous peptides as growth factors and in the destruction of toxic peptides was recognized in 1950 (Fruton and Simmonds, 1950). Subsequent work in several laboratories (summarized by Sussman and Gilvarg, 1971; Simmonds, 1972) has provided ample support for these two functions of intracellular peptidases in *Escherichia coli* K-12. During the 1960's, further interest in bacterial peptidases was stimulated by the realization that bacterial protein biosynthesis may require the participation of a methionine aminopeptidase, and *E. coli* K-12 has been shown to contain several enzymes capable of effecting the removal of an amino-terminal methionine from tri- or dipeptides (Simmonds, 1972)[1] in addition to the "aminopeptidase I" crystallized by Vogt (1970). Whether any of these strain K-12 peptidases, or the "ribosomal-bound aminopeptidase" (Matheson et al., 1970) and the dipeptidases (Brown, 1973; Hayman et al., 1974) purified from *E. coli* strain B, are specifically concerned in protein biosynthesis remains uncertain (cf., Vogt, 1970; Matheson and Dick, 1970; Brown, 1973; Johnson and Brown, 1974). The suggestion has been made that a primary function of such enzymes is their participation in protein turnover, i.e., as catalysts for the hydrolysis of small peptides arising from the degradation of cellular proteins which provides amino acids for the formation of new protein molecules (Simmonds, 1970). Rapid degradation of protein in nongrowing bacteria was first reported by Mandelstam and Halvorson in 1960, and later studies by several investigators (summarized by Payne, 1972b; Goldberg et al., 1974) support the view that intracellular proteolysis may not only supply amino acids for the synthesis of new enzymes in starved *E. coli* cells but also provides a mechanism for the elimination of abnormal cell constituents formed as the result of mutations or mistakes in transcription or translation (Goldberg et al., 1974; Goldberg and Dice, 1974).

Although the enzymic activity of crude *E. coli* extracts toward di- and tripeptides appears to be sufficient to account for the relatively high rates of protein degradation to amino acids which have been observed in starved cells (Payne, 1972b), the existence of several peptidases showing overlapping substance specificity raises the question of whether the full complement of peptidases present in a wild-type strain is essential for this process as well as for cell growth and multiplication. Resolution of this problem would most easily be accomplished by studies on amino acid auxotrophs lacking one or more of the "wild-type peptidases". Most attempts to produce such mutants have been unsuccessful (Payne, 1972b; F. Taylor and S. Simmonds, unpublished data), and only one peptidase auxotroph of strain K-12 has been described: a mutant derived from the lysine

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auxotroph AS013 (Sussman and Gilvarg, 1970). Extracts of the peptidase-deficient lysine auxotroph, strain AJ005, were observed to contain three enzymes active toward lysine homopolymers: a Co^{2+}-dependent dipeptidase, and EDTA-sensitive trilysine-specific tripeptidase, and a tetralysine endopeptidase; the extracts lacked a metal-independent oligopeptidase that was active toward all the lysine homopolymers and was present, together with the other three enzymes, in extracts of the parent strain AS013.

The ability of the peptidase-deficient strain AJ005 to grow readily on lysine or dilysine (but not on trilysine plus or minus lysine) indicates the nonessential nature of the lysine oligopeptidase. Although neither this enzyme nor any of the others was tested for activity toward substrates other than the lysine homopolymers, Sussman and Gilvarg (1970) recognized that the enzymes probably could attack many other peptides. However, in the absence of data concerning the substrate specificity of these enzymes, it was difficult to assess their relative physiological importance in the overall peptide metabolism of *E. coli* K-12. Because previous work in this laboratory had shown strain K-12 extracts contain the metal-dependent dipeptidase DP that is active toward many dipeptides including Lys-Lys and Lys-Gly, the probable identity of the dilysine dipeptidase and dipeptidase DP was proposed (Simmonds, 1972). A study has now been made of strain K-12 and the peptidase-deficient strain AJ005 in order to test this proposal and, at the same time, to determine whether either the trilysine tripeptidase or the lysine oligopeptidase is identical with any of the peptidases previously found in strain K-12; these include two enzymes, aminopeptidases L and AP, characterized by high activity toward methionyl and leucyl tri- and dipeptides (Simmonds, 1972), and tripeptidase TP which lacks dipeptidase activity but rapidly hydrolyzes substrates in the X-Gly-Gly series (where X is Leu, Met, or Phe).[1]

Materials and Methods

E. coli strain AS013 ($Lys^-Thr^-Leu^-$thiamine$^-$) and its peptidase-deficient derivative strain AJ005 were kindly supplied by Dr. Charles Gilvarg, Princeton University. Strain 679-680 (Thr^-Leu^-), like strain AS013, is a mutant of strain K-12 and was used in previous work (summarized by Simmonds, 1970).

Dilysine and trilysine were purchased from Cyclo Chemical, Los Angeles, Calif.; other peptides and amino acids were purchased from Schwarz/Mann, Orangeburg, N.J. All peptides are composed of L-amino acids.

Growth tests were carried out as described previously (Meisler and Simmonds, 1963), but using medium S of Simmonds and Toye (1967) containing 0.2% glycerol and supplemented with, per milliliter, 0.5 μg of thiamine-HCl, 50 μg of DL-threonine, and 0.3 μmol of L-leucine plus the specified amounts of L-lysine or lysine peptides. After inoculation of the test media with a suspension of cells taken from 24-hr yeast extract–peptone–agar slants, the cultures were incubated at 36° without shaking. From the growth curves (based on absorbancy measurements made at approximately 2-hr intervals in an Evelyn photometer fitted with Filter No. 540), an estimate was made of the time required for each culture to reach an absorbancy equivalent to half-maximal growth in the presence of nonlimiting concentrations of all required growth factors.

For use in peptidase studies, strains K-12 and AJ005 were cultured in the high phosphate, peptone-, and glycerol-containing medium B-M of Simmonds and Toye (1967). As in earlier work (Simmonds and Toye, 1967; Simmonds, 1970), bacteria were harvested toward the end of the logarithmic phase of growth, and were washed and resuspended in Tris–KCl–$MgCl_2$ buffer prior to disruption by sonic oscillation. From the resultant broken-cell suspension, the soluble fraction was prepared by centrifugation first to remove cell debris and then to remove ribosomes. Because the cells were disrupted in buffer containing 0.025 *M* KCl, aminopeptidase I remained bound to the ribosomes, and the soluble fractions under study were essentially devoid of the peptidase activity ascribed to that and to other ribosome-bound enzymes (Vogt, 1970; Simmonds, 1972).

Ion-exchange chromatography was carried out on columns of DEAE-Sephadex A-50 equilibrated in a pH 8 buffer composed of 6 m*M* Tris, 6 m*M* β-mercaptoethanol, 0.1 m*M* $MnSO_4$, and 0.1 *M* KCl; columns were developed with a linear concentration of KCl from 0.1 to 0.5 m*M*, with 100 fractions in the gradient. When 0.9 × 30 cm columns were used, 1.5 ml of specified soluble fraction (ca. 5.4 mg of bacterial protein) was applied to the column, and 1-ml fractions were collected. With the 4 × 59 cm column, 41 ml of strain K-12 soluble fraction (146 mg of bacterial protein) was applied and 20-ml fractions were collected. The same preparation of strain K-12 soluble fraction was used for small and large columns.

Peptidase activity was measured at 38° in a 0.2-ml assay mixture (pH 8) that contained 0.1 ml of column effluent fraction (or of a suitably diluted soluble fraction) plus additional components to provide (final concentrations) 2 m*M* substrate, 6.3 m*M* Tris, 0.13 m*M* Mn^{2+}, 0.04 m*M* EDTA, and, where indicated, 15 m*M* β-mercaptoethanol. Column effluent fractions contributed sufficient KCl to give a final concentration in the assay mixture of 0.05–0.25 *M*. As first obtained from the columns, the fractions also could contribute sufficient BME[2] to give a final concentration of 3 m*M*: fractions stored for more than a few days may be assumed to contain little, if any, unoxidized BME because no attempt was made to prevent oxidation of the mercaptan during storage of the fractions in the refrigerator. When the fractions were to be assayed in the presence of additional BME, each fraction was adjusted to 30 m*M* with respect to the mercaptan and kept in a capped test tube for 2 hr in an ice bath prior to use. Where indicated, activity also was measured in assay system "T-M" which was devoid of EDTA and had a Mn^{2+} concentration of 0.35 m*M*. Reactions were initiated by the addition of substrate (0.05 ml of aqueous solution) to an otherwise complete reaction mixture, and were terminated by the addition of 0.6 ml of 15 m*M* acetic acid and immediate immersion of the reaction test tube in an ice bath. Reaction times used to obtain peptidase-activity elution profiles of ion-exchange columns were chosen so as to permit detection of very low levels of enzymic activity although such times often permitted 100% hydrolysis of the substrate by highly active column effluent fractions. Where specific activity was measured, three or more replicate assay mixtures were prepared: one mixture was acidified immediately after the addition of substrate and the others at intervals thereafter in order to ascertain the time required for peptide cleavage in the range of from 5 to about 50%, where the rate of hydrolysis is linear.

