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Substrate Requirements of the *Streptomyces albus* G DD Carboxypeptidase*

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ABSTRACT: *Streptomyces albus* G secretes a carboxypeptidase which hydrolyzes C-terminal D-alanylglycine, C-terminal N^{α}-(D-alanyl)-D, and with a much lower efficiency, C-terminal glycyl-D-alanine linkages. The side chain of the C-terminal D residue may be a long peptide sequence. The K_m values for the binding of peptides ending in L → D-alanyl → D sequences, to the carboxypeptidase are essentially controlled by the structure of the L-amino acid that precedes the C-terminal D-alanyl-D linkages.

The substrate requirements of the soluble DD carboxypeptidase and of the membrane-bound transpeptidase involved in wall synthesis present striking similarities. It appears that the peptides which act as carboxyl donors in the transpeptidation reaction are those which are recognized by the DD carboxypeptidase. It is proposed that the soluble DD carboxypeptidase (or a very closely related enzyme) acts as a transpeptidase when it is integrated in the cell membrane.

Two types of enzymes, transpeptidases and carboxypeptidases, which characteristically recognize peptides ending in C-terminal acyl-D-alanyl-D-alanine sequences are known to occur in prokaryotic cells. Membrane-bound transpeptidases catalyze the last step of the bacterial wall peptidoglycan biosynthesis (Tipper and Strominger, 1965; Wise and Park, 1965). The mechanism of the reaction is such that the carbonyl group which is transferred from one peptide to the amino group of a second peptide always belongs to the penultimate C-terminal D-alanine residue of the peptide donor. As a result, interpeptide bonds are formed and D-alanine residues are released in equivalent amounts (Izaki *et al.*, 1968). DD Carboxypeptidases catalyze the simple hydrolysis of C-terminal D-alanyl-D-alanine linkages, *i.e.*, a reaction which is not coupled with peptide-bond formation. DD Carboxypeptidases have been detected in and partially purified from disrupted cells of *Escherichia coli* (Izaki and Strominger, 1968), *Bacillus subtilis* (Strominger *et al.*, 1969; Matsuhashi *et al.*, 1969), and the blue-green alga *Anabaena variabilis* (Matsuhashi *et al.*, 1969). More recently, a DD carboxypeptidase excreted by *Streptomyces albus* G was also isolated and purified (Ghuysen *et al.*, 1970). The purpose of the present paper is to describe the substrate requirements of this latter enzyme.

Materials and Methods

Analytical Techniques. Reducing groups (Park–Johnson procedure), acetamido sugars (Morgan–Elson reaction), amino acids (fluorodinitrobenzene technique), D-alanine (enzymatic procedure), and N- and C-terminal groups (fluorodinitrobenzene and hydrazinolysis techniques, respectively) were measured as previously described (Ghuysen *et al.*, 1966, 1968).

Electrophoresis was carried out on Whatman No. 3MM paper using an Electrorheophor apparatus Pleuger at pH 6 (acetic acid–pyridine–water 0.33:4:1000 v/v).

Chromatography. The following solvents were used: (I) isobutyric acid–1 N NH_4OH (5:3 v/v); (II) 1-butanol–acetic acid–water (4:1:5 v/v upper phase); (III) chloroform–methanol–acetic acid (88:10:2 v/v); (IV) chloroform–methanol–acetic acid–water (65:25:13:8 v/v). Chromatography was performed on Whatman No. 1 paper, on thin-layer plates of cellulose MN 300 HR (Macherey, Nagel and Co., Düren), and on thin-layer plates of Stahl's silica gel G (Merck). Dinitrophenylamino acids were separated on silica gel plates using solvent III except N^{α}-dinitrophenylornithine and N^{δ}-dinitrophenylornithine which were separated using solvent IV.

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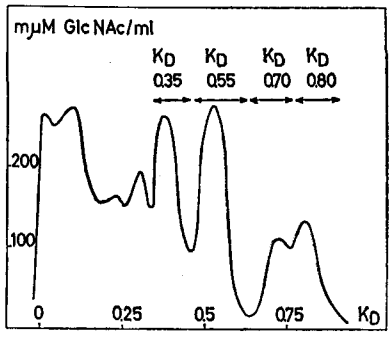


FIGURE 1: Sephadex filtration of walls of *C. poinsettiae* degraded with *Chalaropsis* endo-*N*-acetylmuramidase. Reducing groups are expressed in *N*-acetylglucosamine equivalent.

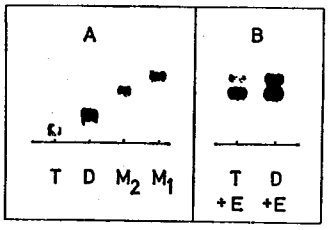


FIGURE 2: Characterization of degraded products from *C. poinsettiae*. (A) Silica gel thin-layer chromatography (solvent I) of fractions K_D 0.35 (T), K_D 0.55 (D), K_D 0.70 (M_2), and K_D 0.80 (M_1) obtained by Sephadex filtration (Figure 1) of degraded walls of *C. poinsettiae:* T, trimer; D, dimer; M_2, monomer (with C-terminal D-alanine); and M_1, monomer (with C-terminal L-homoserine). (B) Degradation of dimer (D + E) and trimer (T + E) into monomers by DD carboxypeptidase. Conditions of incubation: 20 mμequiv of C-terminal N^{α}-(D-alanyl)-D-ornithine linkages (*i.e.*, 20 mμmoles of dimer or 10 mμmoles of trimer) were incubated at 37° with 5 μg of the DD carboxypeptidase preparation in 30 μl of 0.01 M Veronal buffer, pH 9. Reaction was complete after 4 hr. Estimation of terminal N^{α}-ornithine: before treatment = 0.45 and 0.30, per glutamic acid, for dimer and trimer, respectively; after treatment = 1.1 and 1.11.

