Enzymes Involved in Wall Peptide Crosslinking in *Escherichia coli* K12, Strain 44

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By using the glutamate-amidated tetrapeptide L-alanyl-D-isoglutaminyl-(L)-*meso*-diamino-pimelyl-(L)-D-alanine as a probe, there appears to exist in the membranes of *Escherichia coli* K12 strain 44 a DD-carboxypeptidase-endopeptidase system which does not recognize this peptide and a DD-carboxypeptidase-transpeptidase system which recognizes it. The DD-carboxypeptidase-endopeptidase system is essentially hydrolytic. It catalyzes the hydrolysis of UDP-*N*-acetyl-muramyl-pentapeptide into UDP-*N*-acetylmuramyl-tetrapeptide and the hydrolysis of the wall peptidoglycan peptide dimer into monomers. These activities are not inhibited by the glutamate-amidated tetrapeptide. The system may consist either of two enzyme proteins having predominant-ly carboxypeptidase activity and endopeptidase activity, respectively, or of one enzyme protein of which the functioning would depend upon the environmental conditions. The DD-carboxypepti-dase-transpeptidase system (a) catalyzes concomitant hydrolysis (carboxypeptidase activity) and transfer (natural model transpeptidase activity) reactions with the pentapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine. The transfer reaction leads to the synthesis of a dimer that is identical to the one which occurs in the *E. coli* wall peptidoglycan; (b) utilizes the glutamate-amidated tetrapeptide as an acceptor. Simultaneous exposure of the pentapeptide and the glutamate-amidated tetrapeptide to the enzyme system leads to the forma-tion of an hybrid monoamidated peptide dimer and causes a decreased hydrolysis of the penta-peptide; (c) by virtue of its own carboxypeptidase activity, it appears to exert some endopeptid-ase activity. Both carboxypeptidase and endopeptidase activities of this system are inhibited by the glutamate-amidated tetrapeptide, but this represents only a small fraction of the total hydro-lytic activity of the membrane Brij-36T extract. (d) The system catalyzes an unnatural model transpeptidation reaction in which glycine replaces D-alanine at the C-terminal position of the nucleotide UDP-*N*-acetylmuramyl-pentapeptide. This system may also consist either of two enzyme proteins having predominantly natural model transpeptidase activity and unnatural model transpeptidase activity, respectively, or of one enzyme protein of which the functioning would depend upon the environmental conditions. Whatever the exact situation, the *E. coli* DD-carboxypeptidase-transpeptidase system is in many respects, similar to the DD-carboxy-peptidase-transpeptidase single polypeptide enzymes isolated from *Streptomyces* strains R39 and R61.

Escherichia coli possesses three enzymes activities which are involved in the synthesis and the control of the wall peptidoglycan peptide crosslinking [1—5]. The transpeptidase activity hydrolyzes the C-termin-al D-alanyl-D-alanine peptide bond of the penta-peptide sequence L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine with the con-comitant transfer of the carboxyl group of the penultimate D-alanine residue to the (D)-amino group of the *meso*-diaminopimelic acid residue of another pentapeptide, leading to the synthesis of C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid interpeptide bonds. The DD-carboxypeptidase activity hydrolyzes C-terminal D-alanyl-D-alanine peptide bonds without concomitant transpeptidation thus preventing further propagation of the crosslinking system. The endo-peptidase activity [6—7], which is thought to be another manifestation of the DD-carboxypeptidase

activity [8], solubilizes the intact peptidoglycan by hydrolyzing the interpeptide C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkages [9] made by transpeptidation. The membranes isolated from *E. coli* K12 strain 44 were shown to be able to catalyze both the liberation of the C-terminal D-alanine residue from the nucleotide UDP-*N*-acetylmuramyl-pentapeptide (model carboxypeptidase activity) and the incorporation of [14C]glycine into the same UDP-*N*-acetylmuramyl-pentapeptide where it replaced the C-terminal D-alanine residue (unnatural model transpeptidase activity) [10]. After extraction of the membranes by Brij-36T, several fractions exhibiting both activities were isolated [10]. The present paper describes experiments which were devised primarily in order to determine whether this unnatural model transpeptidase was able to catalyze the dimerisation of natural pentapeptide units *in vitro*, *i.e.* a reaction identical to the one which occurs *in vivo* during the last stages of the wall peptidoglycan biosynthesis.

