DISCUSSION PAPER: THE DD-CARBOXYPEPTIDASE-TRANSPEPTIDASE SYSTEM IN *ESCHERICHIA COLI* MUTANT STRAIN 44 *

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INTRODUCTION

The peptide moiety of the wall peptidoglycan of *Escherichia coli* is composed of uncross-linked peptide monomers L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine and peptide dimers in which two peptide monomers are linked together through C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkages [1–3] (FIGURE 1). It has been observed previously that cross-linked peptidoglycan that consists of such peptide dimers could be synthesized when the nucleotide precursors UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine were combined with Mg^{2+} ions and a crude disrupted cellular preparation.[4–8] These authors also postulated that the soluble enzyme that cleaves the terminal D-alanine residue of UDP-*N*-acetylmuramyl pentapeptide (see FIGURE 1) in a DD-carboxypeptidase reaction was distinct from that enzyme in their particulate preparation responsible for transpeptidation. Because of the complexity, however, of such a multienzyme system that did not allow the independent assay of transpeptidase activity, it has not been possible to study the interaction of penicillin with the transpeptidase per se, and thus an understanding at the molecular level of how penicillin kills bacteria has been delayed. Moreover, because two purified single polypeptide chain enzymes isolated from *Streptomyces* species [9, 10] each perform both DD-carboxypeptidase and transpeptidase functions, it was decided to reinvestigate such processes in *E. coli*.

MATERIALS AND METHODS

Strain. Escherichia coli K12 F^-, strain 44 mutated for β-lactamase activity [11] was used throughout the course of these studies.

Membrane Preparations. Membrane envelopes were prepared according to the techniques described by Osborn and colleagues.[12]

* This work was supported by grants from the National Institutes of Health (AI 11489) to J. J. Pollock and from the National Science Foundation (GB-31964X) to M. R. J. Salton. Grants from the Fonds de la Recherche Fondamentale Collective (515 & 1000) and from l'Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (1699 & 2013) to J.-M. Ghuysen are gratefully acknowledged.