Enzymes. Chalaropsis endo-*N*-acetylmuramidase was a gift from Dr. J. H. Hash (Hash and Rothlauf, 1967) (Vanderbilt University, Nashville, Tenn.); *Streptomyces* F_1 endo-*N*-acetylmuramidase, *N*-acetylmuramyl-L-alanine amidase, and aminopeptidase were previously described (Ghuysen *et al.*, 1969); *Streptomyces* DD carboxypeptidase, a preparation containing 100,000 units/mg of protein, was also used (Ghuysen *et al.*, 1970).

Sephadex Filtrations. Gel filtrations of the compounds were expressed in terms of distribution coefficients $K_D = (V_e - V_0)/V_i$ with V_e = elution volume, $V_0 = V_e$ of totally excluded material and $V_i = V_e$ of NaCl $- V_0$.

Wall Peptides. Peptide monomers, dimers, and oligomers were prepared from walls of *S. aureus*, *L. acidophilus*, *B. rettgeri*, *E. coli*, *C. poinsettiae*, and *B. subtilis*.

S. aureus. Walls were prepared from cells grown in the presence of sublethal doses of penicillin G and they were degraded by the *Streptomyces* F_1 endo-*N*-acetylmuramidase. The disaccharide pentapeptide pentaglycine N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl), N^{ϵ}-(pentaglycyl)-L-lysyl-D-alanyl-D-alanine (Table III, **23**), and the bisdisaccharide pentapeptide pentaglycine dimer (Table III, **26**) were isolated from the degraded products as previously described (Muñoz *et al.*, 1966; Ghuysen *et al.*, 1969). Degradation of the disaccharide pentapeptide pentaglycine by *N*-acetylmuramyl-L-alanine amidase yielded the pentapeptide pentaglycine (Table III, **24**). Degradation of the disaccharide pentapeptide pentaglycine by aminopeptidase yielded the disaccharide pentapeptide (Table III, **14**). Degradation of the bisdisaccharide pentapeptide pentaglycine dimer by aminopeptidase yielded a bisdisaccharide pentapeptide dimer containing only one pentaglycine sequence (in endo position) (Table III, **25**). The peptide compounds were purified by filtration in 0.1 M LiCl on two columns of Sephadex G-50 and Sephadex G-25 connected in series and subsequently desalted by filtration in water on Sephadex G-25.

L. acidophilus. The isolation of the disaccharide peptide monomer N^{α}-(*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl), N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanyl-D-alanine, of the bisdisaccharide peptide dimer and of the trisdisaccharide peptide trimer, from a log-phase wall autolysate has been described (Coyette and Ghuysen, 1970). Disaccharide-free peptide monomer (Table III, **21**) and dimer (Table III, **22**) were obtained by *N*-acetylmuramyl-L-alanine amidase treatments.

E. coli and B. rettgeri. The isolation from *E. coli* of the bisdisaccharide peptide dimer (Table I, **8**) (van Heijenoort *et al.*, 1969) from which the peptide dimer (Table I, **9**) was prepared by amidase treatment, and the isolation from *B. rettgeri* of the peptide dimer (Table I, **5**) (Guinand *et al.*, 1969), have been described.

C. poinsettiae. Walls of *C. poinsettiae* (for the structure of the peptidoglycan moiety, see Perkins, 1967), contained per milligram about 400 mμequiv of *N*-acetylglucosaminyl-*N*-acetylmuramylglycyl-γ-D-glutamyl-L-homoseryl-D-alanine units, cross-linked by single D-ornithine residues extending from the C-terminal D-alanine to the α-carboxyl group of D-glutamic acid. D-Ornithine is linked to D-alanine through its α-amino group, and to D-glutamic acid by its δ-amino group. Walls (150 mg) were solubilized with *Chalaropsis* endo-*N*-acetylmuramidase (700 μg in 30 ml of water). Solubilization was complete after 30 min, but the incubation was prolonged up to 5 hr. Complete degradation of the glycan into disaccharide units had occurred. Filtration of the degraded products in 0.1 M LiCl on two identical columns of Sephadex G-50 and Sephadex G-25 connected in series ($V_0 + V_i$ = 750 ml) yielded several fractions (Figure 1). Fractions K_D 0.80, K_D 0.70, K_D 0.55, and K_D 0.35 were further purified and desalted by filtration in water on the Sephadex G-50 and Sephadex G-25 columns system. Each fraction presented a symmetric elution profile and was found to be homogeneous by silica gel thin-layer chromatography using solvent I (Figure 2A). Amino acid composition, estimation of disaccharide units, and of terminal N^{α}-ornithine groups indicated that compound M_1 was the disaccharideglycyl-γ-[N^{δ}-(D-glutamyl)-D-ornithine]-L-homoserine and compound M_2 was the disaccharide-glycyl-γ-[N^{δ}-(D-glutamyl)-D-ornithine]-L-homoseryl-D-alanine. Fraction D was identified as a mixture of two bisdisaccharide peptide dimers (Table I, **6**) (with either D-alanine or L-homoserine as the C-terminal position) and fraction T as a mixture of two trisdisaccharide peptide trimers (Table I, **7**) (with either C-terminal D-alanine or C-terminal L-homoserine). Final proof of the structures was obtained by estimating the

number of N^{α}-(D-alanyl)-D-ornithine interpeptide linkages hydrolyzed by means of the DD carboxypeptidase (*vide infra*, and Figure 2B).

B. subtilis. The peptide dimer shown in Table I, **10** was isolated from walls of vegetative cells of *B. subtilis* (Warth and Strominger, 1968; Warth, 1969). It was a gift of Dr. A. D. Warth (University of Wisconsin, Wis.). The structure of this heptapeptide diamide is identical with that of the *E. coli* octapeptide (Table I, **9**) except that the carboxyl groups located on the D carbon of the *meso*-diaminopimelic acid residues are amide substituted and that the carboxyl group located on the L carbon of one of the *meso*-diaminopimelic acid residues is C terminal.