MATERIALS AND METHODS

Membranes and Membrane Fractions

The following preparations derived from *E. coli* K12 strain 44 [10] were used (Table 1): crude membranes, Brij-36T extracts of the crude membranes, the fraction of the Brij-36T membrane extract unadsorbed on DEAE-cellulose (fraction A'), the fraction unadsorbed on DEAE-cellulose and ampicillin-affinose (fraction A), the fraction fixed on and eluted from DEAE-cellulose (fraction B') and the fraction fixed on and eluted from both DEAE-cellulose and ampicillin-affinose 202 (fraction B).
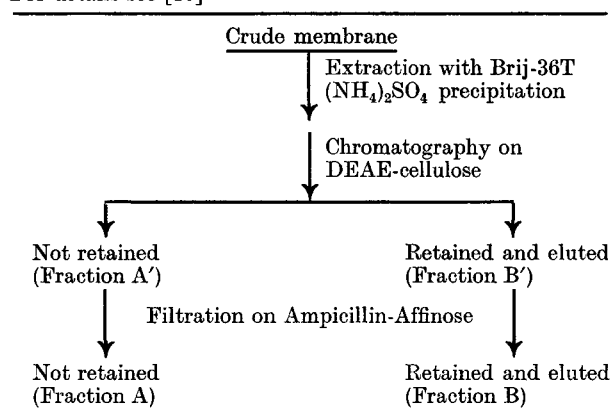
UDP-N-*Acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso-*diaminopimelyl-(L)-D-alanyl-D-alanine and* *UDP*-N-*Acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso-*diaminopimelyl-(L)-D-[*14C*]alanyl-D-[*14C*]-alanine*

These nucleotides were those used previously [10].

*Non-amidated Pentapeptide L-Alanyl-γ-D-glutamyl-(L)-*meso-*diaminopimelyl-(L)-D-[*14C*]alanyl-D-[*14C*]alanine and Glutamate-Amidated Tetrapeptide L-Alanyl-D-isoglutaminyl-(L)-*meso-*diaminopimelyl-(L)-D-alanine*

The radioactive non-amidated pentapeptide was prepared by acid hydrolysis of radioactive UDP-*N*-acetylmuramyl-pentapeptide (5 μmol in 1 ml 0.1 N HCl, 7 min in a boiling water bath), followed by treatment of the liberated *N*-acetylmuramyl-pentapeptide with the *Streptomyces N*-acetylmuramyl-L-alanine amidase [11]. The non-radioactive glutamate-

Table 1. *Fractionation of the membranes of* Escherichia col *K12, strain 44: flow sheet*
For details see [10]

Crude membrane
Extraction with Brij-36T (NH₄)₂SO₄ precipitation

Chromatography on DEAE-cellulose

Not retained (Fraction A')	Retained and eluted (Fraction B')

Filtration on Ampicillin-Affinose

Not retained (Fraction A)	Retained and eluted (Fraction B)

amidated tetrapeptide was prepared from walls of *Streptomyces* R39 [12].

14C-Labeled Bisdisaccharide Peptide Dimer from E. coli

E. coli strain B was grown at 37 °C in minimal growth medium D [13] on a rotary shaker. After two successive overnight cultures, 1.5-l flasks containing 200 ml of minimal medium were inoculated (1%, v/v) and shaken at 37 °C until the absorbance of the cell suspensions was 0.9 at 550 nm. The cells, collected by centrifugation at room temperature, were suspended in the same volume of fresh, prewarmed medium supplemented with L-[14C]alanine (50 μCi per flask; specific activity: 156 mCi/mmol; Amersham). After 3 h at 37 °C of growth, the cells (stationary phase) were collected by centrifugation at 4 °C, washed once with cold distilled water, disrupted with a Bühler disintegrator (Tübingen, Germany). for 5 min at 4 °C, using 9 g wet weight of cells, 70 ml glass beads and 30 ml water containing 5 mg DNase. The cell envelopes were collected by centrifugation and the wall peptidoglycan was purified by treatment with hot sodium dodecylsulfate and trypsin, and solubilized by lysozyme as described by Braun and Rehn [14]. The lysozyme-degraded peptidoglycan was filtered in 0.1 M LiCl on two columns of Sephadex G-50 and Sephadex G-25 connected in series [9], yielding a disaccharide peptide monomer and a bis-disaccharide peptide dimer. Each fraction was desalted by filtration in water on a column of Sephadex G-25. Both monomer and dimer had the same specific activity: 1500 dis. × min⁻¹ × nmol⁻¹ of disaccharide peptide unit.

Enzyme Activities

The enzyme activities were tested as indicated in Table 2. All the incubations were carried out at

Table 2. *Enzyme activities*
A$_2$pm = *meso*-diaminopimelic acid; α-Gln = isoglutamine; MurNAc = N-acetylmuramic acid; GlcNAc-MurNAc, = β-1,4-N-acetyl-glucosaminyl-N-acetylmuramic acid

Activity studied	Reaction	Products
1. DD-Carboxy-peptidase	UDP-MurNAc-L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-DAla + H$_2$O	UDP-MurNAc-tetrapeptide + D-Ala
2. Endopeptidase	GlcNAc-MurNAc-L-Ala-D-Glu(OH) GlcNAc-MurNAc-L-Ala-D-Glu(OH) ⌐L⌐ -D-Ala(OH) A$_2$pm ⌐L⌐ -D-Ala- ⌐D⌐ — (OH) A$_2$pm ⌐D⌐ — (OH) + H$_2$O	GlcNAc-MurNAc-L-Ala-D-Glu(OH) ⌐L⌐ -D-Ala(OH) A$_2$pm ⌐D⌐ — (OH)
3. Unnatural model transpeptidase	UDP-MurNAc-L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-D-Ala + Gly	UDP-MurNAc-L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-Gly + D-Ala
4. Natural model transpeptidase (with concomitant hydrolysis of the donor)	L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-D-Ala (acting both as donor and acceptor) + H$_2$O	Peptide dimer (no 9 in Table 3) + L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala + D-Ala
5. Hybrid model transpeptidase (with concomitant hydrolysis of the donor)	L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-D-Ala- + L-Ala-D-α-Gln-(L)-A$_2$pm-(L)-D-Ala + H$_2$O	Peptide dimer (no 9 in Table 3) + Peptide dimer (no 10 in Table 3) + L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala + D-Ala