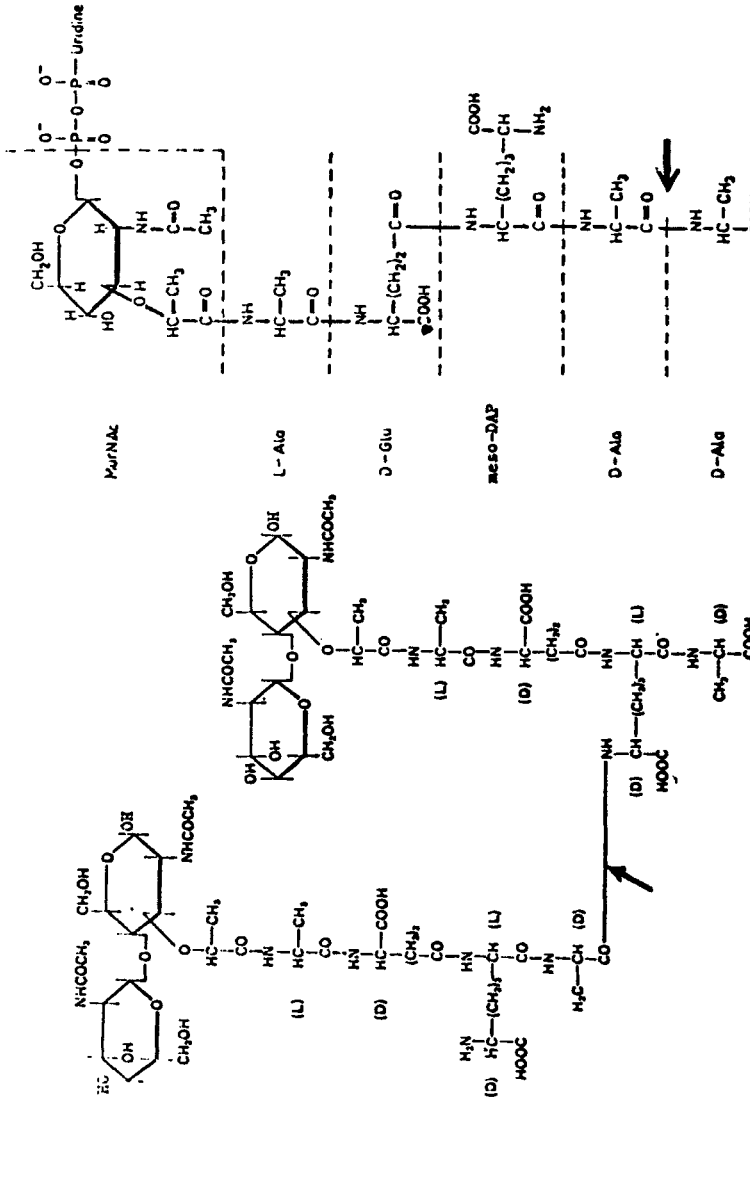


FIGURE 1. Structures of the UDP-*N*-acetylmuramyl pentapeptide and the bisdisaccharide peptide dimer of *E. coli*. Note the position of arrows that indicate DD-carboxypeptidase and endopeptidase catalysis (see Reference 3).

Fractionation Procedures. Details of the techniques used for the fractionation of DD-carboxypeptidase-transpeptidase activities are provided elsewhere.[13] Briefly, membranes were extracted with Brij 36T and the Brij supernatant

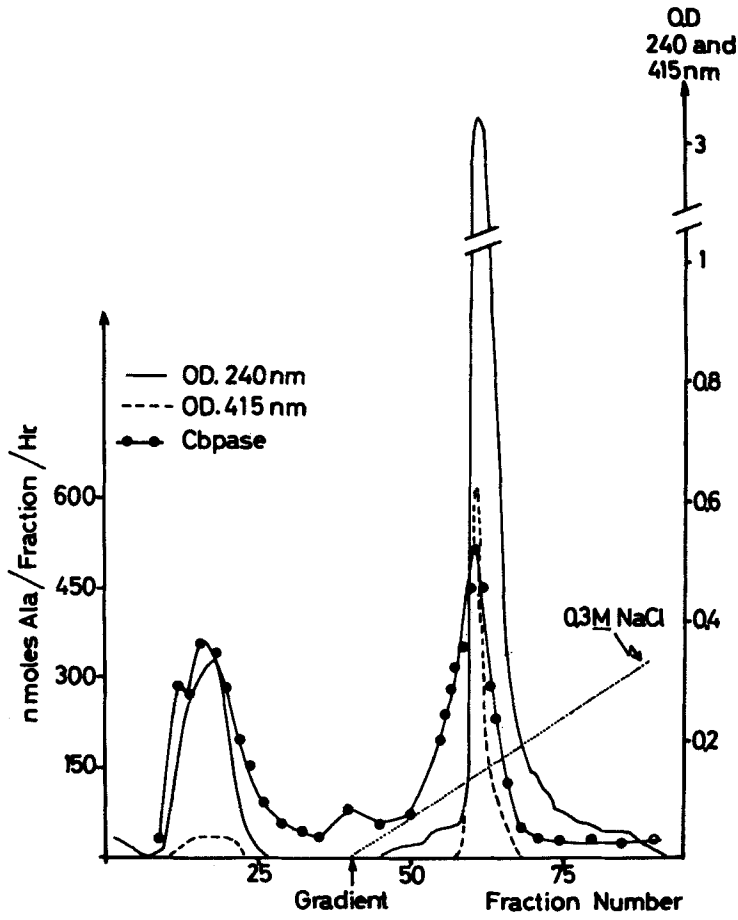


FIGURE 2. DEAE-cellulose chromatography of the DD-carboxypeptidase system of *E. coli* mutant strain 44. Chromatography (2.5×10 cm column) of the 30–60% ammonium sulfate precipitate was performed in Brij 36T with fractions of 3.5 ml being collected at a flow rate of 90 ml/hr. For DD-carboxypeptidase activity, 40–60 μl of the fractions were supplemented with 40 nmoles of unlabeled UDP-*N*-acetylmuramyl pentapeptide and incubated for 4–7 hr at 37° C in the presence of 0.01 M $MgCl_2$ (see Reference 13).

obtained after high-speed centrifugation was treated with solid ammonium sulfate. At 30–60% saturation, a floating layer was found to contain the majority of the catalytic activity and was applied to a DEAE-cellulose column, which yielded two enzyme fractions (FIGURE 2). The fraction of catalytic activity

that was not adsorbed to DEAE-cellulose was also not bound to an ampicillin affinity chromatography column [13] and after ampicillin chromatography was concentrated and designated fraction A. Conversely, the DD-carboxypeptidase activity of the DEAE-cellulose-adsorbed fraction could be bound to and eluted from the affinity column (see FIGURE 3). After an additional filtration on the ampicillin column, active fractions were eluted, pooled, and concentrated to yield fraction B.

