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Fractionation of the DD-Carboxypeptidase-Transpeptidase Activities Solubilized from Membranes of *Escherichia coli* K12, Strain 44

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A transpeptidase activity in *Escherichia coli* was measured independently of other enzymes involved in peptidoglycan synthesis by quantitating the formation of UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-[^{14}C]glycine when UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine was used as donor substrate and [^{14}C]glycine as acceptor in a transfer reaction. After extraction of membrane envelopes with Brij-36T and subsequent ammonium sulfate precipitation, DEAE-cellulose chromatography revealed two major fractions; one not adsorbed to the ion-exchange resin and the other adsorbed. The fraction which was bound to DEAE-cellulose was bound to and could be eluted from an ampicillin affinity chromatography system while the fraction not bound to DEAE-cellulose was also not bound to the ampicillin column. Both unbound and bound ampicillin fractions exhibited DD-carboxypeptidase and transpeptidase activities although for equivalent DD-carboxypeptidase activity, the bound ampicillin fraction required about five times more glycine acceptor to achieve the same amount of transpeptidation as the unbound ampicillin fraction.

It is clear from a number of bacterial systems studied [1–9] that crosslinked peptidoglycan can be generated when the nucleotide precursors, UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-pentapeptide, are incubated with Mg^{2+} ions and either crude disrupted cellular preparations or isolated membranes that contain all the enzymes necessary for bacterial wall synthesis. However, because of its complexity, such a multi-enzyme system does not allow assay of the transpeptidase activity independently of the preceding biosynthetic reactions, so that the interaction of penicillin with the transpeptidase *per se* cannot be studied. It has been proposed that in *Escherichia coli* [1–5], a soluble enzyme cleaving the terminal D-alanine residue of the UDP-*N*-acetylmuramyl-pentapeptide in a DD-carboxypeptidase reaction is distinct from that enzyme in the particulate preparation responsible for transpeptidation. In view of the fact that each of the two purified single polypeptide chain enzymes isolated from the culture filtrates of *Streptomyces* R61 and R39 are able to carry out both DD-carboxypeptidase and transpeptidase functions [10–12], it was decided to reinvestigate such processes in *E. coli*. An abstract of a part of this work has appeared [13]. The experiments in this paper and the following one [14] have been presented at the June 6–8, 1973 meeting of the New York Academy of Sciences on the mode of action of antibiotics.

Abbreviations. Brij-36T, polyoxyethylated ($\bar{n} = 10$) laurylether; Triton X-100, polyoxyethylated ($\bar{n} = 10$) octylphenol.

MATERIALS AND METHODS

Strain

E. coli K12 F$^-$, strain 44, a β-lactamase-negative mutant [14], was used throughout the course of these studies.

Membrane Preparations

Membrane envelopes were obtained from cells grown at 37 °C in 1-liter flasks containing 300 ml Difco antibiotic medium no. 3. Batches of 9 liters were prepared and when the cells reached an absorbance of 0.9 at 550 nm, they were collected by centrifugation and were suspended without washing in 600 ml 0.01 M Tris-HCl buffer pH 7.9 containing

0.75 M sucrose, with the help of a Potter-Elvehjem tissue homogenizer. The cell suspension was cooled to 4 °C, 60 mg egg-white lysozyme (in 30 ml water) were added and the mixture was incubated in ice for 2 min. The suspension was then slowly diluted (10 min) with 1200 ml cold 1.5 mM EDTA pH 7.5. Spheroplasting was virtually complete as previously observed when *Salmonella typhimurium* was treated under identical conditions [15]. The spheroplast suspension was frozen in a bath of solid CO_2-acetone, slowly thawed in cold water and the freezing-thawing procedure was repeated once [16]. The suspension of lysed spheroplasts was centrifuged at $1000 \times g$ for 20 min in order to remove whole cells and then recentrifuged at $48000 \times g$ for 1 h. The membrane pellet was washed twice with 300 ml 0.05 M Tris-HCl buffer pH 7.8 containing 0.2 mM dithiothreitol. The crude membrane preparation could be stored as a pellet at −20 °C for several weeks without loss of activity.

The cytoplasmic and outer membranes of *E. coli* were separated from each other by equilibrium density-gradient centrifugation of the total membrane fraction, exactly as described by Osborn *et al.* for *S. typhimurium* [15].