37 °C in 0.06 M Tris-HCl buffer pH 8.5 containing 0.05 M MgCl$_2$, 0.14 mM dithiothreitol and 0.36°/$_0$ to 8°/$_0$ (w/v) Brij-36T. The products of the reactions were separated by electrophoresis on Whatman 3 MM paper at 60 V/cm, with a Gilson High Voltage Electrophorator DW (10000 volts) either at pH 1.8 (formic acid—water, 20:1000 v/v) or at pH 6.4 (acetic acid—collidine—H$_2$O, 2.7:6.6:1000, v/v/v). The migrations of the compounds are given in Table 3 The eltrophoretograms were scanned and the radioactive spots were counted as described previously [10]. The various enzyme activities were compared by reference to the DD-carboxypeptidase activity. As defined previously [10], one DD-carboxypeptidase unit (1 U) released one μmol D-alanine per min when the nucleotide UDP-N-acetylmuramyl-pentapeptide (1.2 mM) was exposed to the enzyme at 37 °C (35 μl, final volume).

RESULTS

Endopeptidase Activity

When 0.3 mM radioactive bisdisaccharide peptide dimer was exposed for 2 h at 37 °C to 28 μg protein of Brij-36T membrane extract, 17°/$_0$ of the dimer was hydrolyzed to disaccharide peptide monomers, N-Acetylmuramyl-L-alanine amidase activity which would give rise to disaccharide-free peptide dimer

and monomer was not detected under the conditions of the assay. In the course of the fractionation of the membrane extract (Table 1), the endopeptidase activity per DD-carboxypeptidase activity underwent alterations so that the final preparations A and B differed from each other considerably in this respect. At equivalent DD-carboxypeptidase activity, 25°/$_0$ of the bisdisaccharide peptide dimer (0.3 mM) was hydrolyzed into monomers by fraction B after 2 h incubation, and by fraction A after 16 h incubation (Fig.1).

Unnatural Model Transpeptidase

It has been shown [10] that fractionation of the Brij-36T membrane extract caused a partial dissociation between the DD-carboxypeptidase activity and the unnatural model transpeptidase activity (as determined by measuring the transfer reaction between non-radioactive UDP-N-acetylmuramyl-pentapeptide and [¹⁴C]glycine). At equivalent DD-carboxypeptidase activity, fraction B required five times more glycine acceptor than fraction A in order to achieve the same amount of transpeptidation (Fig.5 in [10]). The replacement of [¹⁴C] glycine in the above assay by D-[¹⁴C]alanine led to the same conclusion, *i.e.* that fraction A had a higher unnatural model transpeptidase activity than fraction B, per equivalent DD-carboxypeptidase unit (Fig.2). D-Ala-

Table 3. *Migration of radioactive compounds by electrophoresis on Whatman 3 MM paper (60 V/cm)*

Compound	pH	Time	Migration	Towards
		h	cm	
1. UDP-MurNAc-L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-D-Ala	1.8	2	20.5	Anode
2. UDP-MurNAc-L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-Gly	1.8	2	20.5	Anode
3. UDP-MurNAc-L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala	1.8	2	20.5	Anode
4. Alanine and glycine	1.8	2	68	Cathode
5. Bisdisaccharide peptide dimer (see endopeptidase activity: Table 2)	1.8	2	26	Cathode
6. Disaccharide peptide monomer (see endopeptidase activity: Table 2)	1.8	2	38	Cathode
7. L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala-D-Ala	6.4	4	54	Anode
8. L-Ala-γ-D-Glu-(L)-A$_2$pm-(L)-D-Ala	6.4	4	62	Anode

9.

$$\begin{array}{c} \text{L-Ala-D-Glu(OH)}\\ \text{L-Ala-D-Glu(OH)} \quad \underset{\underset{\text{D}}{|}}{\overset{\text{L}}{\rule{1.5cm}{0.4pt}}}\text{-[D-Ala(OH)]}\\ \underset{\underset{\text{D}}{\overset{|}{\text{A}_2\text{pm}}}}{|}\\ \underset{\underset{\text{D}}{\overset{|}{\text{A}_2\text{pm}}}}{\overset{\text{L}}{\rule{1.5cm}{0.4pt}}}\text{-D-Ala-} \overset{|}{\underset{\text{D}}{}} - (\text{OH}) \end{array}$$

| 6.4 | 4 | 80 | Anode |

10.