For all substrates other than the lysine homopolymers, the extent of peptide hydrolysis was determined (using 0.2-ml aliquots of acidified assay mixture) by the ninhydrin

[2] Abbreviation used BME, β-mercaptoethanol.

method of Matheson and Tatrie (1964). Because tripeptides X-Y-Gly can undergo an aminopeptidase-type cleavage to X and Y-Gly followed by hydrolysis of the dipeptide (Simmonds, 1970), the extent of tripeptide hydrolysis is indicated in terms of the observed increase in ninhydrin-reactive material produced as the result of enzymic reactions and measured in an Evelyn photometer fitted with Filter No. 580. Hydrolysis of di- and trilysine was measured by a modification of the method of Shimura and Vogel (1966) in which 0.4 ml of acidified assay mixture, 0.1 ml of 6 *N* HCl, and 0.5 ml of the 15% ninhydrin solution (placed in 0.75-in diameter Bausch and Lomb Spectronic 20 test tubes) were heated in a boiling water bath for 1 hr. After the solution had been cooled in an ice bath, 5 ml of concentrated H_3PO_4 was added, and the resultant viscous mixture was agitated prior to absorbance measurement in the Spectronic 20 colorimeter–spectrophotometer at 515 nm. In the case of trilysine, which can give rise to either 1 or 3 equiv of lysine (Sussman and Gilvarg, 1970), the extent of hydrolysis is recorded in terms of concentration of lysine liberated. Standard curves used to estimate the extent of hydrolysis of all di- and tripeptides were based on color values obtained with appropriate mixtures of the substrate plus its hydrolysis products.

Results

From the observations of Sussman and Gilvarg (1970), it was expected that strain K-12 would contain two dilysine-cleaving enzymes. To determine whether both enzymes can be detected by the procedures used in this laboratory, a sample of strain K-12 soluble fraction was subjected to chromatography on DEAE-Sephadex A-50 with a linear KCl gradient used for elution. Column effluent fractions were immediately tested for activity toward Lys-Lys, and also toward Phe-Gly to identify the position of dipeptidase DP (Figure 1A, region II) as well as toward Leu-Leu, which is a poor substrate for the dipeptidase but is rapidly hydrolyzed by aminopeptidase L (region I) and aminopeptidase AP (region II) (Simmonds, 1972). Activity toward Lys-Lys was seen in both regions II and III, the latter region being essentially devoid of activity toward Phe-Gly and containing a limited ability to hydrolyze Leu-Leu.

The elution pattern for dilysine cleavage in region II resembles that seen for the Phe-Gly rather than that for Leu-Leu, which is in accord with the hypothesis that dipeptidase DP is responsible for the activity ascribed to the dilysine-specific dipeptidase of strains AS013 and AJ005. Preparations of dipeptidase DP in the Tris–BME–$MnSO_4$ buffer used for column chromatography rapidly lose activity during storage but can be reactivated by the addition of fresh BME (Simmonds, 1972), a property permitting easy differentiation between the dipeptidase and aminopeptidase AP in column fractions containing both enzymes. Thus, when the column fractions were retested after storage for a week in the refrigerator, two regions with Lys-Lys-cleaving activity again were evident (Figure 1B): one (activity peak in fractions 63–65) lacked ability to hydrolyze either Lys-Lys or Phe-Gly in the absence of added BME while the other (activity peak in fractions 77–81) displayed activity toward both Lys-Lys and Leu-Leu that was essentially unaffected by addition of the mercaptan. The BME stimulation of Leu-Leu hydrolysis seen with fractions 72–74 is characteristic of aminopeptidase AP (Simmonds, 1972) and, in the present case, serves to distinguish the elution position of aminopeptidase AP from the positions of the two enzymes acting on dilysine.

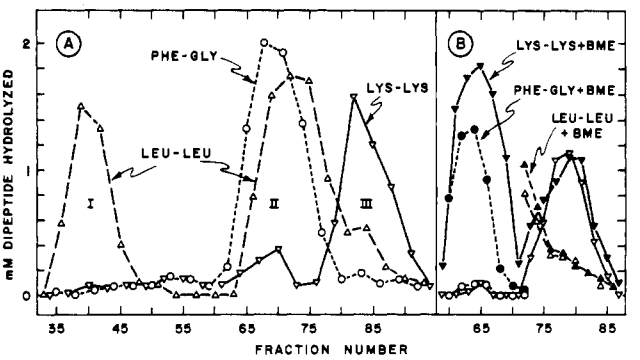

FIGURE 1: Elution patterns showing dipeptidase activity following chromatography of strain K-12 soluble fraction on a 0.9 × 30 cm column of DEAE-Sephadex A-50. Activity (determined as described under Materials and Methods) is expressed as the concentration of dipeptide hydrolyzed by 0.1 ml of effluent fraction in the assay reaction time used, which was: for Leu-Leu, 1 hr in the absence (△) and in the presence (▲) of additional BME; for Lys-Lys, 2 hr in the absence (▽) and in the presence (▼) of BME; for Phy-Gly, 2 hr in the absence (○) and 15 min in the presence (●) of BME. Assays in A were done on the day following the column run and assays in B were done a week later.

Additional evidence that Lys-Lys and Phe-Gly are attacked by the same dipeptidase was obtained in tests carried out on a pool made by combining equal volumes of fractions 61–65. No hydrolysis was observed in the absence of BME during a 3-hr reaction time; in the presence of BME, the specific activity (concentration of peptide hydrolyzed per hour) of 0.1 ml of pool was 1.30 m*M* for Lys-Lys and 10.0 m*M* for Phe-Gly. This ratio of activities agrees well with the ratio previously found with a dipeptidase DP preparation that had been freed of aminopeptidase AP by chromatographic procedures (Simmonds, 1972).

To determine whether the hydrolysis of dilysine by column fractions lacking activity toward Phe-Gly is due to the presence of the lysine oligopeptidase identified by Sussman and Gilvarg (1970) as a constituent of strain AS013, a similar pool was made of fractions 77–82. Under conditions (BME present) in which the specific activity of 0.1 ml of the pool was 0.090 m*M* for dilysine, 0.133 m*M* lysine was released per hour from trilysine. The pool also catalyzed the hydrolysis of Met-Gly-Gly and Met-Leu-Gly. While these data confirm the presence in fractions 77–82 of enzymatic activity toward both di- and tripeptides, the hydrolysis of trilysine might have been due, at least in part, to the activity of the trilysine tripeptidase which Sussman and Gilvarg (1970) reported to emerge from a DEAE-cellulose column at the same position as the oligopeptidase. Likewise, the hydrolysis of methionyl tripeptides could not be ascribed solely to oligopeptidase activity because these compounds are substrates for tripeptidase TP which was known to be present in this region of the column effluent.[1]

Clarification of the behavior to be expected of the trilysine-specific tripeptidase during DEAE-Sephadex chromatography may most readily be accomplished using strain AJ005, the mutant lacking oligopeptidase. Absence of the oligopeptidase might also be expected to lower the total dilysine-cleaving action of the strain AJ005 soluble fraction relative to that of strain K-12 when the two strains are compared using an assay system that minimizes the hydrolysis of Lys-Lys by the dipeptidase and still permits high levels of oligopeptidase activity (cf., Figure 1A, regions II and III). Accordingly, a strain AJ005 soluble fraction was prepared and a sample was diluted 100-fold with the Tris–BME–