*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*-[^{14}C]alanyl-*D*-[^{14}C]-alanine*

The unlabelled nucleotide pentapeptide was prepared either from *Bacillus subtilis* W23 [17] or from *Bacillus megaterium* KM [18]. The radioactive nucleotide was synthesized enzymatically by the addition of D-[^{14}C]alanyl-D-[^{14}C]alanine to UDP-*N*-acetyl-muramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid [18]. Specific activity was 60 Ci/mol.

Paper Electrophoresis

Electrophoresis at pH 1.8 (formic acid-H_2O, 20:1000, v/v) was carried out on Whatman 3MM paper at a constant voltage of 60 volts per cm using a Gilson High Voltage DW Electrophorator. Electrophoretograms were scanned with a Packard Radiochromatogram Scanner and the radioactive peaks were cut into strips and counted with a Packard Tri-Carb liquid Scintillation Spectrometer.

DD-Carboxypeptidase Activity

DD-Carboxypeptidase activity was estimated by measuring the amount of D-alanine liberated from either the unlabelled or the radioactive nucleotide UDP-*N*-acetylmuramyl-pentapeptide. Unlabelled D-alanine was measured by the fluorodinitrobenzene technique [19]. D-[^{14}C]Alanine was separated from UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]alanine by paper electrophoresis at pH 1.8 and the radioactivity was measured as indicated above. One unit of DD-carboxypeptidase (U) released 1 μmol D-alanine per min when UDP-*N*-acetylmuramyl-pentapeptide (1.2 mM, final concentration) was exposed to the enzyme preparation in 35 μl (final volume) of 0.06 M Tris-HCl buffer pH 8.5, containing 0.05 M $MgCl_2$, 0.14 mM dithiothreitol and 0.36% (w/v) or more of Brij-36T. In some cases, Triton X-100 (0.1%, w/v) was used instead of Brij-36T.

Transpeptidase Activity

Transpeptidase activity was estimated by measuring the amount of UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-[^{14}C]glycine formed when 1.2 mM non-radioactive UDP-*N*-acetylmuramyl-pentapeptide (*i.e.* the donor) and [^{14}C]glycine (*i.e.* the acceptor) were exposed to the enzyme preparation at 37 °C under the same conditions as those described for measuring the DD-carboxypeptidase activity. The newly synthesized nucleotide pentapeptide was separated from excess of [^{14}C]glycine by paper electrophoresis at pH 1.8 and the radioactivity was counted as described above.

Buffers

Buffers designate Tris-HCl buffers of specified molarity and pH, supplemented with 0.2 mM dithiothreitol and 0.5% w/v Brij-36T.

Ampicillin-Affinose 202

500 mg ampicillin (Bristol, Pentrexyl) dissolved in 40 ml water was added to 50 ml affinose 202 (Bio-Rad). The pH value was adjusted to 4.6 with 1 N HCl and 1 g 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl (Ott Chemical Co.) dissolved in 5 ml water, was slowly added over a period of 5 min. The mixture was gently stirred for 1 h at room temperature, during which time the pH increased to 6.4 and then at 4 °C overnight. The ampicillin-affinose 202 was extensively washed with ice-cold water and poured into a coarse-sintered glass column at 4 °C. The column was equilibrated against and stored in 0.05 M buffer pH 7.9.

Protein Estimation and Absorbance at 240 nm

The absorbance at 240 nm was used to determine the amount of protein 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 dodecylsulfate. An absorbance of

1.0 at 240 nm corresponded to 0.385 mg membrane protein per ml. At 240 nm, the absorbance of a 0.5% (w/v) solution of Brij-36T (as in the buffers, see above) is about 0.1 and the greatest difference between protein absorbance and Brij-36T absorbance was found at this wavelength. Particulate fractions were first dissolved in 1% sodium dodecylsulfate.

Absorbance at 415 nm

The absorbance at 415 nm was used as an indication of the presence of cytochrome, flavoproteins and other components of membrane.