Uridine-5'-pyrophosphoryl-N-acetylmuramyl Peptides. (1) UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine was a gift of Dr. A. J. Garrett (Medical Research Council, London). It was isolated from *Bacillus subtilis* W_{23} (Garrett, 1969). (2) N^{α}-(UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl)-L-lysyl-D-alanyl-D-alanine (from *S. aureus*) and (3) UDP-*N*-acetylmuramyl-glycyl-γ-D-glutamyl-L-homoseryl-D-alanyl-D-alanine (from *C. poinsettiae*) were obtained as previously described (Chatterjee and Perkins, 1966). The L configuration of the homoserine has been established recently (H. R. Perkins, in preparation). (4) UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L_1)-LL-diaminopimelyl-(L_1)-D-alanyl-D-alanine was obtained from *Streptomyces albus* G as follows. Conidia were grown for 24–48 hr at 25° with shaking in broth medium (Oxoid peptone, 10 g; $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 1 g; KCl, 0.5 g; water, 1 l.). Actively growing cultures (25 ml) were transferred in 1-l. flasks containing 500 ml of medium and after 18 hr of growth, the mycelium from each culture was harvested and resuspended in 150 ml of fresh medium containing 12 μg of Ristocetin/ml. After 5 hr of shaking at 25°, the mycelium of each culture was harvested, washed, and suspended in 50 ml of a cold 10% trichloroacetic acid solution. After shaking at 0° for 1 hr, the trichloroacetic acid supernatants were pooled, extracted with ether, and lyophilized. The residue was filtered on a 75-ml column of Sephadex G-25 in 0.01 N acetic acid and the material that gave a positive Morgan–Elson reaction after acid hydrolysis (5 min at 100° in 0.05 N HCl) was further purified by chromatography on paper Whatman No. 3MM using solvent I. Ultraviolet illumination and ninhydrin revealed the presence of two distinct compounds close to the origin. The compound of higher mobility was submitted to a second chromatography under the same conditions. The yield was 0.3 μmole of nucleotide per l. of *Streptomyces* suspension. Analysis of the nucleotide showed that muramic acid, LL-diaminopimelic acid, glutamic acid, L-alanine, D-alanine, and glycine occurred in the molar ratios 1:1:1:1:1.60:0.25. Attempts to further purify the nucleotide by other means failed. Chromatography of the *N*-acetylmuramyl peptide (obtained after hydrolysis of the nucleotide with 0.05 N HCl, at 100° for 5 min) on cellulose thin-layer plates using solvent II yielded only one band. The amino acid composition of this *N*-acetylmuramyl peptide material was identical with that of the nucleotide. Degradation of the nucleotide preparation by means of the DD carboxypeptidase under given conditions (*vide infra*, Table III, **18**), resulted in the maximal liberation of 0.75 of D-alanine residue/LL-diaminopimelic acid. This result together with the above analytical data were compatible with the presence in the preparation of two nucleotides: UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L_1)-LL-diaminopimelyl-(L_1)-D-alanyl-D-alanine and, possibly, UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L_1)-LL-diaminopimelyl-(L_1)-glycyl-D-alanine, occurring in the molar ratio 1 to 0.30. The involvement of a nucleotide ending in glycyl-D-alanine in the synthesis of a small part of the *Streptomyces* peptidoglycan had been previously postulated (Leyh-Bouille *et al.*, 1970). Owing to the substrate requirements of the DD carboxypeptidase (*vide infra*), such a nucleotide should be much more resistant to the enzyme than the normal one.

In some cases, the *N*-acetylmuramyl peptides were prepared by acid hydrolysis of the nucleotides (0.05 N HCl, 100°, 5 min) and the corresponding peptides were prepared by further treatment with *N*-acetylmuramyl-L-alanine amidase.

Peptide from Sporidesmolide. L-α-Hydroxyisovaleryl-D-valyl-D-leucine from Sporidesmolide I (Russell, 1966) was a gift from Dr. D. W. Russell (Dalhousie University, Halifax).

Synthetic Peptides. Phenylacetylglycyl-D-serine, phenylacetylglycyl-D-leucine, phenylacetylglycyl-D-valine, and phenoxyacetylglycyl-D-valine were gifts from Dr. B. E. Erlanger (Columbia University, New York) (Erlanger and Goode, 1967). N^{ϵ}-Acetyl-L-lysyl-D-alanyl-D-alanine and acetyl-D-alanyl-L-alanyl-D-alanyl-D-alanine were gifts from Dr. A. Loffet (Union Chimique Belge, Bruxelles). All the other peptides were synthesized during the course of the present work. In general, synthesis was by the solid-phase method (Merrifield, 1963; Marshall and Merrifield, 1965). After the peptides had been synthesized and purified the amino groups were acetylated as follows. α-Amino groups were acetylated as previously described (Perkins, 1969). Amino groups, α and ω together, were acetylated by treating 1 mmole of peptide with 3–3.5 mmoles of triethylamine and 2.4 mmoles of acetic anhydride in dioxan–water, 1:1 (3–4 ml), at 0° for 2–3 hr. The acetylated peptide was purified by ion-exchange chromatography on a small column of Zeo-Karb 225 (H^+), which removed unreacted material and base. The yield of diacetylated peptides was 60–80%. Larger quantities of diacetyl-L-lysyl-D-alanyl-D-alanine were also synthesized by conventional methods using both carbodiimide and *N*-hydroxysuccinimide ester coupling procedures (details of peptides syntheses will be published elsewhere; M. Nieto and H. R. Perkins, in preparation).