Substrates. Labeled UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-[^{14}C]alanine was synthesized enzymatically by the addition of D-[^{14}C]alanyl-D-[^{14}C]alanine to UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid.[14] Specific activity was 17 nmoles/μCi. Unlabeled nucleotide-pentapeptide was prepared either from *Bacillus subtilis* W23 [15] or from *Bacillus megaterium* KM.[14] The [^{14}C]bisdisaccharide peptide dimer was prepared from *E. coli* by incorporating L-[^{14}C] alanine into the growth medium.[20]

DD-*Carboxypeptidase Activity.* When unlabeled nucleotide-pentapeptide was used as the substrate, D-alanine release, as measured by the fluorodinitrobenzene technique,[16] indicated enzyme activity. With labeled donor, D-[^{14}C]alanine was separated from UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]alanine by high-voltage paper electrophoresis at pH 1.8,[13] and the respective radioactive peaks were cut into strips and counted with a

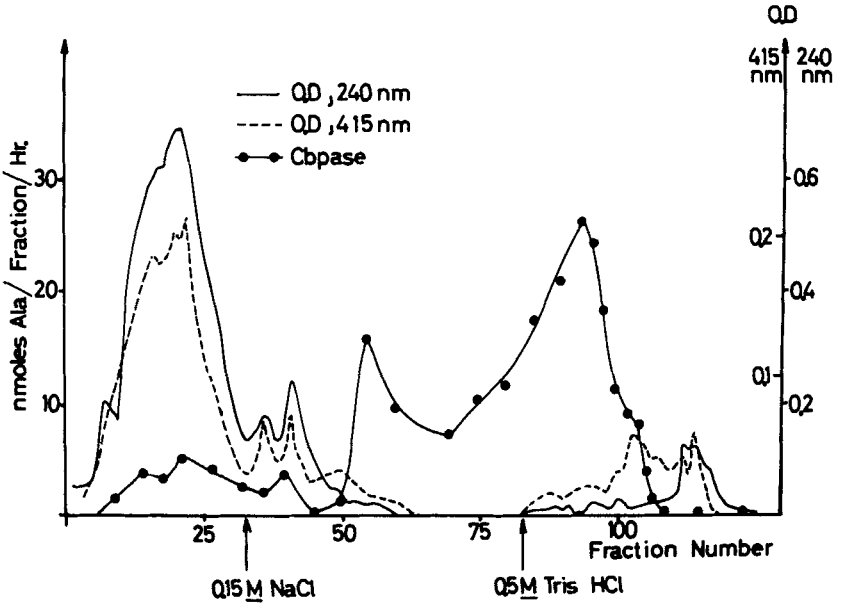


FIGURE 3. Ampicillin affinity chromatography of the DD-carboxypeptidase fraction adsorbed onto and eluted from DEAE-cellulose (see FIGURE 2). Fractions of 3.5 ml were collected at a flow rate of 300 ml/hr. For DD-carboxypeptidase activity, 80 μl of the fractions were incubated for 10 hr at 37° C with 40 nmoles of unlabeled UDP-*N*-acetylmuramyl pentapeptide in the presence of 0.01 M $MgCl_2$ (see Reference 13).

TABLE 1

MIGRATION OF DONORS, ACCEPTORS, AND PRODUCTS

Compound	pH	Hours	Migration * (cm)
UDP-MurNAc-Ala-Glu-Dap-Ala-Ala	1.8	2	20.5 (→+)
UDP-MurNAc-Ala-Glu-Dap-Ala-Gly	1.8	2	20.5 (→+)
UDP-MurNAc-Ala-Glu-Dap-Ala	1.8	2	20.5 (→+)
Alanine and glycine	1.8	2	68 (→−)
Bisdisaccharide peptide dimer	1.8	4	26 (→−)
Disaccharide peptide monomer	1.8	4	38 (→−)
Ala-Glu-Dap-Ala-Ala	6	4	54 (→+)
Ala-Glu-Dap-Ala	6	4	62 (→+)
Ala-Glu-Dap-Ala-(Ala) † | Ala-Glu-Dap-Ala	6	4	80 (→+)
Ala-Glu(amide)-Dap-Ala | Ala-Glu-Dap-Ala	6	4	31 (→+)

* Migration rates are determined after high-voltage electrophoresis at 60 Volts/cm.