RESULTS

Localization of DD-*Carboxypeptidase Activity in* E. coli *K12, Strain 44*

Spheroplasts prepared according to the technique of Osborn *et al.* [15] were lysed by sonic oscillation [15]. The washed membranes were suspended in cold 25% sucrose (w/v) containing 5 mM EDTA (pH 7.5) and 0.2 mM dithiothreitol, to a concentration of about 15 mg protein/ml. Sucrose density-gradient centrifugation was carried out in the presence of 0.2 mM dithiothreitol, in a SW 41 rotor at 38000 rev/min for 15 h at 2—6 °C, and yielded two main visible bands, *i.e.* a heavy white band which in the case of *S. typhimurium* was identified as the lipopolysaccharide-containing outer membrane [15] and a yellow band of low apparent buoyant density. These two fractions were collected by puncturing the bottom of the tube. The yellow fraction contained succinic dehydrogenase activity. On reduction with sodium hydrosulfite, is exhibited two absorption peaks at 530 and 560 nm and the 412-nm peak of the non-reduced state was shifted to 428 nm and considerably amplified. Hence, this fraction was assumed to be derived from the inner membrane. Electron microscopy (Fig. 1 and 2) showed striking differences between the two membrane fractions and especially the occurrence of numerous pili within the plasma membrane fraction. DD-Carboxypeptidase activity in *E. coli* K12, strain 44, was not excreted during growth. It was neither periplasmic (by assaying the dialyzed fraction obtained by spheroplasting the cells) nor intracellular (by assaying the supernatant fraction after lysis of the spheroplasts). It was not associated with the outer membrane of the cell envelope but was bound to the inner plasma membrane fraction. Specific activity was about 6 mU/mg protein (in the presence of 0.1% Triton X-100 or 0.5% Brij-36T).

Extraction of the DD-*Carboxypeptidase Activity from Membrane Envelopes of* E. coli *K12, Strain 44*

Chaotropic agents (NaSCN, $NaClO_4$, $NaNO_3$, NaCl, guanidine-HCl and urea) which increase the water solubility of several particulate proteins [20] and butan-1-ol which solubilizes the membrane-bound DD-carboxypeptidase of *Bacillus stearothermophilus* [18] were not effective means for the resolution of the DD-carboxypeptidase activity of the membrane of *E. coli*, strain 44. Similarly, osmotic shock (using 4 mM Tris-HCl pH 7.8) alone or in combination with 5 mM EDTA did not release the activity.

Neutral detergents such as Triton X-100 (polyoxyethylated ($\bar{n} = 10$) octylphenol and Brij-36T (polyoxyethylated [$\bar{n} = 10$] laurylether) [21] were effective in solubilizing at least part of the DD-carboxypeptidase activity. The action of Triton X-100 (from 1 to 3%, w/v, final concentration) on membranes (2 mg protein/ml, final concentration), at 4 °C in 0.01 M Tris-HCl buffer pH 8, containing 0.2 mM dithiothreitol, followed by centrifugation at $100000 \times g$ for 1 h, yielded supernatant fractions which constantly exhibited 50—60% of the DD-carboxypeptidase activity of the membrane preparation. In the presence of 1 mM EDTA, the concentration of Triton X-100 could be reduced to 0.5% (w/v). The activity which remained associated with the pellet was not released by further treatment with Triton X-100 and EDTA.

The action of Brij-36T (from 0.5 to 3%, w/v) on membrane envelopes (2.5 mg protein/ml, final concentration) for 30 min at 37 °C, in 0.01 M Tris-HCl buffer pH 8 containing 1 mM EDTA and 0.2 mM dithiothreitol, followed by centrifugation at $100000 \times g$ for 2 h at 4 °C, constantly yielded supernatant fractions exhibiting DD-carboxypeptidase activity. The activity recovery varied from 25 to 50%. Prolongation of the incubation at 37 °C and further treatment of the pellet with Brij-36T-EDTA under the same conditions as above did not increase the yield of extraction. Although the Brij-36T-EDTA was less efficient than Triton X-100-EDTA as a solubilizing agent, the former system was finally chosen for large scale extractions because it permitted the accurate determination of the protein content of the fractions by measuring their absorbance at 240 nm (Materials and Methods) and because it resulted in more stable enzyme activities in the final preparations.

Fractionation of the Membrane-Bound DD-*Carboxypeptidase Activity of* E. coli *K12, Strain 44*

Ammonium Sulfate Precipitation. All steps in the fractionation procedure subsequent to Brij-36T extraction were carried out at 4 °C. To the Brij-36T supernatant obtained from the extraction of 500 mg membrane protein (final volume, 200 ml), solid ammonium sulfate was added up to 30% saturation. A floating material was collected by centrifugation and discarded since it contained virtually no DD-