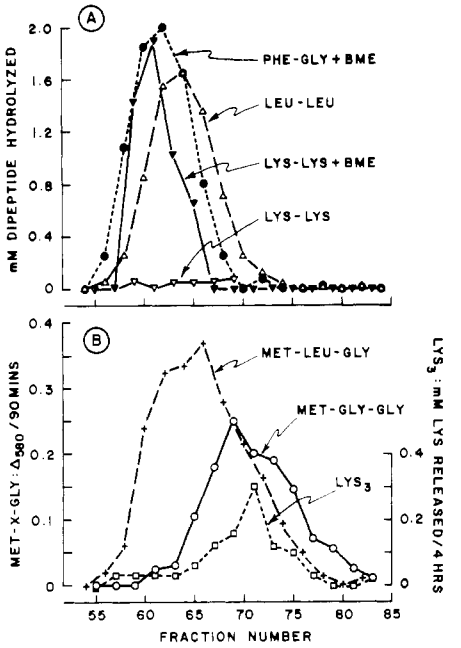

FIGURE 2: Elution patterns showing peptidase activity following chromatography of strain AJ005 soluble fraction as in Figure 1. Preliminary tests indicated the presence of dipeptidase activity in the effluent only at positions corresponding to regions I and II in Figure 1A. Subsequent dipeptidase assays (A), carried out 20 days after the column run, employed reaction times that were: for Leu-Leu (△), 1 hr; for Lys-Lys, 3 hr in the absence (▽) and in the presence (▼) of added BME; for Phe-Gly, 30 min in the presence of BME (●) (in the absence of BME, maximal activity, seen in fraction no. 62, amounted to 0.09 m*M* Phe-Gly hydrolyzed in 3 hr). In the tripeptidase assays (B), which were done 12 days later, activity toward methionyl peptides is expressed as the absorbancy increase (Δ_{580}) resulting from the additional ninhydrin-positive material produced by 0.1 ml of effluent fraction in the designated reaction time: 100% hydrolysis of 2 mM Met-Gly-Gly (○) to Met + Gly-Gly has a Δ_{580} value of 0.96 while complete cleavage to Met + 2Gly has a value of 1.61; the corresponding values for 2 mM Met-Leu-Gly (+) are 0.44 (Met + Leu-Gly) and 1.46 (Met + Leu + Gly). Trilysine hydrolysis (□) is expressed as the concentration of lysine produced.

$MnSO_4$ buffer prior to assay. Comparison of the data obtained in the assay with the data for a similarly diluted preparation of the strain K-12 soluble fraction (Table I) clearly shows strain AJ005 to be partially deficient in dilysine-hydrolyzing activity although its activity toward Phe-Gly closely resembles that of strain K-12. The comparable activity of the two strains toward Leu-Leu may not be used as evidence that the lysine oligopeptidase (i.e., the enzyme responsible for the higher activity of strain K-12 toward dilysine) is unable to attack Leu-Leu. As can be seen from Figure 1, only a very small portion of the total Leu-Leu-cleaving action of strain K-12 is localized in the column fractions carrying the oligopeptidase (region III) and its absence from strain AJ005 may not be detectable in tests on the soluble fraction per se. The validity of this conclusion became evident when a sample of the strain AJ005 soluble fraction was examined by the chromatographic procedures used with strain K-12.

The initial tests on the effluent fractions from the strain AJ005 column gave activity curves for Leu-Leu, Lys-Lys, and Phe-Gly that resemble the curves shown in Figure 1A only for regions I and II; no activity toward any dipeptide was evident at positions analogous to region III of the strain K-12 column effluent. In subsequent assays, the tests for dipeptide-hydrolyzing action were repeated (Figure 2A) and

Table I: Dipeptidase Activity of Strains K-12 and AJ005.[a]

Dipeptide Substrate	m*M* Peptide Hydrolyzed per Hour	
	Strain K-12	Strain AJ005
Leu-Leu	0.734	0.728
Lys-Lys	0.380	0.130
Phe-Gly	1.040	0.946

[a] Each freshly prepared soluble fraction, diluted 100-fold with the BME-containing buffer used for column chromatography, was assayed with 2 m*M* dipeptide as described under Materials and Methods. Enzyme activity is given as the effect produced by 1 μl of undiluted soluble fraction (approximately 3.5 μg of bacterial protein).

activity toward trilysine and methionyl tripeptides also was measured (Figure 2B). Again ability to hydrolyze Lys-Lys and Phe-Gly was limited to a single region of the column effluent (activity peak in fractions 60–62, Figure 2A) and required the presence of BME, observations confirming the presence in strain AJ005 of dipeptidase DP. Limitation of ability to hydrolyze Leu-Leu to the same region (activity peak in fractions 62–64) confirms not only the absence from strain AJ005 of both the dilysine- and dileucine-hydrolyzing activities associated with region III of the strain K-12 elution pattern (Figure 1) but also the fact that strain AJ005 contains aminopeptidase AP.

Although the activity curve for Lys-Lys-Lys (Figure 2B) overlaps the curves for the dipeptides, the position of peak activity toward trilysine (fraction 71) is clearly distinguishable from the peaks for the dipeptides and indicates the presence of a trilysine-specific tripeptidase. If the curves for Lys-Lys-Lys and Leu-Leu are assumed to delineate the fractions containing the tripeptidase and aminopeptidase AP, respectively, then the ability of both enzymes to hydrolyze methionyl tripeptides[3] is evident from the asymmetry of their activity curves. For example, the ascending limb of the curve for Met-Leu-Gly [an excellent substrate for the aminopeptidase AP (Simmonds, 1972)] is superimposable on that of the curve of Leu-Leu in the region of fractions 56–64, while in the region of fractions 71–80 the descending limb of the Met-Leu-Gly curve closely follows that of the trilysine curve, and the high activity of fractions 65–70 is readily explained by the presence of significant amounts of either or both aminopeptidase and tripeptidase. In the Met-Gly-Gly curve significant hydrolysis of this tripeptide (a poor substrate for aminopeptidase AP[1]) first becomes evident only in fraction 65, which is rich in aminopeptidase AP but contains little lysine tripeptidase activity; the curve rises steadily until fraction 69, which still contains an appreciable amount of the aminopeptidase together with a high level of the tripeptidase; and after the peak of activity toward trilysine has been reached the curve for Met-Gly-Gly falls in much the same manner as the curves for the other tripeptides. An estimate of the relative activity of the tripeptidase toward Met-Gly-Gly and Lys-Lys-Lys can be made from the data for fraction 71: the Δ_{580} value for the former is equivalent to the conversion of about 0.4 m*M*

[3] Hydrolysis of Met-X-Gly substrates occurs via the initial removal of the Met residue and only rarely is the new formed X-Gly subject to cleavage. As the Δ_{580} values for the initial step in the hydrolysis of Met-Gly-Gly and Met-Leu-Gly differ by a factor of approximately two (Figure 2, legend), a rough measure of the relative activity of a given fraction to these methionyl tripeptides can be obtained by doubling the Δ_{580} values for Met-Leu-Gly which are shown in Figures 2–4.

Met-Gly-Gly to Met + Gly-Gly in a reaction time of 1.5 hr whereas a reaction time of 4 hr permitted the hydrolysis of 0.3 m*M* trilysine to Lys + Lys-Lys. While fraction 71 probably hydrolyzed about 0.85 m*M* Met-Leu-Gly in 1.5 hr, some of the hydrolysis probably was catalyzed by the small amount of aminopeptidase AP that was present.

After completion of the tests described above, equal volumes of fractions 54–70 were combined and the resultant pool, containing all the fractions that showed activity toward Phe-Gly and Lys-Lys, was assayed to measure the relative specific activity of the strain AJ005 dipeptidase DP. The ratio of the specific activity values obtained (0.660 m*M* for dilysine and 4.20 m*M* for Phy-Gly) is almost identical with the ratio previously found for the strain K-12 enzyme.

Comparison of the elution patterns for strains K-12 and AJ005 suggested that the tripeptidase (as indicated by the trilysine curve in Figure 2) should emerge from a DEAE-Sephadex A-50 column at a position between those of dipeptidase DP and the oligopeptidase (as indicated by the dilysine curves in Figures 1 and 2). Consequently, demonstration of the presence in strain K-12 of both tri- and oligopeptidases should be possible, especially if the column effluent fractions are tested for activity toward lysine homopolymers under conditions in which dipeptidase DP is inactive. The resultant data then could be correlated with the results of tests for activity toward Met-Gly-Gly which now appeared to serve as a substrate for the tri- and oligopeptidases as well as for aminopeptidase AP and for aminopeptidase L (Simmonds, 1970, 1972). Such tests were carried out using fractions from a large-scale column to which was applied a sample of the strain K-12 soluble fraction used in the earlier work.