$$\begin{array}{c} \text{L-Ala-D-Glu(amide)}\\ \text{L-Ala-D-Glu-(OH)} \quad \underset{\overset{|}{\text{A}_2\text{pm}}}{\overset{\text{L}}{\rule{1.5cm}{0.4pt}}}\text{-D-Ala(OH)}\\ \underset{\underset{\text{D}}{\overset{|}{\text{A}_2\text{pm}}}}{\overset{\text{L}}{\rule{1.5cm}{0.4pt}}}\text{-D-Ala-} \underset{\text{D}}{\overset{|}{\rule{0pt}{0.4pt}}}\text{-D-Ala-(OH)}\\ \underset{\text{D}}{\overset{|}{\rule{0pt}{0.4pt}}} - (\text{OH}) \end{array}$$
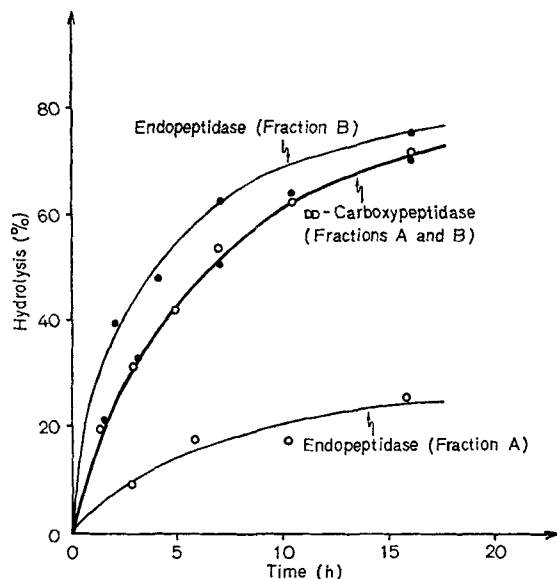
| 6.4 | 4 | 31 | Anode |

Fig. 1. *DD-Carboxypeptidase activity vs endopeptidase activity of fractions A and B: time course.* Fractions A and B (120 μU) were incubated with UDP-N-acetylmuramyl-pentapeptide (1.2 mM) for carboxypeptidase activity and with radioactive bisdisaccharide peptide dimer (0.3 mM) for endopeptidase activity. Final reaction mixtures (35 μl) were 0.06 M Tris-HCl buffer pH 8.5, 0.05 M MgCl$_2$, 0.14 mM dithiothreitol and 3—8% Brij-36T

nine was a better acceptor than glycine and this property was true for both fractions A and B. L-Alanine was not used as an acceptor (Fig. 2). It should be mentioned that when isolated membranes or Brij-36T membrane extracts were used as enzyme sources, the transpeptidation assays with D-[^{14}C]alanine, instead of [^{14}C]glycine, were unsuccessful. This failure is probably attributable to the fact that these preparations metabolized D-[^{14}C]alanine into several radioactive compounds which were separable by paper electrophoresis but were not identified. Enzymes metabolizing D-alanine were also detected in *E. coli* K12 by Miyakawa *et al.* [15].

Natural Model Transpeptidase Activity with Radioactive Pentapeptide L-Alanyl-γ-D-glutamyl-(L)-meso-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-[^{14}C]alanine

The above pentapeptide is the one which is involved in peptide crosslinking during the biosynthesis of the wall peptidoglycan of *E. coli* (see Introduction). When this pentapeptide (1.43 mM) was exposed to either fractions A or B, concomitant hydrolysis (yielding the tetrapeptide no 8 in Table 3) and transfer (yielding the dimer no 9 in Table 3) occurred (Table 4). Fig. 3 shows the separation by paper

electrophoresis of the various radioactive compounds present in the reaction mixtures, *i.e.* free D-alanine, tetrapeptide, dimer and residual pentapeptide. The peptide dimer formed under these conditions had the same migration as the peptide dimer either isolated through enzymic degradation of the *E. coli* wall peptidoglycan (Materials and Methods) or synthesized *in vitro* from the same pentapeptide through the action of the DD-carboxypeptidase-transpeptidase from *Streptomyces* R39 [16]. Under the conditions used (where less than 50% of the pentapeptide was utilized for hydrolysis and transpeptidation altogether) and within experimental error, the ratio of transpeptidation to hydrolysis of the pentapeptide was lower and the yield of dimer formation was

higher with fraction B than with fraction A. When expressed in terms of the ratio of transpeptidation to hydrolysis, the efficiency of fraction B in the natural model transpeptidation reaction was about 5 times higher than that of fraction A. In fact, the relative efficiency of fraction B was probably underestimated since fraction B, when compared to fraction A, had a higher endopeptidase activity (Fig. 1) by virtue of which the dimer made by transpeptidation could be rehydrolyzed into monomers. The results shown in Table 4 were obtained with amounts of enzyme preparation corresponding to 120 μU for fraction A and 40 μU for fraction B.