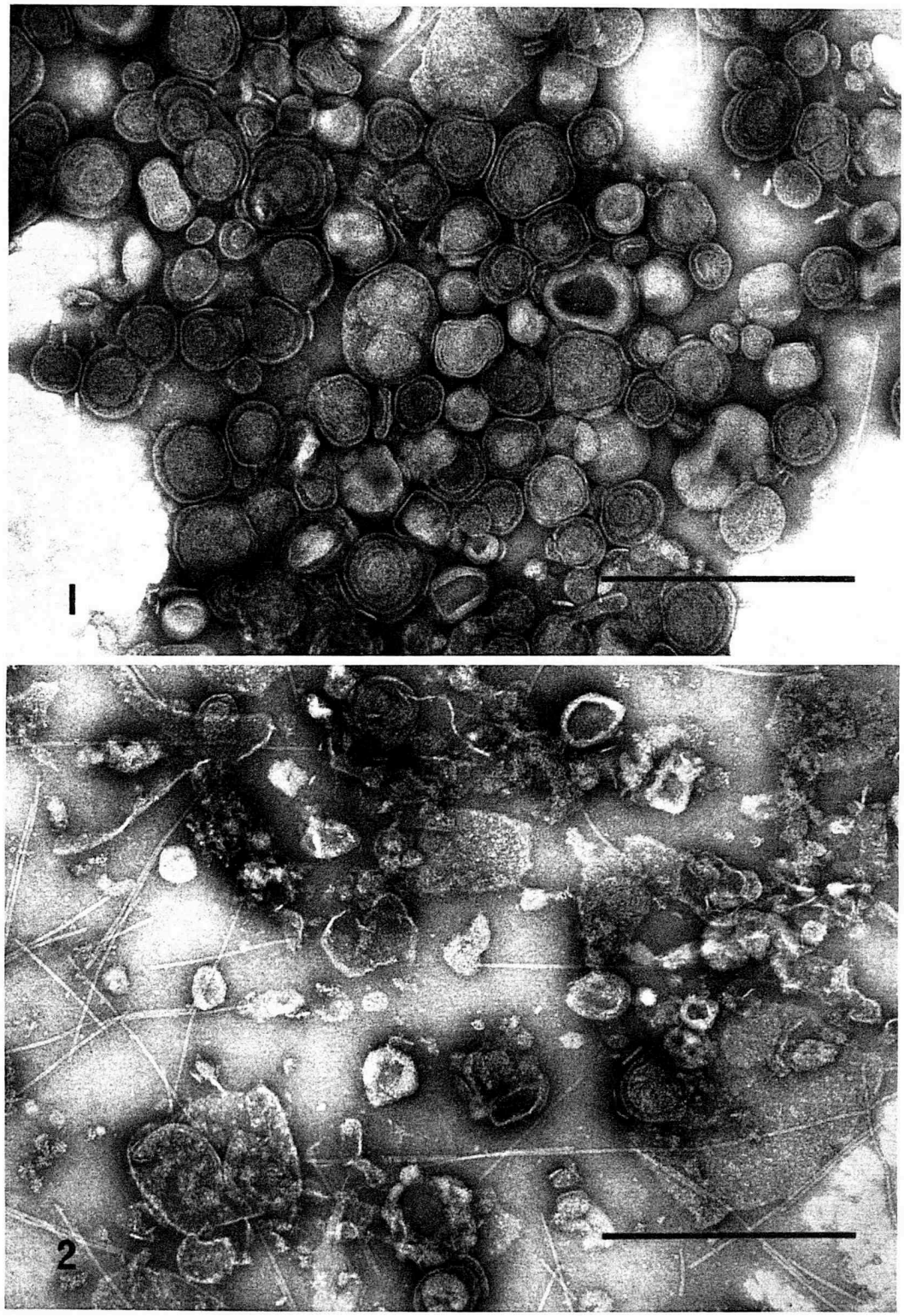

Fig. 1. *Electron micrograph of the outer membrane of* E. coli *K12, mutant strain 44.* Note the spherical shape of the membranes containing concentric structures which is typical of isolated lipopolysaccharide. Magnification, ×78000, bar indicates 0.5 μm

Fig. 2. *Electron micrograph of the inner membrane of* E. coli *K12, mutant strain 44.* The plasma membrane fragments are covered with small particles. Pili also occur in the preparation. Magnification, ×78000, bar indicates 0.5 μm