Experimental Section

Specificity Profile of the Enzyme. The sensitivity of the various peptides to the *Streptomyces* DD carboxypeptidase was routinely determined by incubating 15 mμmoles of peptide in 34 μl, final volume, of 0.01 M Veronal buffer, pH 9, in the presence, depending upon the peptide, of 0.2–10 μg of enzyme protein, for 10 min up to 24 hr, at 37°. The results were expressed in mμequiv of linkage hydrolyzed per hr, per mg of enzyme (specific activities). Kinetics of the reactions were determined by estimating the amount of C-terminal amino acid released or, in the case of the peptide dimer and oligomers by estimating the amount of terminal amino groups exposed (Ghuysen *et al.*, 1969; Figure 2B). Degradation into monomers was also followed by appropriate paper electrophoresis or chromatography techniques (Figure 2B). Activities were always calculated on a 50% extent of hydrolysis. With several peptides, the tests were also per-

TABLE I: Influence of the C-Terminal Residue on Carboxypeptidase Activity.

No.	Compd	Specific Activity[a]
1	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala → L-Ala	0
2	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala → Gly	5,000
3	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala → D-Leu	11,000
4	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala → D-Ala	24,000
5	H-L-Ser → D-Glu (→ L-Orn → D-Ala → D-Lys or D-Orn —OH; L-Orn: H)	250
6	Disacch → Gly → D-Glu (→ L-homoSer → D-Ala → D-Orn-OH; D-Glu → L-homoSer → (D-Ala-OH)[b])	7,000
7	Same as peptide 6, but trimer instead of dimer	12,000
8	Disacch → L-Ala → D-Glu-OH (L → D-Ala-OH; DAP → D-Ala → OH (D); DAP: H — OH (D))	12,500
9	Same as dimer 8, but without disaccharide substituents	20,000
10	H-L-Ala → D-Glu-OH (L → OH; DAP → D-Ala → NH_2 (D); DAP: H — NH_2 (D))	750

[a] Expressed in mμequivalent of linkage hydrolyzed per mg of enzyme, per hr, in 0.01 M Veronal buffer, pH 9. Heavy arrows show the sensitive linkages. [b] A mixture of two dimers with either C-terminal D-alanine or C-terminal L-homoserine (peptide **6**) or C-terminal L-ornithine (peptide **5**). Origin of the peptides: **1**, **2**, **3**, **4**: synthetic peptides; **5**: *B. rettgeri* walls; **6**, **7**: *C. poinsettiae* walls; **8**, **9**: *E. coli* walls; **10**: *B. subtilis* walls. Degradation of the oligomers into monomers was also followed by electrophoresis at pH 6 (compounds **6** and **7**), and by silica gel thin-layer chromatography in solvent I (compound **5**). Disacch = β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl.

TABLE II: Influence of the Penultimate C-Terminal Residue on Carboxypeptidase Activity.

No.	Compd	Specific Activity[a]
1	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala ➡ D-Ala	24,000
2	N^{α},N^{ϵ}-Bisacetyl → L-Lys → Gly ➡ D-Ala	1,500
3	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Leu → D-Ala	0
4	N^{α},N^{ϵ}-Bisacetyl → L-Lys → L-Ala → D-Ala	0
5	N^{α},N^{ϵ}-Bisacetyl → L-Lys → L-Ala → L-Ala; phenylacetyl → Gly → D-Ser; phenylacetyl → Gly → D-Leu; phenylacetyl → Gly → D-Val; phenoxyacetyl → Gly → D-Val; L-α-isohydroxyvaleryl → D-Val → D-Leu	0 0

[a] Expressed in mμequiv of linkage hydrolyzed per mg of enzyme, per hr, in 0.01 M Veronal buffer, pH 9. Heavy arrow show the sensitive linkages. Origin of the peptides: synthetic peptides except L-α-isohydroxyvaleryl → D-Val → D-Leu which was prepared from Sporidesmolide I (Russell, 1966).

formed in the presence of 0.02 M Tris-HCl buffer, pH 7.5, supplemented with 0.002 M $MgCl_2$. A twofold increase of the specific activity was generally observed when the Tris-Mg^{2+} buffer was used instead of the Veronal buffer. Results are reported in Tables I to III. They show the influence exerted on the carboxypeptidase activity by the C-terminal residue (Table I), by the penultimate C-terminal residue (Table II), and by the residue that precedes the C-terminal D-alanyl-D linkage (Table III).

Michaelis constant and maximal velocity (Table IV) were determined at 37° in 0.02 M Tris–0.002 M $MgCl_2$ buffer, pH 7.5, for several peptides that were selected owing to the large differences observed for the corresponding enzyme sensitivities. These determinations were based on initial velocity measurements.

Peptide Inhibitors. N^{α},N^{ϵ}-Bisacetyl-L-lysyl-D-alanyl-D-alanine (15 mμmoles) was incubated at 37°, for 20 min, in 30 μl (final volume) of 0.02 M Tris–0.002 M $MgCl_2$ buffer, pH 7.5, with 1 μg of enzyme and in the presence of the following peptides (up to 150 mμmoles): N^{ϵ}-acetyl-L-lysyl-D-alanyl-D-alanine, N^{α}-acetyl-L-lysyl-D-alanyl-D-alanine, N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-leucyl-D-alanine, N^{α},N^{ϵ}-bisacetyl-L-lysyl-L-alanyl-D-alanine, and the hexapeptide diamide (Table I, **10**). No inhibition of the carboxypeptidase activity was observed. Similarly, phenylacetylglycyl-D-serine, phenylacetylglycyl-D-leucine, phenylacetylglycyl-D-valine, and phenoxyacetylglycyl-D-valine did not inhibit the enzyme activity on N^{α}-(*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl), N^{ϵ}-(pentaglycyl)-L-lysyl-D-alanyl-D-alanine.

Discussion

(1) Substrate Requirements of the Streptomyces Carboxypeptidase. From the foregoing studies, the substrate requirements of the enzyme can be discussed using the peptide $R_4NHCHR_3CONHCHR_2CONHCHR_1COOH$ as a general model.