Based on these data, fraction B appeared to have, per DD-carboxypeptidase unit, a much higher natural model transpeptidase activity than fraction A, whereas the reverse was true for the unnatural model transpeptidase activity.

Model Transpeptidase Activity with Radioactive Pentapeptide L-Alanyl-γ-D-glutamyl-(L)meso-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-[^{14}C]alanine and Non-Radioactive Glutamate-Amidated Tetrapeptide L-Alanyl-D-isoglutaminyl-(L)-meso-diaminopimelyl-(L)-D-alanine

The pentapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine possesses both the donor group (the C-terminal D-alanyl-D-alanine) and the acceptor group (the free amino group located on the D-center of *meso*-diaminopimelic acid in α-position to a free carboxyl group) required for transpeptidation reactions. The glutamate-amidated tetrapeptide L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine lacks the donor group but has the proper acceptor amino group. Hence, pentapeptide and glutamate-amidated tetrapeptide when exposed simultaneously to an active preparation, should compete for the same acceptor site on the enzyme. Moreover, in the presence of large amounts of glutamate-amidated tetrapeptide, the pentapeptide should lose its bifunctional property, being utilized under these conditions, exclusively as peptide donor. In these experiments, a glutamate-amidated tetrapeptide was used instead of the non-amidated tetrapeptide which could be readily obtain-
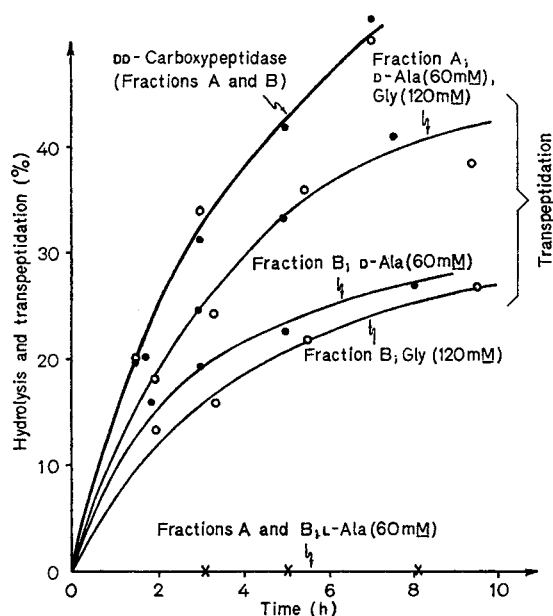

Fig. 2. *Unnatural model transpeptidase activity of fractions A and B with D-[^{14}C]alanine, L-[^{14}C]alanine and [^{14}C]glycine as acceptors: time course.* Fractions A and B (120 μU) were incubated under the same conditions as those described in Fig. 1 with non radioactive UDP-*N*-acetylmuramyl-pentapeptide (1.2 mM) in the absence of amino acid acceptor (carboxypeptidase assay) and in the presence of (O) 120 mM [^{14}C]glycine, (●) 60 mM D-[^{14}C]alanine and (×) 60 mM L-[^{14}C]alanine (transpeptidase assays)
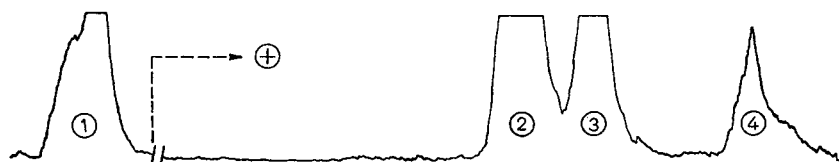

Fig. 3. *Separation of radioactive compounds by paper electrophoresis at pH 6.5 (4 h; 60 V/cm).* Mixture of, from left to right, (1) D-[^{14}C]alanine (product of total reaction: hydrolysis + transpeptidation); (2) residual pentapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-

[^{14}C]alanine; (3) tetrapeptide L-analyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]alanine; (4) radioactive non-amidated peptide dimer (product of transpeptidation no 9 in Table 3). For details, see text and legend of Table 3

Table 4. *Concomitant hydrolysis and transfer reactions with pentapeptide L-alanyl-γ-D-glutamyl-(L)-meso-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-[^{14}C]alanine by fractions A and B*

Radioactive pentapeptide (1.5 mM) was incubated at 37 °C with fraction A (120 μU) and fraction B (40 μU). For composition of the reaction mixtures (35 μl, final volume), see Materials and Methods. Brij-36T concentration was 5—8%. Yields are in percentages pentapeptide converted. It is not known whether this dimer (peptide no 9) which originates through dimerisation of the radioactive pentapeptide has one or two radioactive D-alanine residues since the C-terminal D-[^{14}C]alanine in the peptide dimer might be removed by carboxypeptidase action. The yield of conversion was calculated by multiplying the percentage of radioactivity found in the isolated peptide dimer by a factor of 1.5