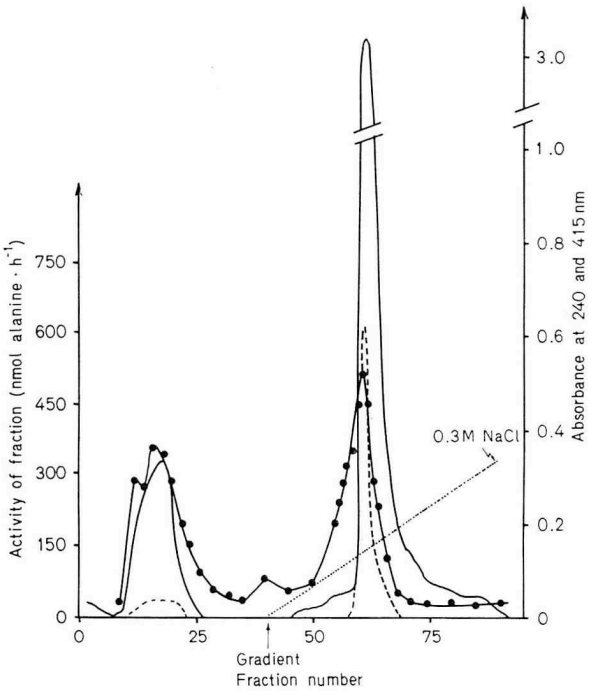

Fig. 3. *DEAE-cellulose chromatography in the presence of Brij-36T of the* DD-*carboxypeptidase system of* E. coli *K12, mutant 44.* For condition of chromatography, see text. Fractions (3.5 ml) were collected at a flow rate of 90 ml per hour. For DD-carboxypeptidase activity, 40 to 60 µl fractions were supplemented with 40 nmol unlabelled UDP-*N*-acetyl-muramyl-pentapeptide and incubated for 4 to 7 h at 37 °C in the presence of 0.01 M $MgCl_2$. Results are expressed in nmol alanine liberated × fraction^{-1} × h^{-1}. Absorbance at 415 nm is indicative of the presence of cytochromes. (——) Absorbance at 240 nm, (----) absorbance at 415 nm and (●——●) DD-carboxypeptidase activity

carboxypeptidase activity. The solution was then brought up to 60% saturation by the further addition of ammonium sulfate. A floating layer containing most of the enzyme activities was collected after centrifugation, dissolved in 0.05 M buffer pH 7.9 (Materials and Methods) and dialyzed against and diluted with the same buffer to 35 ml. Absorbance of the solution at 240 nm was 2.2 (*i.e.* 0.84 mg protein per ml).

Fractionation on DEAE-Cellulose. The dialyzed ammonium-sulfate precipitate fraction (35 ml containing 30 mg protein in 0.05 M buffer pH 7.9) was applied to a DEAE-cellulose column (2.5 × 10 cm) previously equilibrated against the same buffer. The column was washed with the buffer until the effluent had a very low absorbance at 240 nm. The non-adsorbed fractions which were active (no. 10—26, Fig. 3) were pooled. The DEAE-cellulose column was then treated with a linear gradient of NaCl (0—0.3 M) in 0.05 M buffer pH 7.9. The eluted fractions which had carboxypeptidase activity (no. 55—64, Fig. 3) were pooled.

Filtration on Ampicillin-Affinose 202 of the DD-*Carboxypeptidase Activity Non-Adsorbed on DEAE-Cellulose.* The pooled fractions which had not been adsorbed on DEAE-cellulose, were filtered on a ampicillin-affinose 202 column (2.5 × 2.5 cm) previously equilibrated against 0.05 M buffer pH 7.9. The column was washed with the same buffer. The active fractions of the effluent were pooled and concentrated by dialysis against dry carbowax 4000, yielding preparation A.

Chromatography on Ampicillin-Affinose 202 of the DD-*Carboxypeptidase Activity Adsorbed on and Eluted from DEAE-Cellulose.* The pooled fractions which had been adsorbed on DEAE-cellulose and eluted from it, were diluted 2-fold in 0.05 M buffer pH 7.9. The solution (absorbance of 0.8 at 240 nm, 0.3 mg protein per ml) was filtered through a ampicillin-affinose 202 column (2.5 × 11 cm) previously equilibrated against 0.05 M buffer pH 7.9. The column was washed with the same buffer until the effluent exhibited a low absorbance at 240 nm. These fractions (no. 1—30, Fig. 4) had very little activity and were discarded. If the applied protein concentration was increased from 0.3 mg/ml to 0.8 mg/ml or higher, considerably more DD-carboxypeptidase activity was found in fractions 1—30. The column was then sequentially treated first with 0.15 M NaCl in 0.05 M buffer pH 7.9 and then with 0.5 M buffer pH 8.6 (Fig. 4). The 0.15 M NaCl active fractions (no. 30—80) were pooled and diluted with 0.05 M buffer pH 7.9 until the concentration of NaCl had been lowered to 0.03 M. Similarly, the active fractions obtained by elution of the column with 0.5 M buffer (no. 80—125) were pooled and diluted 10-fold with 0.2 mM dithiothreitol—0.5% Brij-36T. The two active solutions were combined and submitted to an additional filtration on a ampicillin-affinose 202 column (2.5 × 2.5 cm) previously equilibrated against 0.05 M buffer pH 7.9. The enzyme activity was retained on the column and, after washing with the 0.05 M buffer pH 7.9, it was eluted with 0.5 M buffer pH 8.6. The active fractions were pooled, concentrated by dialysis against dry carbowax 4000 and finally dialyzed against 0.05 M buffer pH 8.6, yielding preparation B.