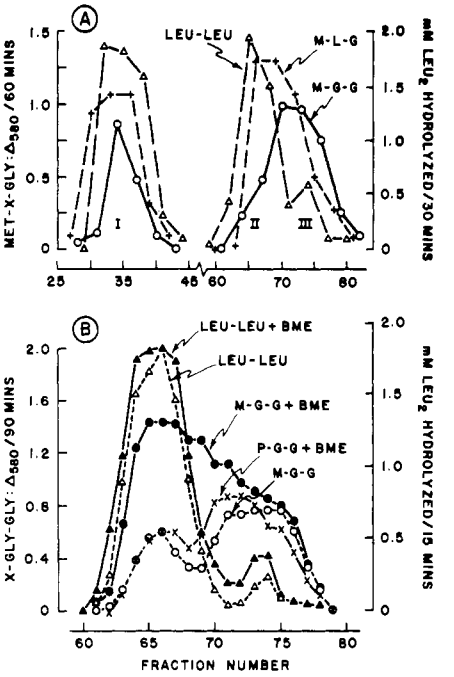

FIGURE 3: Elution patterns showing peptidase activity following chromatography of strain K-12 soluble fraction on a 4 × 50 column of DEAE-Sephadex A-50. Activity toward Leu-Leu in the absence (△) and in the presence (▲) of added BME, toward Met-Gly-Gly in the absence (○) and the presence (●) of BME, and toward Phe-Gly-Gly in the presence of BME (×) is expressed as in Figure 2 (Δ_{580} values of Phe-Gly-Gly are similar to those specified for Met-Gly-Gly). Tests in A were done on the day following the column run and those in B were done a week later. Reaction times were as indicated for each substrate on the appropriate ordinate.

The large column originally had been run as part of a study of strain K-12 methionine aminopeptidases,[1] and the first assays defined the distribution of hydrolytic activity toward methionyl tripeptides and toward Leu-Leu as shown in Figure 3A; activity toward Phe-Gly was found to be limited to region II, with the activity peak being present in fraction 64. Data from a second set of assays on the fractions spanning regions II and III suggested the presence of three enzymes active toward substrates in the X-Gly-Gly series (Figure 3B). This is most easily seen by inspection of the curves for "Phe-Gly-Gly + BME" and for Met-Gly-Gly tested in the absence of BME, both of which show two distinct areas of enzymic activity. One area in each curve is characterized by a relatively sharp maximum at about fraction 66, which also has maximal activity toward Leu-Leu and represents the activity peak for aminopeptidase AP. The other area in each X-Gly-Gly curve has a broad maximum which is first attained at fractions 71–72; these fractions may be assumed to contain tripeptidase TP because they display little Leu-Leu-hydrolyzing activity. The curves for Leu-Leu hydrolysis show a rise to a second activity peak in fractions 73–74 and these fractions appear to contain an "oligopeptidase". Although not illustrated in Figure 3B, the activity curve for Phe-Gly-Gly hydrolysis in the absence of BME also had a low maximum at fraction 66 (the absorbancy value being about one-third that indicated for Phe-Gly-Gly + BME curve) and, after fraction 68, the curve rose sharply so that for fractions 69–79 the curve was almost identical with the curve shown for Met-Gly-Gly cleavage in the absence of BME.

While the shape of the "Met-Gly-Gly + BME" curve in Figure 3B differs markedly from the others, it too suggests the presence of three enzymes. Whereas the ascending limb rises in parallel with the Leu-Leu curves, the descending limb differs from those of the dipeptide curves, and includes an area of high activity coincident with the tripeptidase TP maximum before it becomes identical with the other tripeptide curves in the region of the "oligopeptidase".

At the activity peak for aminopeptidase AP (fraction 66), the absorbancy value for Met-Gly-Gly hydrolysis in the presence of BME (Δ_{580} of ca. 1.50) is much greater than the value characterizing 100% cleavage to Met + Gly-Gly and indicates that some of the Gly-Gly also is hydrolyzed. This is due largely to BME activation of dipeptidase DP which has a low, but significant, activity toward Gly-Gly. For example, fraction 66 showed a specific activity toward Gly-Gly of 0.02 m*M* in the absence of BME and 0.90 m*M* in the presence of the mercaptan; in the same assay, the corresponding specific activity values for Phe-Gly were 1.0 and 12.0 m*M*, respectively.

The use of assay conditions (absence of BME) that limit the extent of hydrolysis of Met-Gly-Gly and, especially, Phe-Gly-Gly by column fractions containing dipeptidase DP–aminopeptidase AP mixtures thus made it possible to demonstrate the presence in strain K-12 of tripeptidase TP and of an oligopeptidase, both of which have relatively high activity toward X-Gly-Gly substrates. From the work with strain AJ005 (Figure 2), it became apparent that tripeptidase TP must be identical with the trilysine tripeptidase and that the oligopeptidase activity toward Leu-Leu and X-Gly-Gly substrates could be a function of the strain AS013 lysine oligopeptidase. Confirmation of these conclusions was sought by further tests on the fractions from the large column.

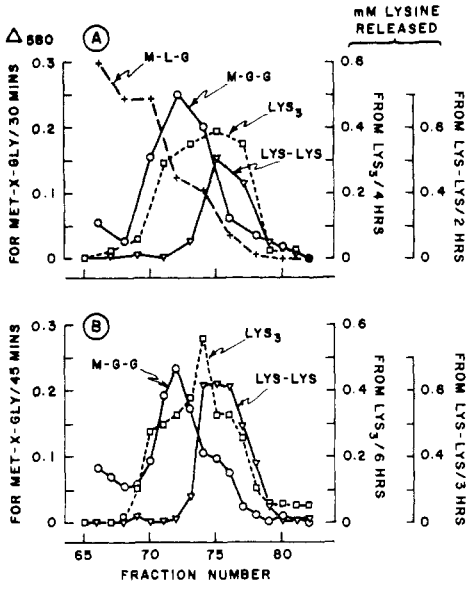

FIGURE 4: Further peptidase-activity elution patterns for the strain K-12 column described in Figure 3. After storage of the column effluent fractions for 3 months, activity toward Lys-Lys (▽), Lys-Lys-Lys (□), Met-Gly-Gly (○), and Met-Ley-Gly (+) was measured in the usual EDTA-containing assay system (A) and in assay system T-M (B).

In these tests (Figure 4), BME was omitted from the assay mixtures in order to prevent dipeptide cleavage by any dipeptidase DP present in the fractions and to keep the apparent activity of aminopeptidase AP at a minimal level. Met-Leu-Gly was included among the substrates tested in order to verify the elution position of aminopeptidase AP (cf. Figures 2 and 3) as well as to observe its hydrolysis by the fractions acting on the lysine peptides. As expected, the curves in Figure 4 describing the hydrolysis of di- and trilysines show that the trilysine-sepcific tripeptidase (fractions 69–72) emerges from the DEAE-Sephadex column just prior to the lysine oligopeptidase (fractions 73–79), and the amount of lysine released from the tripeptide is especially high where fractions contain both enzymes (fractions 73–75). The identity of trilysine tripeptidase and tripeptidase TP is attested by the shapes of the curves for Met-Leu-Gly and Met-Gly-Gly. Moreover, the Met-Gly-Gly curve has its peak at fraction 72 (Figure 4B) coincident with the highest point in the trilysine curve for the effluent region carrying fractions that lack activity toward dilysine. Again as expected, the methionyl tripeptides also are hydrolyzed by fractions containing lysine oligopeptidase activity. The latter type of activity toward Lys-Lys is maximal in fractions 74–76 (Figure 4B), and this portion of the Met-Gly-Gly curve has a shoulder not unlike the shoulder in the trilysine curve. In addition, the shapes of the Met-Gly-Gly curves in Figure 4 indicate that the storage of fractions for about 3 months between the assays illustrated in Figures 3B and 4, respectively, led to a selective loss of oligopeptidase activity relative to tripeptidase activity.