(i) There is a strict requirement that the C-terminal amino acid should have either a free carboxyl group (see Figure 1, Chart I, and Table II in Ghuysen *et al.*, 1970) or $-CONH_2$. Even amidation of the carboxyl group makes it a poor substrate for the enzyme. The inability of the heptapeptide diamide (Table I, **10**) to inhibit the action of the enzyme upon N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine at molar ratios of 10 to 1 shows that amidation markedly depresses the enzyme affinity to the peptide.

(ii) There is no requirement for a specific structure of the R_1 residue (Table I). The C-terminal amino acid can be, for example, glycine, leucine, alanine, ornithine, lysine, and diaminopimelic acid. If the C-terminal amino acid is not glycine, then the asymmetric carbon must be D (Table I, **1** and **4**; see also the action upon isolated walls: Chart I and Table II in Ghuysen *et al.*, 1970). The nature of the C-terminal D-amino acid, however, influences the enzyme efficiency. Replacement of D-alanine by D-leucine (Table IV, **2** and **3**) does not effect the K_m value but induces a threefold decrease of the V_{max} value. It was also observed that the R_1 side chain may be extremely bulky. Peptides exhibiting a high sensitivity to the carboxypeptidase and in which, for example, the ε-amino group of the C-terminal D-lysine, the δ-amino group of the C-terminal D-ornithine, or the amino group located on the L carbon of a C-terminal *meso*-diaminopimelic acid, are substituted by long peptide chains, are listed in Table I, **5** to **9**. Kinetics of hydrolysis of the pentapeptide nucleotide and of the disaccharide peptide dimer both isolated from *C. poinsettiae* (Table IV, **8** and **9**) show that the replacement at the C-terminal position of D-alanine by a N^{δ}-peptide-substituted D-ornithine has little effect on the K_m value but induces a fivefold increase of the V_{max} value. This of course, explains how the carboxypeptidase seemingly behaves as an endopeptidase and how it exerts lytic activities on certain bacterial walls (Ghuysen *et al.*, 1970).

(iii) There is a strict requirement for the R_2 side chain to be either an hydrogen atom or, much better, a methyl group, in which case the asymmetric carbon must be D (Table II). The replacement of the penultimate D-alanine in the peptide N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine by a glycine (Table IV, **3** and **4**) markedly increases the K_m value (15,000 μM) but does not modify the V_{max} value. The replacement of the penultimate D-alanine by L-alanine or by D-leucine (Table II) makes the modified peptides resistant to the enzyme and prevents them from being inhibitors. In an early work (Ghuysen *et al.*, 1965) dealing with the enzymatic degradation of the walls of *S. aureus*, it had been observed that the complex

TABLE III: Influence of the Residue that Precedes C-Terminal D-Alanyl → D-Alanine on Carboxypeptidase Activity.[a]

No.	Compd	Specific Activity[b]
1	Acetyl → D-Ala ➞ D-Ala	20
2	Acetyl → Gly → D-Ala ➞ D-Ala	180
3	Acetyl → L-Ala → D-Ala ➞ D-Ala	160
4	Acetyl → D-Ala → L-Ala → D-Ala ➞ D-Ala	200
5	Acetyl → L-Tyr → D-Ala ➞ D-Ala	300
6	UDP → MurNAc → Gly → γ-D-Glu → L-homoSer → D-Ala ➞ D-Ala	1,250
7	MurNAc → Gly → γ-D-Glu → L-homoSer → D-Ala ➞ D-Ala	300
8	Gly → γ-D-Glu → L-homoSer → D-Ala ➞ D-Ala	1,100
9	N^{α},N-$^{\gamma}$-Bisacetyl → L-DAB → D-Ala ➞ D-Ala	8,100 (22,000)[c]
10	N^{α},N^{δ}-Bisacetyl → L-Orn → D-Ala ➞ D-Ala	7,000 (18,000)[c]
11	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala ➞ D-Ala	24,000 (40,000)[c]
12	N^{α}-Acetyl → L-Lys (H) → D-Ala ➞ D-Ala	200 (600)[c]
13	N^{α}-(UDP → MurNAc → L-Ala → γ-D-Glu) → L-Lys (H) → D-Ala ➞ D-Ala	100
14	N^{α}-[Disacch → L-Ala → γ-D-Glu(NH_2)] → L-Lys (H) → D-Ala ➞ D-Ala	30
15	N^{α}-(MurNAc → L-Ala → γ-D-Glu) → L-Lys (H) → D-Ala ➞ D-Ala	30
16	N^{α}-(L-Ala → γ-D-Glu) → L-Lys (H) → D-Ala ➞ D-Ala	200
17	N^{α}-(γ-D-Glu) → L-Lys (H) → D-Ala ➞ D-Ala	110
18	UDP → MurNAc → L-Ala → γ-D-Glu → (L_1)-LL-DAP-(L) → D-Ala ➞ D-Ala	840[d]
19	UDP → MurNAc → L-Ala → γ-D-Glu → (L)-*meso*-DAP-(L) → D-Ala ➞ D-Ala	3,000 (6,000)[c]
20	MurNAc → L-Ala → γ-D-Glu → (L)-*meso*-DAP-(L) → D-Ala ➞ D-Ala	800
21	N^{α}-[L-Ala → γ-D-Glu(NH_2)], N^{ϵ}-[β-D-Asp(NH_2)] → L-Lys → D-Ala ➞ D-Ala	450
	N^{α}-[L-Ala → γ-D-Glu(NH_2)] → L-Lys → D-Ala ➞ D-Ala	220
22	N^{α}-[L-Ala → γ-D-GluNH_2)] → L-Lys → D-Ala → D-Asp(NH_2) (H-D-Asp(NH_2))	
23	N^{α}-[Disacch → L-Ala → γ-D-Glu(NH_2)] → L-Lys (H-(Gly)$_5$) → D-Ala ➞ D-Ala	4,000 (9,000)[c]
24	N^{α}-[L-Ala → γ-D-Glu(NH_2)] → L-Lys (H-(Gly)$_5$) → D-Ala ➞ D-Ala	5,000
25	N^{α}-[Disacch → L-Ala → γ-D-Glu(NH_2)] → L-Lys → D-Ala ➞ D-Ala; N^{α}-[Disacch → L-Ala → γ-D-Glu(NH_2)] → L-Lys (H) → D-Ala → (Gly)$_5$	1,200
26	N^{α}-[Disacch → L-Ala → γ-D-Glu(NH_2)] → L-Lys → D-Ala ➞ D-Ala; N^{α}-[Disacch → L-Ala → γ-D-Glu(NH_2)] → L-Lys (H-(Gly)$_5$) → D-Ala → (Gly)$_5$	3,000
27	H-L-Lys (Acetyl) → D-Ala ➞ D-Ala	40[c]