The final preparations, A and B, contained 5 to 10% (w/v) Brij-36T (as a result of their concentration on dry carbowax) and exhibited specific activities of about 5 mU/mg protein (see Table 1).

The same ampicillin columns have been used for more than one year at 4 °C to give identical results providing that the protein concentration applied to the column was kept at about 0.3 mg per ml. After each elution of the column with 0.5 M buffer which was performed in less than 1 h, the column was immediately washed and stored in the 0.05 M buffer pH 7.9.

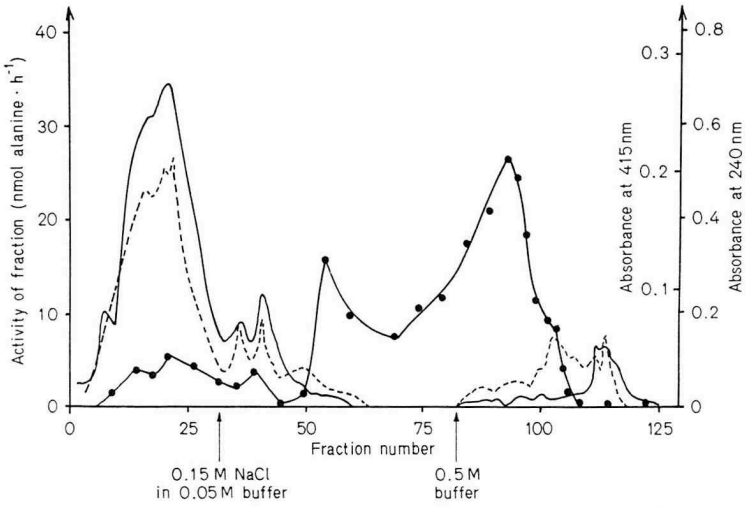

Fig. 4. *Ampicillin-affinose 202 chromatography in the presence of Brij-36T of the* DD-*carboxypeptidase fraction adsorbed on and eluted from DEAE-cellulose (Fig. 3).* For conditions of chromatography, see text. Fractions (3.5 ml) were collected at a flow rate of 300 ml per hour. For DD-carboxypeptidase activity, 80 µl of the fractions were incubated for 10 h at 37 °C with 40 nmol unlabelled UDP-*N*-acetylmuramyl-pentapeptide in the presence of 0.01 M $MgCl_2$. Results are expressed in nmol alanine liberated $\times$fraction$^{-1}\times h^{-1}$. Absorbance at 415 nm is indicative of the presence of cytochromes. For symbols see Fig. 3

Table 1. *Fractionation of the* DD-*carboxypeptidase activity of the membrane Brij-36T extract of* E. coli *K12, mutant 44*
For conditions, see Materials and Methods. $(NH_4)_2SO_4$ precipitate obtained after dissolution and dialysis against 0.05 M buffer pH 7.9. It should be noted that the (100%) recovery in the Brij-36T extract represents 30—50% of the available enzyme activity of the membrane fraction

Step	Specific activity	Enzyme recovery	Protein recovery
	mU/mg protein	%	
Brij-36T, membrane extract	3.3	(100)	(100)
0—30% $(NH_4)_2SO_4$ precipitate	0.23	3	42
30—60% $(NH_4)_2SO_4$ precipitate	13.3	70	13
Fraction not adsorbed on DEAE-cellulose	5	4	2
Fraction A	5	4	2
Fraction adsorbed on and eluted from DEAE-cellulose	2.5	8	10
Fraction B	5	2	1

Transpeptidase and DD-*Carboxypeptidase Activities of Fractions A and B*

In addition to DD-carboxypeptidase activity, the isolated membrane envelopes in the absence or presence of Brij-36T exhibited transpeptidase activity when exposed to the system UDP-*N*-acetylmuramyl-pentapeptide and glycine (Materials and Methods). In the course of fractionation, the ratio or transpeptidase activity to DD-carboxypeptidase activity underwent considerable variation so that fractions A and B differed in this respect. For equivalent DD-carboxypeptidase activity, fraction B required about 5 times more glycine acceptor to achieve the same amount of transpeptidation as fraction A (Fig. 5).