Use of EDTA-free assay system T-M (Figure 4B) was prompted by the report of Sussman and Gilvarg (1970) that tests in phosphate buffer devoid of added metal ions (Co^{2+}) showed the trilysine tripeptidase to be EDTA-sensitive and the oligopeptidase to be metal independent. In preliminary tests with strain AJ005 tripeptidase-containing fractions (from the column of Figure 2), the data obtained indicated that omission of EDTA from the Tris-Mn^{2+} buffer causes a small decrease in activity toward trilysine. This effect again is evident in the work on strain K-12 where tests were made of activity toward Met-Gly-Gly as well as toward trilysine. Thus, with column fractions believed to contain tripeptidase uncontaminated by either the oligopeptidase or aminopeptidase AP, the amounts of Met-Gly-Gly hydrolyzed per hour by fraction 72 are equivalent to 1.0 m*M* in the presence of EDTA (Figure 4A) and 0.65 m*M* in system T-M (Figure 4B), and the amounts of trilysine hydrolyzed per hour by fraction 71 are equivalent to 0.073 m*M* in the presence of EDTA and 0.051 m*M* in system T-M. Under the conditions used here, the tripeptidase appears to be inhibited either by the relatively high level of Mn^{2+} of assay system T-M or by a metal-ion contaminant whose effect can be reversed by addition of EDTA but not by raising the Mn^{2+} concentration from 0.13 to 0.35 m*M*. Based on its ability to hydrolyze Lys-Lys, the activity of the oligopeptidase is not altered by changes in the EDTA and Mn^{2+} content of the assay system, e.g., fraction 75 effected the hydrolysis of 0.15 m*M* dilysine per hour in the presence of EDTA (Figure 4A) and 0.14 m*M* in its absence (Figure 4B).

The oligopeptidase and the tripeptidase are the only enzymes displaying significant activity toward trilysine. In contrast to the methionyl tripeptides, trilysine is not attacked by aminopeptidase AP (Figures 2 and 4). Nor does it serve as a substrate for aminopeptidase L which is known to act on a variety of tripeptides including Met-Gly-Gly and Met-Leu-Gly (Simmonds, 1970, 1972; see also Figure 3A, region I). For example, fraction 35 of the strain K-12 column illustrated in Figure 3A released less than 0.03 m*M* lysine from trilysine in a 3-hr reaction time whereas in the same assay, 0.35 m*M* Met-Gly-Gly and 1.6 m*M* Leu-Leu were hydrolyzed in 1 hr. Consequently, the absence of the oligopeptidase from strain AJ005 places a restriction on the ability of that lysine auxotroph to grow on trilysine which is evident in the growth tests reported by Sussman and Gilvarg (1970) and in those described below.

The tests described by Sussman and Gilvarg involved precedures differing in several respects from those routinely used in this laboratory. The minimal medium S used here is poorly buffered (due to its low phosphate concentration), contains a relatively high NH_4^+ concentration, with nitrogen also available in the form of nitrate ions and asparagine, and has glycerol (rather than glucose) as the carbon source. In their tests, Sussman and Gilvarg used inocula sufficiently large to permit visible growth within 4–6 hr, whereas the size of the inocula for medium S is chosen so that growth becomes visible only after an overnight incubation period and may readily be followed until the end of the growth cycle.

The growth experiments summarized in Table II included simultaneous tests on the lysine auxotrophs AS013 and AJ005 and on strain 679-680 which resembles the other strains in requiring for growth both leucine and threonine. As may be seen in the table, strains 679-680 and AS013 respond to media containing free or peptide-bound lysine in a similar manner, thereby providing further evidence that *E. coli* growth is normally unaffected by the presence of trilysine. In contrast, the growth of strain AJ005 in the presence of trilysine is markedly inhibited, and shows an increase in the $T_{0.5}$ value (mainly a reflection of the lag time of the growth cycle) of about 12 hr when trilysine is compared to lysine and 19 hr when the tripeptide is compared to dilysine. While the growth curves given in the paper of Sussman and

Table II: Growth Response of Auxotrophs to Lysine and Lysine Peptides.[a]

Lysine Source Added		$T_{0.5}$ (hr)		
Compound	Concn (m*M*)	679-680	AS013	AJ005
None		16	*b*	*b*
Lysine	0.1	21.5	20.5	47
	0.3	22.5	20.5	53
Lys-Lys	0.1	23.5	21.5	43
Lys-Lys-Lys	0.1	20.5	21.5	62[c]

[a] The time required for a culture to attain half-maximal growth, $T_{0.5}$, was estimated as described under Materials and Methods. All cultures grew to the same final absorbancy value of 0.160. [b] No growth was observed during the 72-hr incubation. [c] When tested for loss of lysine requirement by transfer of 0.1 ml of the 72-hr culture to medium devoid of any lysine source, no growth was observed after a 72-hr incubation period.

Gilvarg (1970) indicated that strain AJ005 failed to grow on trilysine, their trilysine-containing culture apparently was followed for no more than 5 or 6 hr after initiation of visible growth on lysine and on dilysine.

In this connection, it may be added that growth of strain AJ005 in trilysine-containing cultures has been observed in tests carried out at intervals over a 2-year period during which the strain was subjected to repeated serial transfers on yeast extract–peptone–agar slants. Retesting of the culture at the end of 2 years by the procedures described for Figure 2 has shown that the mutation causing loss of oligopeptidase activity is a stable one.

The data in Table II also suggest that growth of strain AJ005 on lysine and on dilysine is characterized by lag times that are unusually long in comparison to the lag times seen with the other two strains. Such a difference between strains AJ005 and AS013 may be inferred from the growth curves given by Sussman and Gilvarg, i.e., the lag time for AJ005 appears to have been about 4 or 5 hr and that for AS013 no more than 2 hr. The slow initiation of growth in cultures of AJ005 as compared to other auxotrophs of strain K-12 has been noted also on yeast extract–peptone–agar slants and in the phosphate–peptone–glycerol-containing medium used to prepare bacteria for peptidase studies.

Discussion

The work of Sussman and Gilvarg (1970) led to the recognition of four enzymes in extracts of the lysine auxotroph AS013 which catalyze the hydrolysis of lysine homopolymers. While one enzyme was active toward all the homopolymers tested, each of the others displayed strict substrate specificity acting respectively only on di-, tri-, or tetralysine. The experiments reported here provide evidence that extracts of the parent wild-type strain K-12 also contain the oligopeptidase as well as the dilysine-specific and trilysine-specific enzymes, and confirm the fact that strain AJ005 is a peptidase-deficient mutant lacking oligopeptidase activity toward di- and trilysines (Table III).

The present studies, like those of Sussman and Gilvarg, involved the use of ion-exchange chromatography to separate the complex mixture of peptidases present in cell-free extracts. However, the extracts examined by Sussman and Gilvarg were subjected to ammonium sulfate fractionation prior to chromatography. Because this treatment removed

Table III: Enzymes Shown to be Present in Strains AS013 and K-12.

Strain AS013 Enzyme	Strain K-12 Enzyme	Known Substrates[a]
Lys_2-specific enzyme (Co^{2+}-dependent)	Dipeptidase DP (BME- and Mn^{2+}-dependent)	A-Gly, Gly-Gly, A-Leu, Lys-Lys, and other dipeptides[b]
Lys_3-specific enzyme (EDTA-sensitive)	Tripeptidase TP (may be metal dependent)	B-Gly-Gly, Phe-Gly-Gly, B-Gly-B, B-Leu-Gly, Lys-Lys-Lys; inactive toward dipeptides[c]
Oligopeptidase active toward Lys_2, Lys_3, and Lys_4	Oligopeptidase (shows aminopeptidase activity)	Leu-Leu, Lys-Lys, (Phe-Gly); Met-Gly-Gly, Phe-Gly-Gly, Lys-Lys-Lys, Met-Leu-Gly
	Aminopeptidase L	A-Gly, B-Leu; A-Gly-Gly, B-Leu-B, Met-Ala-Ser; other peptides[c,d]
	Aminopeptidase AP	(B-Gly), B-Leu; (B-Gly-Gly, Phe-Gly-Gly, B-Gly-B), B-Leu-Gly[d]