Enzyme fraction	Time of incubation	Yields from		Ratio of transpeptidation to hydrolysis
		Hydrolysis (tetrapeptide)	Transpeptidation (peptide no 9, Table 3)	
	h	%		
A	2	19	1.3	0.07
	4	41	2	0.05
	8	36	2.7	0.07
B	4	15	4.9	0.32
	8	22	7.2	0.33

Table 5. *Concomitant hydrolysis and transfer reactions occurring when radioactive pentapeptide L-alanyl-γ-D-glutamyl-(L)-meso-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-[^{14}C]alanine and non-radioactive Glu-amidated tetrapeptide L-alanyl-D-isoglutaminyl-(L)-meso-diaminopimelyl-(L)-D-alanine are simultaneously exposed to fractions A' and B'*

Radioactive pentapeptide (1.5 mM) and increasing amounts of non-radioactive Glu-amidated tetrapeptide (from 0 to 75 mM) were incubated at 37 °C with fraction A' (12×10⁻⁵ DD-carboxypeptidase units, 8 h) and fraction B' (24×10⁻⁵ DD-carboxypeptidase units, 2 h). For composition of the reaction mixtures (35 μl, final volume), see Materials and Methods. Brij-36T concentration was 5—8% (final concentration). See Table 4 about the peptide dimer

Enzyme frcction	Glu-amidated tetrapeptide	Yields			Ratio of transpeptidation to hydrolysis		
		Hydrolysis (tetrapeptide)	Transpeptidation		Non-amidated dimer to tetrapeptide	Mono-amidated dimer to tetrapeptide	Total dimers to tetrapeptide
			Non-amidated peptide dimer (no 9, Table 3)	Mono-amidated peptide dimer (no 10, Table 4)			
	mM	%					
A'	0	36.7	2.7	0	0.075	—	0.075
	1.5	29	2.1	1.3	0.07	0.045	0.115
	15	21	1.2	2.1	0.06	0.1	0.16
	30	18	0.9	3.2	0.05	0.18	0.23
	75	15	0.8	2.9	0.05	0.19	0.24
B'	0	27	6	0	0.23	—	0.23
	15	13	2.2	11	0.17	0.84	1.01
	30	10	1.5	11	0.15	1.10	1.25

ed by carboxypeptidase action on the free pentapeptide. Indeed, the presence of an amide group on the α-carboxyl group of D-glutamic acid made it possible to separate the non-amidated peptide dimer (formed by dimerisation between two non-amidated pentapeptides; dimer no 9 in Table 3) from the mono-amidated peptide dimer (formed by dimerisation between one non-amidated pentapeptide and one amidated tetrapeptide; dimer no 10 in Table 3). Fixed amounts of radioactive pentapeptide (1.5 mM) were exposed to the enzyme preparation in the presence of increasing amounts of glutamate-amidated tetrapeptide (up to 75mM). The experimental

conditions were such that in the absence of glutamate-amidated tetrapeptide, about 30—40% of the pentapeptide was utilized for the transpeptidation and the hydrolysis reactions altogether. The presence of increasing amounts of the glutamate-amidated tetrapeptide in the reaction mixtures had the three following effects (Table 5): (a) increasing amounts of mono-amidated peptide dimer were formed at the expense of the non-amidated peptide dimer, (b) the hydrolysis of the pentapeptide was inhibited and (c) the observed decrease in hydrolytic activity was greater than could be accounted for by the yield of the transpeptidation reactions, thus resulting in a

progressive increase of the ratios of transpeptidation over hydrolysis. This property of the peptide acceptor to inhibit the hydrolysis of the peptide donor was a feature of the kinetics of concomitant transfer and hydrolysis reactions catalyzed by the single poly-peptide DD-carboxypeptidase-transpeptidase enzymes excreted by *Streptomyces* R61 and R39 [12,17,18]. This similarity strongly suggested that a DD-carb-oxypeptidase-transpeptidase enzyme similar to the *Streptomyces* ones, occurred in *E. coli* and was present in both membrane fractions A and B. Finally, the data of Table 5 showed that, when expressed in terms of ratios of transpeptidation (*i.e.* formation of the hybrid mono-amidated peptide dimer) to hydrolysis, fraction B was about 5 times more efficient than fraction A, *i.e.* a value which was identical to that obtained when fractions A and B were compared with respect to their natural model transpeptidase activity (Table 4).

Effects of Glutamate-Amidated Tetrapeptide on (a) Hydrolysis of Radioactive UDP-N-Acetyl-muramyl-pentapeptide (Carboxypeptidase Assay), (b) Hydrolysis of Bisdisaccharide Peptide Dimer into Disaccharide Peptide Monomer (Endopeptidase Assay) and (c) Transfer Reaction between UDP-N-Acetylmuramyl-pentapeptide and [¹⁴C]Glycine (Unnatural Model Transpeptidase Assay)