Effects of pH and Molarity of Tris-HCl buffer on DD-*Carboxypeptidase and Transpeptidase Activities*

Fig. 6 shows the effects of the pH value (8—9.5 in 0.18 M Tris-HCl buffer) and the molarity of the Tris-HCl buffer (0.05—0.5 M, at pH 8.6) of the reaction mixture (in the presence of 50 mM $MgCl_2$ and Brij-36T) on the hydrolysis of radioactive 0.017 mM UDP-*N*-acetylmuramyl-pentapeptide (carboxypeptidase activity) and the transfer reaction between non-radioactive 0.8 mM UDP-*N*-acetylmuramyl-pentapeptide and 4 mM [^{14}C]glycine (transpeptidase activity), by fraction A. Both activities were maximal at about pH 9.2 in 0.1 to 0.2 M Tris-HCl buffer.

DISCUSSION

DD-Carboxypeptidase activity resides exclusively in the inner membrane of *E. coli* K12, mutant strain 44. After extraction of membrane envelopes with Brij-36T and precipitation with ammonium sulfate, two fractions exhibiting DD-carboxypeptidase activity were obtained by DEAE-cellulose chromatography (Fig. 3). As indicated in Table 1, both the non-adsorbed and adsorbed fractions obtained by chromatography on DEAE-cellulose had lower specific activities than the 30—60% ammonium sulfate precipitate even though more than 90% of the pro-

tein was recovered in the two fractions. One possible explanation for this loss of specific activity and consequently the poor yield of enzyme recovery, is the redistribution or modification of certain lipid components within these fractions. It was observed when detergent was omitted from the buffer system during various fractionation procedures that no catalytic activity could be detected in the isolated fractions. However, when a total lipid extract from *E. coli* was added back to these fractions, some reconstitution of enzyme activity was noted although this was not quantitatively reproducible (unpublished results). It is noteworthy that the "purified", soluble DD-carboxypeptidase isolated from extracts of *E. coli* strain B obtained by sonic oscillation of the cells [4, 5] also had a very low specific activity (5 nmol D-alanine liberated $\times$ mg protein^{-1} $\times$ min^{-1} when the concentration of UDP-*N*-acetylmuramyl-pentapeptide used was 0.01 mM).

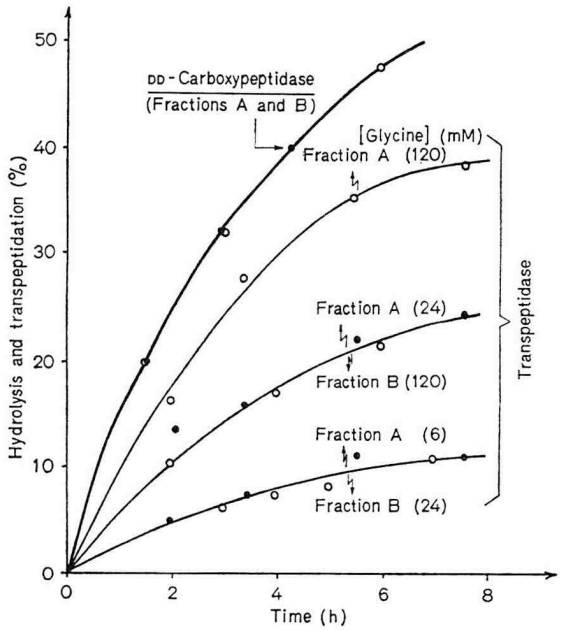

Fig. 5. *DD-Carboxypeptidase activity* vs *transpeptidase activity of fractions A and B: time couse.* Fractions A and B (120 μU) were incubated with unlabelled UDP-*N*-acetylmuramyl-pentapeptide (1.2 mM) in the absence of glycine for DD-carboxypeptidase activity and in the presence of [^{14}C]glycine (from 6 mM 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 activity, see Materials and Methods