TABLE III: Footnotes

[a] Heavy arrows show the sensitive linkages. [b] Expressed in mμequiv of linkage hydrolysis per mg of enzyme, per hr, in 0.01 M Veronal buffer, pH 9. [c] Specific activity in 0.02 M Tris-HCl buffer, pH 7.5, containing 0.002 M $MgCl_2$. [d] At apparent completion of the reaction (using 12 μg of enzyme), 11 mμmoles of D-alanine was liberated. Owing to the absolute activity of the enzyme upon N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine (24,000) and upon N^{α},N^{ϵ}-bisacetyl-L-lysylglycyl-D-alanine (1500) (Table II, **1** and **2**), the hydrolysis of the glycyl-D-alanine nucleotide of which the presence in the preparation is postulated (Materials and Methods) would not occur. Origin of the peptides: **1, 2, 3, 4, 5, 9, 10, 11, 12, 17, 27**: synthetic peptides; **6, 7, 8**: *C. poinsettiae* (nucleotide); **13, 15, 16**: *S. aureus* (nucleotide); **18**: *S. albus* G (nucleotide); **19, 20**: *B. subtilis* (nucleotide); **13, 23, 24, 25, 26**: *S. aureus* walls; **21, 22**: *L. acidophilus* walls. MurNAc = *N*-acetylmuramyl; Disacch = β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl.

TABLE IV: Kinetics of Hydrolysis of Various C-Terminal Linkages by *Streptomyces* Carboxypeptidase.

No.	Compd	V_{max}[a]	K_m (μM)
1	N^{α}-Acetyl → L-Lys → D-Ala ➡ D-Ala	20	6,000
2	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala ➡ D-Ala	100	330
3	N^{α},N^{ϵ}-Bisacetyl → L-Lys → D-Ala ➡ D-Leu	33	330
4	N^{α},N^{ϵ}-Bisacetyl → L-Lys → Gly ➡ D-Ala	107	15,000
5	N^{α}-[Disacch → L-Ala → γ-D-Glu(NH_2)] → L-Lys → D-Ala ➡ D-Ala H-$(Gly)_5$ ↑	9	280
6	UDP → MurNAc → L-Ala → γ-D-Glu → (L)-*meso*-DAP-(L) → D-Ala D-Ala	10	400
7	N^{α}-[L-Ala → γ-D-Glu(NH_2)], N^{ϵ}-[β-D-Asp(NH_2)] → L-Lys → D-Ala D-Ala	12	2,500
8	UDP-MurNAc → Gly → γ-D-Glu → L-homoSer → D-Ala D-Ala H-D-Orn-OH ↑	16	1,000
9	Disacch → Gly → D-Glu → L-homoSer → D-Ala ➡ D-Orn-OH	78	1,500
	Disacch → Gly → D-Glu → L-homoSer → (D-Ala-OH)		

[a] In μmoles of linkage hydrolyzed per mg of enzyme per hr. For conditions see text. The substrate concentrations used for the assays and expressed in K_m equivalents, varied from 0.2 up to 3. Heavy arrows show the sensitive linkages. MurNAc = *N*-acetylmuramic acid; Disacch = β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid.

secreted by *Streptomyces* sp. exhibited two carboxypeptidase activities: a D-alanine carboxypeptidase hydrolyzing D-alanyl-D-alanine sequences at the C termini of the peptide moiety of the peptidoglycan, and a glycine carboxypeptidase hydrolyzing C-terminal D-alanylglycine sequences which had appeared as a result of the action of a glycyl–glycine bridge-splitting enzyme (then called peptidase 2 and subsequently identified as the MR endopeptidase; Ghuysen, 1968). In more recent works dealing with the structure of wall peptidoglycans in *E. coli* and *B. rettgeri* (Bricas, 1968; van Heijenoort *et al.*, 1969; Guinand *et al.*, 1969; Ghuysen *et al.*, 1969; Ghuysen *et al.*, 1970), it was recognized that the "KM endopeptidase" exerted its lytic action upon these walls through the hydrolysis of DD linkages that were in position α to a free carboxyl group. It is now clear that all these activities can be assigned to the carboxypeptidase described here (Ghuysen *et al.*, 1970).

(iv) The length, the structure, and the polarity of the R_3 side chain of the substrate are of prime importance for the enzyme activity (Table III).

Studies carried out on synthetic peptides have shown that the presence at the R_3 position of a glycine residue (R_3 = H), an L-alanine residue (R_3 = CH_3) or of an L-tyrosine residue (R_3 = hydroxybenzyl) confers some sensitivity to the corresponding peptides (Table III, **1–5**). Quite markedly, however, the presence at the same position, of L-homoserine (R_3 = CH_2CH_2OH), of N^{γ}-acetyl-L-diaminobutyric acid (R_3 = $CH_2CH_2NHCOCH_3$) or N^{δ}-acetyl-L-ornithine (R_3 = $(CH_2)_2CH_2NHCOCH_3$), and of N^{ϵ}-acetyl-L-lysine (R_3 = $(CH_2)_3CH_2NHCOCH_3$), is paralleled by a progressive and spectacular increase of the rate of hydrolysis of the terminal D-alanyl-D-alanine linkage (Table III, **6–11**).