† The parentheses indicate that it has not been determined whether the terminal alanine of the acceptor portion of the dimer is still present or has been cleaved enzymatically.

Packard scintillation counter. Details of the reaction mixtures are given in Reference 13.

Transpeptidase Activity. Three different donor-acceptor systems were used to measure transpeptidation. In one system, unlabeled nucleotide-pentapeptide (donor) was mixed with [^{14}C]glycine (acceptor) and enzyme,[13] and the amount of UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-[^{14}C]glycine formed was quantitated by pH 1.8 electrophoresis (see TABLE 1). In a second system, nonamidated pentapeptide, L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-[^{14}C]alanine [20] served as both donor and acceptor to yield a peptide dimer that could be separated by pH 6 paper electrophoresis (TABLE 1). With the third system, nonamidated labeled pentapeptide served as the donor, and the *Streptomyces* R39 amidated tetrapeptide [9] was used as the acceptor. In the latter system, a hybrid dimer (see TABLE 1) could be formed, in addition to the usual peptide dimer of transpeptidase system two (see above). Presumably, the only structural difference between these dimers is that the acceptor portion of the hybrid dimer has an amide group on the glutamic acid residue.

Endopeptidase Activity. This was estimated by following the cleavage of radioactive bisdisaccharide peptide dimer into radioactive disaccharide peptide monomer [20] (FIGURE 1 & TABLE 1).

Optical Density. Absorbance at 240 nm was used to determine the amount of protein present in the enzyme fractions on the basis of the correlation found between the membrane protein as determined by Lowry's method and the membrane absorbance at 240 nm. In both determinations, the membranes were first dissolved in 1% sodium dodecyl sulfate. An optical density of 1.0 at 240 nm corresponded to 0.385 mg membrane protein per milliliter. The absorbance at 415 nm was used as an indication of the presence of cytochrome.

TDB Buffers. TDB designates Tris-HCl buffers of specified molarity and pH, supplemented with 0.2 mM dithiothreitol and 0.5% Brij 36T.

Results and Discussion

Fractionation. After extraction of membrane envelopes with Brij 36T and precipitation with ammonium sulfate, two fractions that exhibited DD-carboxypeptidase activity were obtained by DEAE-cellulose chromatography (Figure 2). Both fractions displayed transpeptidase activity (not shown in Figure) when tested in the nucleotide-pentapeptide-[^{14}C]glycine system, but only the fraction that adsorbed onto and eluted from DEAE-cellulose could be bound to and eluted from the ampicillin affinity column (Figure 3). When the DEAE-adsorbed fraction was applied to the ampicillin column, most of the protein and its marker cytochrome were not bound to the column, but the majority of the DD-carboxypeptidase activity was fixed (Figure 3). After a 0.05 M TDB buffer wash, the addition of 0.15 M sodium chloride in 0.05 M TDB buffer resulted initially in the release of more protein and cytochrome and again a small amount of enzyme. After the protein had dropped off to virtually zero absorbance, a considerable amount of activity was eluted from the column. If the elution with 0.15 M sodium chloride was allowed to continue past fraction 80 (Figure 3), a slow release of DD-carboxypeptidase activity was noted. Subsequent to the initial release of contaminating protein, the sodium chloride elution appeared to be very specific. If 0.15 M sodium chloride elution was not permitted to continue past fraction 80, the addition of 0.5 M TDB buffer enhanced the elution of the enzyme, with very little protein being released (Figure 3). When 0.5 M sodium chloride in 0.05 M TDB buffer was used instead of 0.15 M sodium chloride, more DD-carboxypeptidase was released, but additional activity, although less, could still be eluted by the 0.5 M TDB buffer.