Of the two fractions, only that one which was adsorbed on to and eluted from DEAE-cellulose could be bound to the ampicillin-affinose affinity column. During this process most of the protein and the marker cytochrome were not bound to the ampicillin column while the majority of the DD-carboxypeptidase activity was fixed on it (Fig. 4). After washing the column with 0.05 M buffer pH 7.9, elution with 0.15 M NaCl in 0.05 M buffer pH 7.9 resulted initially in the release of a considerable quantity of protein together with cytochrome and of a small amount of enzyme, and later in the release of the majority of the available enzyme activity and of very little protein. If the elution with 0.15 M NaCl was allowed to continue past fraction 80 (Fig. 4) a slow release of DD-carboxypeptidase activity was still noted. At this point, better release of activity was produced in changing the eluent to 0.5 M buffer pH 8.6 and again very little protein was eluted. When 0.05 M buffer pH 7.9 was supplemented with 0.5 M NaCl instead of 0.15 M NaCl, more DD-carboxypeptidase was released. Additional activity, although less than before, could still be eluted by the 0.5 M buffer pH 8.6.

An analysis of Fig. 4 indicates that the affinity chromatography system removed about 85% of the

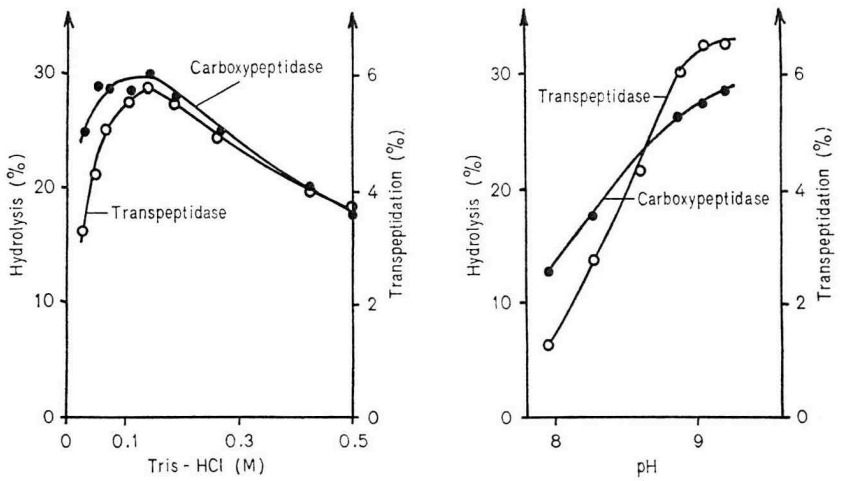

Fig. 6. *Effect of pH and molarity of Tris-HCl buffer on DD-carboxypeptidase and transpeptidase activities of fraction A.* For conditions, see text

contaminating protein but fraction B (Table 1) did not show a commensurate increase in specific activity. Again the explanation may be related to certain lipid-activating components or to increased instability as purification proceeds. Upon analysis of fraction B by sodium dodecylsulfate-polyacrylamide-gel electrophoresis, 3—4 bands were revealed by staining with coomassie blue, indicating that the procedure had resulted in considerable purification. The fact that both DD-carboxypeptidase and transpeptidase activities could be eluted with 0.15 M NaCl (Fig. 4) suggests that the *E. coli* transpeptidase was not irreversibly bound to ampicillin [3, 5]. However, Tris-HCl buffer is a weak nucleophile which might cleave an acylated complex formed between the β-lactam ring of ampicillin and a protein sulfhydryl or hydroxyl group. It should also be noted that membrane proteins and membrane lipids are known to bind avidly to one another. Under these conditions, the affinity column should not be used as an indication of what type of complex is formed.

For the equivalent DD-carboxypeptidase activity, there was a variation in the amounts of transpeptidation product formed by fractions A and B (Fig. 5). Despite these quantitative differences, there is no reason at this stage to suspect that the catalytically active proteins present in the two fractions are different. When dealing with membrane proteins, it is exceedingly difficult to establish whether or not multiple catalytic systems occur in the membrane [22] (as sometimes proposed [23]). Indeed, the same active proteins can be located in different physical environments (especially with regard to the lipids). Moreover, with the exocellular DD-carboxypeptidases-transpeptidases from *Streptomyces* R39 and R61, it was shown that the proportion of the enzyme activity that could be channelled into the transpeptidation and the hydrolysis pathways depended very much upon the environmental conditions [10—12]. A more detailed study of the enzyme activities exhibited by the two fractions A and B isolated from the membranes of *E. coli*, strain 44, is presented in the following paper [14].

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