The acyl substitution of the ω-amino group at the end of the R_3 side chains appears to be another requirement of the enzyme (Table III, **11** to **17**). For example, the enzyme ef-

ficiency (Table IV) upon N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine is much higher than that upon N^{α}-monoacetyl-L-lysyl-D-alanyl-D-alanine. Because of its large K_m value, N^{α}-monoacetyl-L-lysyl-D-alanyl-D-alanine is a poor substrate, and it is not an inhibitor. Similar observations were made with the L-ornithine-containing peptides. The depressing effect exerted by a free ω-amino group located at the end of the R_3 side chain was also observed when the carboxypeptidase acted upon peptide oligomers. For example, the peptide dimer of *B. rettgeri* (Table I, **5**) contains C-terminal L-ornithyl-D-alanyl-D-lysine and C-terminal L-ornithyl-D-alanyl-D-ornithine sequences with free δ-amino groups. It is 30 times less sensitive to the enzyme than the disaccharide peptide dimer of *C. poinsettiae* (Table I, **6**) that contains a C-terminal L-homoseryl-D-alanyl-D-ornithine sequence deprived of any free amino group. Evidently, this requirement of the carboxypeptidase well explains why the walls of *B. rettgeri* are so much less sensitive to its lytic action than those of *C. poinsettiae* and of *C. insidiosum* (Table II, in Ghuysen *et al.*, 1970).

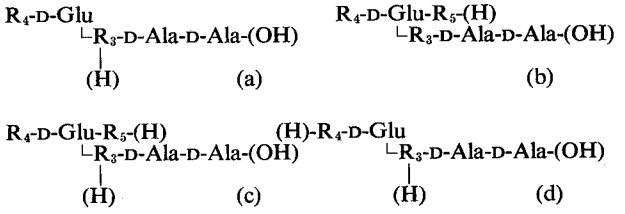
Studies with the natural peptides, however, revealed that the presence of a free amino group at the end of the R_3 side chain is compatible with a high enzyme activity under certain conditions. The following example suggests that there would be a requirement for the terminal amino group at the R_3 side chain to be acylated or in α position to a carboxyl group. Indeed, it was observed that the transformation of the ϵ-amino group of L-lysine to an α-amino group by introduction of a carboxyl group in α position (as occurs when L-lysine is replaced by diaminopimelic acid) (Table III, **18–20**) or its substitution by a pentaglycine sequence (as occurs when L-lysine is replaced by N^{ϵ}-(pentaglycyl)-L-lysine) (Table III, **23–26**) are paralleled by a large increase of the sensitivity of the corresponding peptides to the carboxypeptidase. Actually, the K_m values for these two latter peptides (but not the V_{max} values) are identical with that for N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine (Table IV, **5**, **6**, and **2**). These increases in activity could be due either to a requirement for an oxygen atom (as in R'CO·NHR–, $NH_2(COOH)CH \cdot R$–, or $HOCH_2 \cdot R$–) or to a requirement that a side-chain amino group, if present, should be mainly uncharged at the pH of the reaction. This condition would be met by an α-amino group such as in diaminopimelic acid as opposed to an ω-amino group as in lysine.

Other peptides in which the ϵ-amino group of L-lysine is substituted by a D-isoasparagine residue or by a very long peptide chain are also listed in Table III (**21**, **22**, **25**, **26**). Sometimes, but not always, the presence of these substituents enhances the rate of hydrolysis of the C-terminal D-alanyl-D-alanine linkage. UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine was found to be about 3 times more sensitive than the corresponding LL-diaminopimelic acid containing peptide (Table III, **18**, **19**). Hence, the activating effect seems to be higher when the carboxyl group in α position to the amino group confers a D configuration to the carbon atom. Much remains to be done in order to explore fully the structural features of the R_3 side chain that fulfill the carboxypeptidase requirements.

The data of Table IV show that the K_m values for peptides ending in L → D-alanyl → D sequences (and of which the α-amino group of the L residue is substituted, *vide infra*) is essentially under the control of the L residue that precedes the linkage specifically hydrolyzed by the *Streptomyces* carboxypeptidase. Low K_m values of about 400 μM coincide with the presence at the R_3 position of N^{ϵ}-acetyl-L-lysine, N^{ϵ}-pentaglycyl-L-lysine or *meso*-diaminopimelic acid. L-Homoserine confers a K_m value of 1000 to 1500 μM, N^{ϵ}-D-isoasparaginyl-L-lysine a K_m value of 2500 μM, and N^{ϵ}-L-lysine a K_m value of 6000 μM.

(v) There is a strict requirement for the R_4 residue not to be an hydrogen atom. N^{ϵ}-Acetyl-L-lysyl-D-alanyl-D-alanine (Tavle III, **27**) is a very poor substrate and it is not an inhibitor. However, the nature of the substituent at the α-amino terminus of the peptide may vary. Acetic acid, γ-linked D-glutamic acid, D-isoglutamine, L-alanyl-γ-D-glutamic acid, L-alanyl-D-isoglutamine, and UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamic acid seem not to exert any specific influence on the enzyme activity (Tables I and III). Surprisingly, however, it was repeatedly observed that the replacement of UDP-*N*-acetylmuramic acid in the latter sequence by *N*-acetylmuramic acid or by β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid had some depressing influence on the enzyme activity (Table I, **8** and **9**; Table III, **6–8**; **13–16**; **19** and **20**; **23** and **24**).