An analysis of Figure 3 indicates that the affinity chromatography system removed approximately 85% of the contaminating protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fraction B (Materials and Methods), however, revealed four coomassie blue-stained bands, which suggested that the preparation was not homogeneous. Because DD-carboxypeptidase-transpeptidase activity had been eluted simply with 0.15 M sodium chloride, it might be concluded that the *E. coli* transpeptidase was not irreversibly bound to ampicillin, as suggested by Izaki and coworkers,[4–6] but that it was in fact reversibly bound. Conversely, Tris could be considered a weak nucleophile, and it might be argued that it would cleave any irreversibly acylated complex between the β-lactam ring of ampicillin and a protein sulfhydryl or hydroxyl group. Since, however, membrane proteins and membrane lipids avidly bind to one another, it should be emphasized that the affinity column should not be used as an indication of what type of complex is formed. In our laboratory, the same affinity column has thus far been used for more than one year and therefore seems quite stable to the procedures used.

DD-Carboxypeptidase-Transpeptidase Activities of Fractions A and B. Although D-alanine and glycine served as acceptors in the transpeptidation reaction, L-alanine did not (Figure 4), which is analogous to the situation with purified *Streptomyces* enzymes.[17] For both fractions A and B, D-alanine appeared to be about twice as efficient an acceptor as glycine (see Figure 4).

For equivalent DD-carboxypeptidase activity, fraction B required approximately five times more glycine acceptor to achieve the same amount of transpeptidation as fraction A (FIGURE 5). Although this variation existed, there is no reason a priori to suspect that the catalytically active proteins in these fractions are different. When working with membrane proteins, it is not uncommon to find the same enzymatic activities in different areas of a DEAE-cellulose or sephadex column. Moreover, if the activities are located in different lipid environments, then even protein band differences observed upon migration in

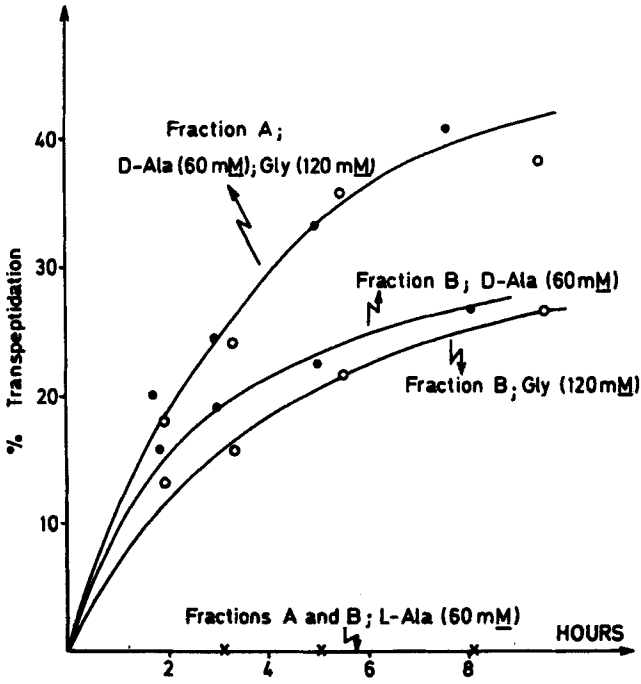


FIGURE 4. Time course of transpeptidase activity of fractions A and B with the acceptors D-[^{14}C]alanine, L-[^{14}C]alanine, and [^{14}C]glycine. Fractions A and B at equivalent DD-carboxypeptidase activity were incubated with unlabeled UDP-*N*-acetylmuramyl pentapeptide (1.2 mM) in the presence of [^{14}C]glycine (120 mM, ○—○), D-[^{14}C]alanine (60 mM, ●—●), and L-[^{14}C]alanine (60 mM, ×—×). For estimation of activities, see MATERIALS AND METHODS (see also Reference 20).

sodium dodecyl sulfate gel electrophoresis [18] would not allow the conclusion that multiple catalytic systems existed, as proposed by some workers.[19] Such a difference in physical environment, however, may be a natural one that exists in the intact membrane and could lead not only to differences in the extent of transpeptidation but also to differences in the antibiotic sensitivities of the catalytically active fractions.[11]

Endopeptidase Activities of Fractions A and B. At equivalent DD-carboxypeptidase activity, 25% of the bisdisaccharide peptide dimer was hydrolyzed

into monomer after 2 hr of incubation in the presence of fraction B and after 16 hr in the presence of fraction A (FIGURE 6). When the fractionation procedure was performed in the presence of Triton X-100 instead of Brij 36T, the fraction not adsorbed to DEAE-cellulose did not show endopeptidase activity under the conditions tested. This may suggest that there is an endopeptidase enzyme distinct from the DD-carboxypeptidase-transpeptidase system, with the former acting as a lytic enzyme and functioning in cell wall extension proc-