[a] In substrates, A is Ala, Leu, Met, or Phe, and B is Leu or Met. Very poor substrates are listed in parentheses. Strain AS013 enzymes are those described by Sussman and Gilvarg (1970); the strain also contains an endopeptidase cleaving tetralysine to 2 equiv of dilysine. Data for strain K-12 are limited to enzymes in the soluble fraction and, unless otherwise noted, are taken from this paper. Strain K-12 was not examined for the presence of the endopeptidase. [b] Highest activity seen with Met-Ala and Met-Met; low activity seen with Lys-Lys and Leu-Gly; almost no activity seen with Leu-Leu (Simmonds, 1972). The dipeptidase probably is the strain K-12 counterpart of the strain B Mn^{2+}- and sulfhydryl-activated dipeptidase M (Brown, 1973) rather than of the Zn-dipeptidase (Hayman et al., 1974). [c] Data taken in part from S. Simmonds et al. (manuscript in preparation). [d] Data taken in part from Simmonds (1972). Neither aminopeptidase shows activity toward di- or trilysine.

at least 75% of the metal-dependent activity toward dilysine, the subsequent analyses may have failed to detect all the dilysine-cleaving enzymes present in the crude extracts. As described in Material and Methods, we examined the soluble fraction made from cell extracts that had been prepared and freed of ribosomes by methods effecting the removal of "ribosome-bound" peptidase activity. Although the ability of the ribosomal fraction of strain K-12 to hydrolyze dilysine has not been measured, the activity toward a variety of dipeptides is known to represent a very small portion of the activity seen with the unfractionated extract, ranging from 13% in the case of Leu-Leu to less than 1% for Phe-Gly (Simmonds, 1970). Hence, the observation that the soluble fraction of strain K-12 contains only two enzymes active toward dilysine (Figure 1), one of which is lacking in strain AJ005 (Figure 2), indicates that the dilysine-specific peptidase and the lysine oligopeptidase probably are the only enzymes in strains K-12 and AS013 having significant activity toward dilysine. The oligopeptidase together with the trilysine-specific enzyme may be assumed to be responsible for most, if not all, of the trilysine-cleaving activity of strain AS013 since the material applied to chromatography columns contained essentially all the activity present in the crude extract prior to ammonium sulfate treatment (Sussman and Gilvarg, 1970). A similar conclu-

sion may be drawn from the work on strain K-12 in view of the fact that aminopeptidases L and AP (Simmonds, 1970, 1972) have been found to lack activity toward trilysine (Figures 2 and 4). While the lysine oligopeptidase activity first observed in strain AS013 must be ascribed to an enzyme not previously detected in the soluble fraction of strain K-12, the lysine tripeptidase and dipeptidase activities now are seen to be functions of strain K-12 tripeptidase TP and dipeptidase DP, respectively.

At the beginning of the work described here, it already was known that partially purified preparations of dipeptidase DP contain a BME- and metal-dependent enzyme active toward a wide variety of dipeptides, including Lys-Lys and Lys-Gly, in the presence of Tris buffer with Mn^{2+} (0.13 m*M*) as the metal-ion activator (Simmonds, 1972). In tests with Met-Leu and Met-Gly which, like Phe-Gly, are excellent substrates for the dipeptidase, very little activity was seen when Co^{2+} was used in place of Mn^{2+}, and enzyme activity was somewhat inhibited by the addition of 1 m*M* Co^{2+} to an assay mixture containing 0.13 m*M* Mn^{2+}. The observation of Sussman and Gilvarg (1970) that 1 m*M* Co^{2+} is an excellent activator of dipeptidase activity toward dilysine as measured, in a phosphate buffer, with either the crude extracts or with chromatography column effluent fractions was therefore unexpected. In the present studies, the use of a Tris–Mn^{2+} buffer in column chromatography precluded the testing of effluent fractions in the phosphate–Co^{2+} assay system of Sussman and Gilvarg. That system could be used with the soluble fraction per se, and when 1 μl of the strain AJ005 soluble fraction was tested, the resultant hydrolysis of Lys-Lys was characterized by a specific activity value about twice the value (noted in Table I) obtained in the usual Tris–Mn^{2+} system. Thus, the relative activity of dipeptidase DP to different peptides may depend upon the composition of the assay mixture. In this connection, it is noteworthy that a similar phenomenon obtains with the Zn-containing dipeptidase isolated from *E. coli* B by Hayman et al. (1974; see also, Patterson et al., 1975). Lys-Gly is a poor substrate for the Zn-dipeptidase per se, and the addition of Co^{2+} markedly increased the rate of hydrolysis without altering the K_m for the dipeptide; activation also resulted upon addition of Mn^{2+}, but with this metal ion the K_m value also increased. However, the addition of either metal ion inhibited the hydrolysis of substrates that are rapidly cleaved in their absence, e.g., Met-Gly.

In their study of strain AS013, Sussman and Gilvarg (1970) were obliged to use gel filtration on Sephadex G-200 in order to separate the trilysine-specific tripeptidase from the lysine oligopeptidase because the two enzymes were eluted at the same position during development of the DEAE-cellulose column with an exponential KCl gradient. In the work with strain K-12, partial separation of the tri- and oligopeptidases was obtained by development of the DEAE-Sephadex A-50 column with a linear KCl gradient (Figure 4), and complete separation should be possible with a very shallow salt gradient, a procedure that permits complete separation of dipeptidase DP from aminopeptidase AP upon rechromatography of mixtures containing those enzymes (Simmonds, 1972).

The availability of peptidase-mutant AJ005 facilitated study of the trilysine tripeptidase which now has been found also to hydrolyze Met-Gly-Gly and Met-Leu-Gly and to do so at rates significantly faster that it hydrolyzes trilysine. The presence of Met-Gly-Gly-cleaving activity in column effluent fractions that are devoid of activity toward Leu-Leu (Figure 2) is characteristic of strain K-12 tripeptidase TP. Previous experiments had shown tripeptidase TP to be responsible for most of the activity toward substrates in the X-Gly-Y series which is present in crude preparations of aminopeptidase AP (Simmonds, 1972), and gel filtration of such crude preparations on Sephadex G-150 gave two enzymes: aminopeptidase AP and a smaller enzyme, tripeptidase TP, that hydrolyzes Leu-Gly-Leu, Met-Gly-Met, and Leu-, Met-, and Phe-Gly-Gly but lacks demonstrable activity toward dipeptides such as Leu-Leu and Ala-, Gly-, and Phe-Gly.[1] Preliminary estimates placed the particle weight of the strain K-12 tripeptidase in the range of 10000 to 150000, in fair agreement with the estimated weight of the strain AS013 trilysine-specific enzyme (Sussman and Gilvarg, 1970). Because methionine is the only free amino acid detected among the hydrolysis products of Met-Leu-Gly and Met-Gly-Gly, tripeptidase TP has been classified as an aminopeptidase, and the enzyme may be assumed also to attack trilysine from the amino-terminal end. Whether the tripeptidase is a metalloenzyme is still uncertain: dependence of activity on the presence of a metal ion is indicated by the EDTA sensitivity observed by Sussman and Gilvarg (1970) and our results also suggested a need for an appropriate metal-ion activator.

Although little information is currently available about the properties of the oligopeptidase, the enzyme appears capable of hydrolyzing both Lys-Lys and Leu-Leu as well as trilysine, Met-Leu-Gly, and Met- and Phe-Gly-Gly. Preliminary data indicate that it also has some activity toward Phe-Gly but little toward Gly-Gly, and that, like tripeptidase TP, the oligopeptidase acts as an aminopeptidase. Because it is the only known peptidase whose presence is not required for *E. coli* growth, a complete characterization of the oligopeptidase and, in particular, a detailed study of its substrate specificity will be necessary in order fully to elucidate its role in metabolism.