Since the glutamate-amidated tetrapeptide was recognized by the natural model DD-carboxypeptid-ase-transpeptidase (*i.e.* was an acceptor for the transpeptidation reaction and an inhibitor for the hydrolysis pathway of the pentapeptide donor), experiments were carried out in order to determine the possible inhibitory effects of the same glutamate-amidated tetrapeptide on the other activities ex-hibited by the membrane fractions (endopeptidase, DD-carboxypeptidase and unnatural model trans-peptidase). Radioactive bisdisaccharide peptide dimer (0.33 mM; for endopeptidase assay), radioactive UDP-N-acetylmuramyl-pentapeptide (1.5 mM; for carboxypeptidase assay) and non-radioactive UDP-N-acetylmuramyl-pentapeptide (1.5 mM supplemented with 7.5 mM [¹⁴C]glycine; for unnatural transpepti-dase assay) were incubated with the preparation A (120 μU) in the absence and in the presence of in-creasing concentrations of glutamate-amidated tetra-peptide (up to 77 mM). The scanning of the electro-phoretograms did not reveal the presence of any peculiar radioactive compound which could have been produced by a transfer of the glutamate-amidated tetrapeptide to the substrates used (trans-peptidation with the bisdisaccharide peptide dimer would give rise to an hybrid mono-disaccharide mono-amidated peptide dimer; transpeptidation with UDP-N-acetylmuramyl-pentapeptide would
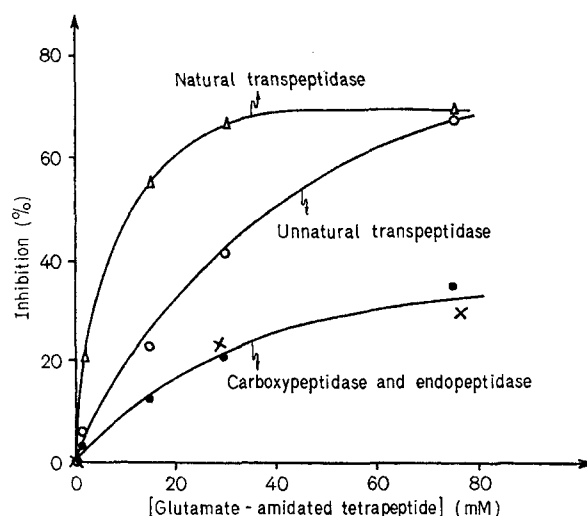

Fig. 4. *Effects of increasing concentrations of glutamate-amidated tetrapeptide on the hydrolysis of radioactive UDP-N-acetylmuramyl-pentapeptide (carboxypeptidase assay), the hydrolysis of the radioactive bisdisaccharide peptide dimer (endopeptidase assay), the transfer reaction between non-radioactive UDP-N-acetylmuramyl-pentapeptide and [¹⁴C]glycine (un-natural model transpeptidase assay) and the transfer reaction between two radioactive pentapeptide units (natural model transpeptidase assay).* Fraction A (120 μU per 35 μl), final volume) was used. For composition of the reaction mixtures, see Materials and Methods. Concentration of Brij-36T was 5—8% (final concentration). For the concentrations of the substrates, see text. Under the experimental conditions used and in the absence of glutamate-amidated tetrapeptide, the yields of hydrolysis were 32% in the carboxypeptidase assay and 17% in the endopeptidase assay. The yield of un-natural model transpeptidase (expressed in terms of UDP-N-acetylmuramyl-pentapeptide converted into radioactive nucleotide) was 4.5% and the yield of natural model trans-peptidase (expressed in terms of radioactive pentapeptide converted into radioactive non-amidated peptide dimer) was 2.7% (see Table 5)

give rise to an hybrid mono-amidated UDP-N-acetyl-muramyl-octapeptide). Nevertheless, a partial inhibi-tion of the endopeptidase, carboxypeptidase and unnatural model transpeptidase occurred (Fig. 4). However, the decrease observed with the unnatural model transpeptidase was considerably higher than those observed with both the carboxypeptidase and the endopeptidase. The results suggested that a high proportion (60—70%) of these two latter activities might occur in a form (or forms) that would not recognize the glutamate-amidated tetrapeptide and, consequently, would not be affected by its presence in the reaction mixture. The effect of glut-amate-amidated tetrapeptide on the unnatural model transpeptidase was also compared with the effect of the same compound on the natural model trans-peptidase (*i.e.* on the dimerisation between two pentapeptides L-alanyl-γ-D-glutamyl-(L)-*meso*-di-aminopimelyl-(L)-D-alanyl-D-alanine; Table 5). At low

concentrations, the glutamate-amidated tetrapeptide was a much more potent inhibitor of the natural model transpeptidase (Fig. 4). Interestingly, however, at high concentrations of glutamate-amidated tetrapeptide (70 mM), both unnatural and natural model transpeptidase were inhibited to the same extent (70%, Fig. 4).

Influence of the Ampicillin-Affinose Treatment on the Fractionation of the E. coli *Enzyme Activities*

The partition of the various activities between fraction A' and B', *i.e.* before treatment with ampicillin-affinose (Table 1), was virtually identical to that found between fractions A and B that were either filtered through the ampicillin-affinose column or fixed on and eluted from it, respectively. Hence, the chromatography on DEAE-cellulose was the essential step of the fractionation procedure responsible for the observed distribution of the activities studied.