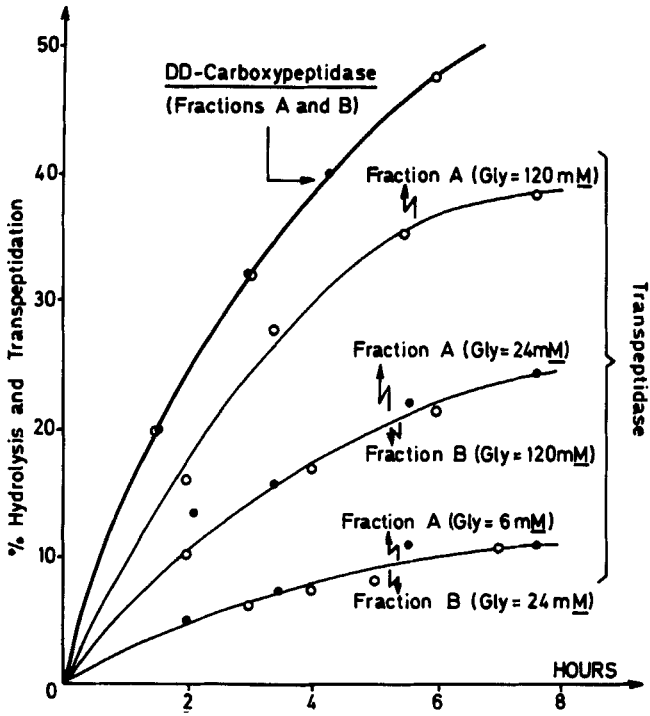


FIGURE 5. Time course of DD-carboxypeptidase versus transpeptidase activities of fractions A and B. Both fractions were incubated with nucleotide-pentapeptide (1.2 mM) for DD-carboxypeptidase activity and in the presence of [^{14}C]glycine (from 3 to 120 mM) for transpeptidase activity. Final reaction 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. For estimation of activities, see MATERIALS AND METHODS (see also Reference 13).

esses.[1] As one control in the amounts of cross-bridge found in bacterial cell walls, it may be that once cross-bridge is formed by transpeptidation, the transpeptidase cannot recleave the bridge, even if it is of the *E. coli* DD-type. To accomplish cleavage, one would then require the *E. coli* endopeptidase or a comparable lytic enzyme found in other bacteria.

Effects of the R39 Amidated Tetrapeptide. Increasing the amount of R39 tetrapeptide relative either to the nucleotide-pentapeptide (FIGURE 7) or to

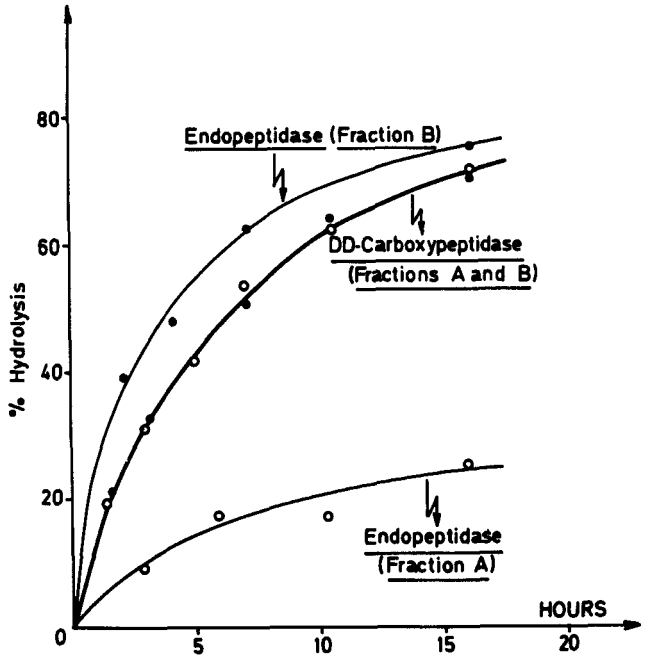
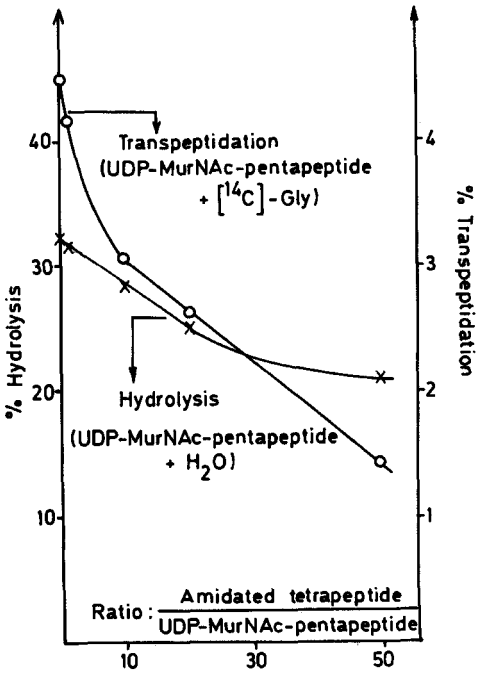