It is becoming increasingly clear that the soluble fraction prepared by centrifugation of cell-free extracts of *E. coli* K-12 contains a number of enzymes catalyzing the hydrolysis of small peptides. The residual ribosomal fraction contains other peptidases including aminopeptidase I, described by Vogt (1970), and at least one enzyme with activity toward dipeptides not readily attacked by aminopeptidase I (Simmonds, 1970). The ribosome-bound enzymes are active toward many of the peptides that also are substrates for soluble enzymes, and the specific activity of the ribosomal fraction per se to a given peptide may approach that of the soluble fraction (Simmonds, 1970, 1972). However, for all peptides studied the potential contribution of the ribosomal fraction to the total activity of the crude cell extract is far less than that of the soluble fraction. Most of the tripeptides studied to date can be hydrolyzed by enzymes present in each subcellular fraction. Even Phe-Gly-Gly, which initially appeared to be resistant to hydrolysis by strain K-12 soluble and ribosomal fractions (Simmonds, 1970), is cleaved not only by enzymes TP and AP and the oligopeptidase (Figure 3B) but also, albeit very slowly, by aminopeptidase I (Vogt, 1970) and aminopeptidase L (Simmonds, 1972). Trilysine is an exception: the initial hydrolytic attack on this tripeptide apparently is limited to the action of the soluble tri- and oligopeptidases. As noted above, aminopeptidase L preparations lack activity toward trilysine although, like the tri- and oligopeptidases, they show relatively high activity toward Met-Gly-Gly (Figure 3A). Nor is it probable that

trilysine is readily hydrolyzed by aminopeptidase I: while this enzyme rapidly hydrolyzed Met-Gly-Gly, it displays the same very low activity toward Lys-Gly-Gly as it does toward Phe-Gly-Gly (Vogt, 1970). The potential importance of the soluble metal-dependent dipeptidase in the final stage of polypeptide degradation is illustrated by the data of Sussman and Gilvarg (1970) describing the hydrolysis of lysine homopolymers by the crude extract of strain AJ005: release of lysine from the tetra- and trilysines, as well as from dilysine, was greatly stimulated by the addition of Co^{2+} to the extract. A similar though less marked Co^{2+} activation was observed with the strain AS013 extract.

As has now been found for dipeptidase DP from both strains AJ005 and K-12, the ability of the enzyme to hydrolyze Lys-Lys is relatively poor compared to its action on several other dipeptides such as Phe-Gly. Nevertheless, the ability of the lysine-requiring AJ005 to grow as readily on dilysine as a lysine (Sussman and Gilvarg, 1970, and Table II) indicates that dipeptidase DP activity in vivo is sufficient to satisfy the needs of growing cultures for the free amino acid. In strains other than AJ005 any failure of dipeptidase-catalyzed hydrolysis of Lys-Lys could be compensated by the action of the oligopeptidase. Indeed, evidence that the oligopeptidase can account for a major portion of dilysine hydrolysis effected by cell extracts under conditions in which dipeptidase DP is not fully active is provided by the data comparing the relative extent of dilysine cleavage by the soluble fractions of strains AJ005 and K-12 (Table I) and also by the relative amounts of dilysine-cleaving activity present in the oligo- and dipeptidase regions of the strain K-12 column effluent (Figure 1A). A not dissimilar situation exists in the case of Leu-Leu which is hydrolyzed by the dipeptidase even more slowly than is Lys-Lys (Simmonds, 1972). For Leu-Leu, however, failure of dipeptidase-catalyzed hydrolysis would be little consequence even in strain AJ005 since this compound is very rapidly hydrolyzed by aminopeptidases L and AP (Figures 1–3, and Simmonds, 1972). In contrast to dileucine and dilysine, Phe-Gly is readily attacked only by dipeptidase DP[4] (Simmonds, 1972, and Figure 1), although other equally good substrates of the dipeptidase are also good substrates for aminopeptidases, e.g., Met-Leu, which is very rapidly hydrolyzed by both aminopeptidase L and AP, and Met-Gly, which is hydrolyzed fairly rapidly by aminopeptidase L but, like other X-Gly compounds, is a poor substrate for aminopeptidase AP (Simmonds, 1972). Consequently, no correlation may be drawn between the rates at which dipeptides are hydrolyzed by dipeptidase DP and their susceptibility to cleavage by other peptidases.

The fact that every peptide studied can serve as a substrate for two or more enzymes should enable *E. coli* K-12 to cope with most metabolic problems arising from the specific inactivation of a given peptidase or from a mutation-induced loss of the ability to synthesize a peptidase. The only known example of the latter situation is provided by strain AJ005, but in this case the inability to synthesize the oligopeptidase results in such a profound disturbance in peptide metabolism that the mutant is unable to grow readily on trilysine. That the trilysine growth inhibition occurs even in the presence of free lysine clearly points to the requirement for both oligo- and tripeptidase activities in order to prevent intracellular accumulation of trilysine to a toxic level (Sussman and Gilvarg, 1970). It also is probable that the oligopeptidase, as well as the dipeptidase, can play an important role in tetralysine metabolism since the data of Sussman and Gilvarg (1970) indicate that Co^{2+}-supplemented extracts of strain AJ005 are partially deficient in ability to release lysine from the tetrapeptide, a process effected in this mutant only via the endopeptidase-catalyzed hydrolysis of tetralysine to yield two molecules of dilysine which then are hydrolyzed by dipeptidase DP. Several examples of a similar cooperative action involving aminopeptidases and the dipeptidase have been observed with tripeptides in the Met-X-Y and Leu-X-Y series (Simmonds, 1970, 1972), and further study of the substrate specificity of the endopeptidase undoubtedly will reveal other polypeptides whose complete degradation depends on the sequential action of the endopeptidase and dipeptide-cleaving enzymes. While the ability of strain AJ005 to grow equally well on dilysine and on lysine established the nossential nature of the oligopeptidase, the absence of the enzyme may cause some changes in the rates of reactions associated with the initiation of rapid culture growth, i.e., the growth cycles of strain AJ005 in media devoid of trilysine appear to include unusually long lag periods. Consideration therefore must be given to the possibility that some peptidases may be important catalysts for metabolic reactions specifically associated with the transition of a bacterial culture from the stationary to the exponential phase.

The extensive prolongation of the lag phase observed with cultures of strain AJ005 provided with trilysine as the sole exogenous source of lysine (Table II) is reminiscent of the bacteriostatic effect of peptides containing one or more residues of L-leucine (Fruton and Simmonds, 1950). Although the growth inhibition caused by both types of peptides may be relieved by hydrolysis of the inhibitory compounds, the toxicity of leucine peptides is not correlated with a loss of peptidase activity (Meisler and Simmonds, 1963; Simmonds, 1966). Rather, as was recently shown by Umbarger and his collaborators (Vonder Haar and Umbarger, 1972; Wasmuth and Umbarger, 1974), a peptide such as Gly-Leu is a heterotropic negative effector of the threonine deaminase catalyzing the initial step in isoleucine biosynthesis; under certain conditions Gly-Leu also can block threonine deaminase production. These findings provide an impressive illustration of the close relationship possible between enzymes directly involved in peptide degradation and those concerned in other aspects of metabolism in *E. coli* K-12.

From the currently available data, it is clear that the study of peptidase mutants can provide information about the physiological functions of peptidases in *E. coli*. The difficulty encountered in attempts to isolate such mutants is evident from the fact that, in addition to the strain K-12 mutant AJ005, only one other has been reported. The later, strain W864, was derived from a glycine auxotroph of *E. coli* W by Kessel and Lubin (1963) and lacked the ability to grow on Gly-Gly. A crude extract of the mutant was almost completely devoid of hydrolytic activity toward Gly-Gly when tested in a phosphate buffer containing Co^{2+}, although it showed activity toward Leu-Gly and Leu-Leu resembling that of an extract made from the parent strain. Little information has been published about strain W peptidases except for the brief report that strain W resembles the strain K-12 mutant AS013 in containing a lysine oligopeptidase, a trilysine tripeptidase, and a Co^{2+}-dependent dilysine dipeptidase (Sussman and Gilvarg, 1970). If strain

[4] Phe-Gly can be hydrolyzed by ribosomal-bound enzymes as well as by dipeptidase DP (Simmonds, 1970).

W also contains enzymes similar to the strain K-12 aminopeptidases L and AP, the "Gly-Gly peptidase mutant" would appear to lack dipeptidase DP since the aminopeptidases account for most of the activity of strain K-12 extracts toward Leu-X dipeptides (Simmonds, 1972) whereas dipeptidase DP appears to be responsible for most of the activity toward Gly-Gly.[1] However, another interpretation of the data for strain W864 is possible, based on information concerning the peptidases present in *E. coli* B. According to Payne (1972a), crude extracts of strain B appear to contain two Gly-Gly cleaving enzymes: one which is activated by Mn^{2+} and the other which is activated by Co^{2+} and is responsible for the vast bulk of extract activity toward Gly-Gly. The Mn^{2+}-dependent activity of the crude extract may be ascribed to the action of "dipeptidase M", isolated from strain B by Brown (1973), which is similar to the strain K-12 peptidase DP in its activation by BME, requirement for Mn^{2+}, and relatively low level of activity toward Gly-Gly. The Co^{2+}-dependent activity of the extract probably resides in the Zn-dipeptidase whose activity toward Gly-Gly is stimulated by the addition of Co^{2+} (Hayman et al., 1974, Patterson et al., 1975). Thus, if strain W resembles strain B, the Co^{2+}-containing assay system used to compare extracts of strain W864 and its parent may have failed to detect the presence of dipeptidase M. Consequently, the inability of the mutant to grow on Gly-Gly and the inability of the extract to hydrolyze Gly-Gly could be explained by the absence of Zn-dipeptidase. No strain K-12 counterpart of the strain B Zn-enzyme has as yet been observed.