DISCUSSION

E. coli K12, strain 44, contains an enzyme system which, operationally, can be defined as follows. When acting on the pentapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine, it catalyzes a transfer reaction leading to the formation of a peptide dimer (natural model transpeptidase), concomitantly with the hydrolysis of the pentapeptide into tetrapeptide. The glutamate-amidated tetrapeptide L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine which has the proper amino group for transpeptidation, acts as a competitor of the pentapeptide for the acceptor site on the enzyme system, causes the formation of an hybrid mono-amidated peptide dimer and decreases the extent of hydrolysis of the pentapeptide donor. Such activities can be attributed to a DD-carboxypeptidase-transpeptidase enzyme analogous to the purified DD-carboxypeptidase-transpeptidase single polypeptide enzymes isolated from the the culture filtrates of *Streptomyces* R39 and R61 [12,17,18]. This interpretation, if verified by the actual isolation and purification of the *E. coli* DD-carboxypeptidase-transpeptidase system, would support the concept of the ubiquitous occurrence of such a system among the bacteria. By virtue of their carboxypeptidase activity, the *Streptomyces* R39 and R61 DD-carboxypeptidase-transpeptidase enzymes also catalyze the liberation of the C-terminal D-alanine residue from the UDP-*N*-acetylmuramyl-pentapeptide and, although with a weak efficiency, the hydrolysis of peptide dimers into monomers [19,20]. Hence, it seems reasonable to assume that the *E. coli* DD-carboxypeptidase-transpeptidase system is also able to perform such activities and, more precisely, to propose that it is responsible for that part of the total hydrolytic

(carboxypeptidase and endopeptidase) activity which can be inhibited by the glutamate-amidated tetrapeptide.

The fact that the unnatural model transpeptidase which catalyzes the replacement of D-alanine by glycine at the C-terminal position of the UDP-*N*-acetylmuramyl-pentapeptide is inhibited by high concentrations of the glutamate-amidated tetrapeptide, strongly suggests that this activity is also attributable to the DD-carboxypeptidase-transpeptidase system, However, fraction A has predominantly the unnatural model transpeptidase activity whereas fraction B has predominantly the natural model transpeptidase activity. It is possible that one is dealing with distinct DD-carboxypeptidase-transpeptidase proteins that are characterized by specific differences in their relationships between binding and/or catalytically active sites. Another possibility is that *E. coli* possesses one single DD-carboxypeptidase-transpeptidase enzyme protein but that differences in the microenvironment of fraction A/A' and fraction B/B' (either naturally preexisting in the membranes or artificially created by the fractionation procedure) would alter the functioning of the enzyme. The fact that the proportion of the activity of the *Streptomyces* enzymes that can be channelled into the transpeptidation or the hydrolysis pathways depends upon the environmental conditions [12,17,18] is consistent with this latter interpretation.

A high proportion of the DD-carboxypeptidase and endopeptidase activities in *E. coli* K12, strain 44, appears not to be affected by the glutamate-amidated tetrapeptide and, for this reason, is probably catalyzed by an enzyme system which is distinct from the DD-carboxypeptidase-transpeptidase one(s). Fraction A has predominantly the carboxypeptidase activity and fraction B has predominantly the endopeptidase activity. Again, these two activities might be catalyzed either by distinct proteins or by one single protein of which the functioning would depend upon the microenvironmental conditions.

The composition of the *E. coli* "DD-carboxypeptidase-transpeptidase-endopeptidase" complex, as revealed by the present studies, is summarized in Table 6. Relative to DD-carboxypeptidase activity, fraction B (or B') had predominantly the natural model transpeptidase (and low unnatural model transpeptidase and endopeptidase activities) whereas the contrary was true for fraction A. The fact that partition of the enzyme activities was not clear-cut seemingly indicated that an appreciable extent of cross-contamination occurred between the two fractions. However, the fractions were not cross-contaminated, as demonstrated in the following paper [13], where the inhibitory action of cephalothin and ampicillin was used as a probe for further characterization of the enzyme activities.

Table 6. *The* DD*-carboxypeptidase-transpeptidase-endopeptidase complex in* Escherichia coli *K12, strain 44*
See Table 2 about activity

System	Activity	Action of glutamate-amidated tetrapeptide	Enzymes involved
DD-Carboxypeptidase-transpeptidase system	Natural transpeptidase (++)	Acceptor → hybrid dimer and Inhibitor of the hydrolysis pathway	Distinct enzyme proteins or one enzyme protein occurring in different active forms
	Unnatural transpeptidase (++)	Acceptor function (?): not detected Inhibitor	
	DD-Carboxypeptidase (±) Endopeptidase (±)	Inhibitor Inhibitor	
DD-Carboxypeptidase-endopeptidase system	DD-Carboxypeptidase (++) and endopeptidase (±)	No effect	Distinct enzyme proteins or one enzyme protein occurring in different active forms
	Endopeptidase (++) and DD-carboxypeptidase (±)	No effect	

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