FIGURE 6. Time course of DD-carboxypeptidase versus endopeptidase activities of fractions A and B. Both fractions were incubated with unlabeled nucleotide-pentapeptide (1.2 mM) for DD-carboxypeptidase activity and with labeled bisdisaccharide peptide dimer (0.3 mM) for endopeptidase activity. The composition of the reaction mixtures are the same as in FIGURE 5 (see Reference 20).

FIGURE 7. Effect of the R39 tetrapeptide on transpeptidation in the nucleotide-pentapeptide system. Labeled nucleotide-pentapeptide (1.4 mM) for DD-carboxypeptidase assay and unlabeled nucleotide-pentapeptide (1.4 mM) plus [^{14}C]glycine (7 mM) for transpeptidase assay were exposed to fraction A for 4 hr at 37° C in the absence of Glu-amidated tetrapeptide (R39 tetrapeptide) and in the presence of increasing concentrations (up to 70 mM) of the tetrapeptide. For other conditions, see FIGURE 5.



the nonamidated pentapeptide (FIGURE 8) caused progressive loss in the extent of transpeptidation in the respective systems. When the nucleotide-pentapeptide was the donor, the R39 tetrapeptide would not serve as an acceptor; it would, though, when the nonamidated pentapeptide was used as donor substrate [20] (FIGURES 7 & 8). The fact that inhibition of transpeptidation could be achieved in the two types of assays would suggest that catalytically one is working with one transpeptidase but that synthesis depends upon the structural specificity of both the donor and the acceptor molecules. The synthesis of a nonamidated

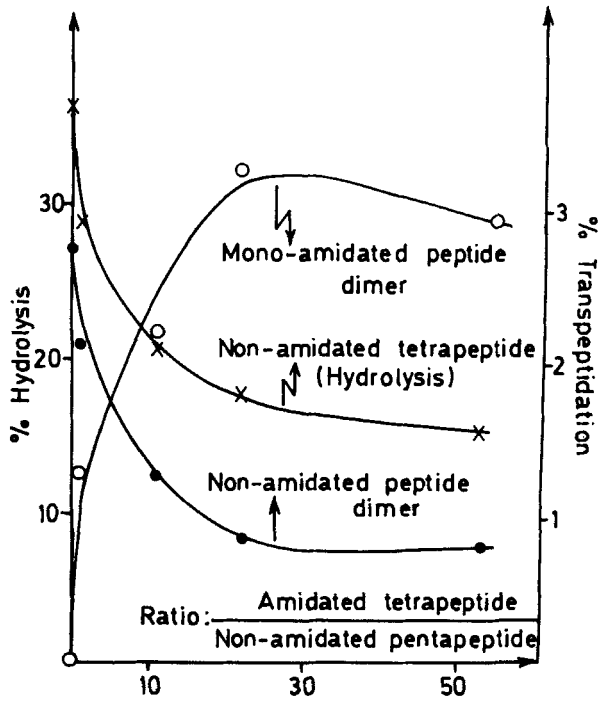


FIGURE 8. Effect of the R39 tetrapeptide on transpeptidation in the nonamidated pentapeptide system. Labeled pentapeptide (1.4 mM) either alone or in the presence of unlabeled Glu-amidated tetrapeptide (1.4–70 mM) were incubated with fraction A for 8 hr at 37° C. The various radioactive compounds were separated by paper electrophoresis (see TABLE 1). For other conditions, see FIGURE 5.

peptide dimer that is identical to the dimer found in the *E. coli* peptidoglycan would further allow the suggestion that this transpeptidase is the physiological one.

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