The difficulty in isolating peptidase-deficient mutants probably results from the use of inadequate screening methods for their detection. Detection of a "dipeptidase mutant" by plating onto media containing dipeptides following mutagenic treatment of amino acid auxotrophs, as attempted by Payne (1972b), would be complicated by the presence in cells of so many enzymes capable of hydrolyzing dipeptides. In this connection, mention may be made of the unsuccessful attempts in this laboratory by Mr. F. Taylor to convert the oligopeptidase-deficient strain AJ005 into a mutant incapable of growth on dilysine. Because such a "double peptidase mutant" would lack dipeptidase DP as well as the oligopeptidase, it is possible that loss of the ability to produce dipeptidase DP represents a lethal mutation in strain K-12. Peptidase-mutant isolation involving tests for the growth response of an amino acid auxotroph to a tripeptide as was done by Sussman and Gilvarg (1970) was successful mainly because trilysine proved to be toxic to strain AJ005. However, the use of tripeptides to screen for peptidase mutants may be a generally applicable procedure if suitable care is taken in the selection of the amino acid auxotroph to be mutagenized and the tripeptide chosen for the screening test. For example, it should be possible to isolate a "Phe-Gly-Gly peptidase mutant" by first converting strain AJ005 to a phenylalanine or glycine auxotroph capable of growth on Phe-Gly-Gly and by testing for subsequently induced peptidase mutants with a screening procedure that permits differentiation between rapid and slow growth on the tripeptide as compared to growth on the free amino acids. Indeed, the introduction into strain AJ005 of a phenylalanine or glycine requirement might be unnecessary in view of the fact that each of these amino acids, and also Phe-Gly and Gly-Gly, can serve as the sole source of nitrogen for the growth of strain K-12 (S. Simmonds, unpublished experiments). Thus, detection of mutants deficient in the ability to hydrolyze Phe-Gly-Gly might be accomplished by replicate plating onto media containing as the nitrogen source either the tripeptide or an appropriate mixture of phenylalanine plus glycine or Gly-Gly. The choice of Phy-Gly-Gly, rather than the corresponding methionyl or leucyl tripeptide, is dictated by the knowledge that it is almost resistant to attack by the soluble and ribosomal fractions of strain K-12 and that it is hydrolyzed relatively slowly by the relevant strain AJ005 enzymes, i.e., tripeptidase TP, aminopeptidase L, and aminopeptidase AP. As more information about the number of peptidases and, especially, about the substrate specificity of each peptidase become available, it should become easier to devise better detection methods which will permit the isolation of mutants useful in the study of the role of peptidases in bacterial metabolism.

The recent work on *Salmonella typhimurium* clearly illustrates how such information can be used for the isolation and genetic mapping of peptidase-deficient mutants (Miller and MacKinnon, 1974; Miller, 1975). In their study, Miller and McKinnon (1974) subjected a crude extract of a leucine auxotroph to polyacrylamide gel electrophoresis and tested the gel for the presence of activity toward Leu-Gly. Four bands showing enzymic activity were identified, i.e., peptidases A, B, D, and N; a further test with Leu-Gly-Gly as substrate indicated that all but one band in the gel (peptidase D) also contained activity toward tripeptides. The test with Leu-Gly-Gly revealed two additional bands of activity (no. 2 and 4), one of which (no. 4) appeared completely devoid of activity toward Leu-Gly. Of the six enzymes so identified, three (peptidases A, B, and N) hydrolyzed Leu-Ala-NH_2, and only one (peptidase N) also was active toward alanyl-β-naphthylamine. Based on these data, it was possible to select for a mutant incapable of hydrolyzing the naphthylamine (shown to lack peptidase N) from which were isolated double, triple, and quadruple mutants of genotypes *pepN*$^-$*pepA*$^-$ (unable to grow on Leu-Ala-NH_2 in place of leucine), *pepN*$^-$*pepA*$^-$*pepD*$^-$ (unable to grow on the dipeptide amine or on Leu-Gly), and *pepN*$^-$*pepA*$^-$*pepD*$^-$*pepB*$^-$ (unable to grow on the amide, on Leu-Gly, or on Leu-Leu). Because the material responsible for peptidase A activity was heat stable, Miller and MacKinnon suggested it may be similar to the heat-stable aminopeptidase I of *E. coli* strain K-12 (Vogt, 1970). From the brief description that is given of the behavior of the *S. typhimurium* extract during ion-exchange chromatography, peptidase A appears to be eluted from the chromatography column by a buffer of low ionic strength, another similarity to aminopeptidase I (Simmonds, 1972) and to the corresponding strain B ribosomal-bound aminopeptidase (Matheson and Murayama, 1966). Similarities between other *Salmonella* and *E. coli* peptidases become evident on comparison of their behavior during gel electrophoresis. Thus, the banding positions of the *Salmonella* "dipeptidase" (enzyme D), "tripeptidase" (band no. 4), and peptidases B and N roughly correspond to the positions of the strain K-12 dipeptidase DP, tripeptidase TP, aminopeptidase AP, and oligopeptidase, respectively [as observed on polyacrylamide gel electrophoresis of the enzyme mixture comprising regions II and III of Figures 1A and 3A (C. Hermsdorf, unpublished data)]. It may also be noted that band no. 2 from the *Salmonella* extract displays high activity toward Leu-Gly-Gly and Met-Ala-Ser but is only weakly active toward Leu-Gly, properties characteristic of the strain K-12 aminopeptidase L.[1] These similarities between the peptidases of *Salmonella typhimurium* and *E. coli* merit further investigation and especially with respect to the ability of the for-

mer to hydrolyze homopolymers of lysine and of ornithine, in view of the report that a *S. typhimurium* strain is naturally inhibited not only by triornithine but also by trilysine (Sussman and Gilvarg, 1970).

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Replacement of Metal in Metalloenzymes. A Lead–Alkaline Phosphatase†

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ABSTRACT: Lead ions can interact with calf intestine alkaline phosphatase. Experiments using ^{203}Pb-labeled Pb^{2+} ions showed that Pb^{2+} ions bind the native protein in a molar ratio of Pb/protein of 1:5 with moderate inhibition of its biochemical activity. The 4 g-atoms of Zn per mol present in the native enzyme may be removed by dialysis against EDTA. The inactive apoenzyme is capable of incorporating Pb^{2+} ions in a Pb/protein molar ratio of 2:1, giving a lead–protein complex still enzymatically active also when genetic material, such as nucleotides or DNA, has been used as a substrate. The reconstituted lead–protein is capable of binding Zn^{2+} ions without any release of the Pb^{2+} ions and with an increase in the catalytic activity of only 10–15%. The absence of Zn in the inactive apoenzyme as well as in the reconstituted lead–protein, the incorporation of Pb^{2+} ions in stoichiometric amounts in the apoenzyme, and the weak influence of the Zn^{2+} ions on the enzymatic assay of the lead–enzyme suggest that lead ions partially reactivate the calf intestine alkaline phosphatase apoenzyme.

It has been found that Zn atoms can be removed from the *Escherichia coli* alkaline phosphatase with a consequent complete loss of the enzymatic activity (Lazdunski and Lazdunski, 1969). The obtained apoenzyme is still capable of incorporating divalent metals such as Zn, Co, Cd, Ni, Mn, Hg, and Cu. However, only Zn- and Co-alkaline phosphatase were found to be significantly active enzymes, while the other metalloproteins show a negligible catalytic activity (Plocke and Vallee, 1962). Limited studies have been carried out on the replacement of Zn with other metal ions in mammalian alkaline phosphatase (Thoai et al., 1947; Harkness, 1967). In particular, we have found nothing in the literature dealing with the interaction of lead with mammalian alkaline phosphatase, although Kosmider established a relationship between the toxic effect of lead and the diminished activity of the enzyme in experimental animals and man (Kosmider, 1963).

In this paper the replacement of the native metal constituent (Zn) by lead in calf intestine alkaline phosphatase resulted in an enzymatically active Pb-enzyme. The study

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