(*2*) *Physiological Significance of the* DD *Carboxypeptidase.* An approach to the understanding of the physiological significance of the DD carboxypeptidases is suggested by the remarkable similarity between the substrate requirements of the transpeptidases involved in wall peptidoglycan synthesis and those of the *Streptomyces* carboxypeptidase as they are revealed by the present studies. The tetrapeptide units in all bacterial wall peptidoglycans have the general sequence L-alanyl(or L-seryl or glycyl)-γ-D-glutamyl-L-R_3-D-alanine. These tetrapeptides substitute the glycan strands and, in turn, they are cross-linked through specialized bridges. From a study of the nature and location of these interpeptide bridges, there appear 4 different chemotypes of bacterial peptidoglycans (Ghuysen, 1968; see also Leyh-Bouille *et al.*, 1970). The cross-linking of the peptide units is introduced by transpeptidation reactions and, depending upon the bacterial species, the peptide units which undergo transpeptidation have one of the following general sequences.[1] In



[1] The following peptides are pertinent to the present discussion: (a) one amino group at the R_3 position: *N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine in *E. coli*; N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl), N^{ϵ}-(pentaglycyl)-L-lysyl-D-alanyl-D-alanine in *S. aureus*; N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl), N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanyl-D-alanine in *L. acidophilus*; *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-(L_1); glycyl-(L_2)-LL-diaminopimelyl-(L_1)-D-alanyl-D-alanine in *Streptomyces* sp. and *Cl. welchii*; (b) one amino group at the R_5 position: *N*-acetylmuramyl-glycyl-γ-[N^{δ}-(D-glutamyl)-D-ornithine]-L-homoseryl-D-alanyl-D-alanine in *C. poinsettiae*; (c) two amino groups at the R_3 and R_5 positions, respectively; *N*-acetylmuramylglycyl-γ-[N^{γ}-(D-glutamyl)-D-diaminobutyric acid]-L-diaminobutyryl-D-alanyl-D-alanine in *C. insidiosum*;

all cases, the carbonyl group of the penultimate C-terminal D-alanine residue of one peptide donor is transferred to the amino group of a second peptide acceptor. Interpeptide bonds are formed and the D-alanine residues of the donor peptides are released in equivalent amounts. In order to be recognized by the transpeptidase, the donor and acceptor peptides must be located at specific positions. There is no evidence about the specificity of the acceptor amino group. It may just be any one that is available in the structure. Often, the donor peptide is also the acceptor peptide and only one kind of amino group located either at the R_3 (as in *S. aureus* and in *E. coli*, *vide infra*) or R_5 (as in *C. insidiosum*, *vide infra*) position, can act as acceptor. In *M. lysodeikticus*, however, there seem to be two kinds of amino acceptors located at the R_3 and R_4 positions (Ghuysen, 1968). From an integration of structural and biosynthetic studies and from the data presented in this report, it appears that the specific composition required for the donor peptide in the transpeptidation reaction is one that confers a low Michaelis constant value to the peptide-DD carboxypeptidase combination reaction, *i.e.*, that the peptides which act as donors are those which are recognized by the DD carboxypeptidase.

(i) In *E. coli*, the peptides L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine directly undergo transpeptidation and the amino group located on the D carbon of *meso*-diaminopimelic acid serves as acceptor (Peptidoglycans of Chemotype I; Ghuysen, 1968). Parallel to this, the above peptide is known to readily interact with the DD carboxypeptidase (K_m = 400 μM) (Table IV).

(ii) Peptides with a free γ-amino group of L-ornithine or a free ε-amino group of L-lysine at the R_3 position, are poorly recognized by the carboxypeptidase. Strikingly, there are no examples known that such peptides are substrates for those bacterial transpeptidases which are involved in the biosynthesis of the wall peptidoglycans of chemotype II (Ghuysen, 1968). Prior to their transfer into the growing-wall peptidoglycan, the L-ornithine- or L-lysine-containing peptide units undergo modifications which consist in the substitution of the ω-amino group by one or several additional amino acids residues. In *S. aureus*, for example, the lysine residue of N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanyl-D-alanine is replaced by N^{ϵ}-(pentaglycyl)-L-lysine. As a consequence, the modified peptide fufills the requirements of the transpeptidase and its interaction with the DD carboxypeptidase exhibits a low K_m value (280 μM) (Table IV).

(iii) Transpeptidation reactions do not involve large changes in free energy and, consequently, they do not reach completion. The efficiency of the transpeptidation reaction in a given organism should thus be reflected by the extent of peptide cross-linking in the completed wall peptidoglycan. Such a relationship is valid, however, only for those bacteria such as *S. aureus* (Tipper and Berman, 1969) and *L. acidophilus* 63 AM Gasser (Coyette and Ghuysen, 1970) in which the residual noncross-linked C termini of the wall peptide moieties have retained the D-alanyl-D-alanine sequence, thus demonstrating that peptide hydrolases are not involved in the regulation of the size of the peptide (Ghuysen *et al.*, 1970). The extent of wall peptide cross-linking indicates that the transpeptidation reaction is most efficient in *S. aureus* (80 to 90%) whereas it is poorly efficient in *L. acidophilus* (30 to 40%). Parallel to this, the combinations of the *S. aureus* and of the *L. acidophilus* peptides (Table IV, **5** and **7**) with DD carboxypeptidase exhibit K_m values of 280 μM and of 2.500 μM, respectively, that is, that the efficiency of the transpeptidation appears to be related to the affinity of the DD carboxypeptidase for the donor peptide.

(iv) In a major part of the wall peptidoglycan of *Streptomyces* sp. and of *Clostridium welchii* (Leyh-Bouille *et al.*, 1970), cross-linking between L-alanyl-D-isoglutaminyl-(L_1)-LL-diaminopimelyl-(L_1)-D-alanine is mainly mediated *via* D-alanylglycyl-LL-diaminopimelic acid bridges (*i.e.*, a peptidoglycan of chemotype II. The same structure occurs in wall of *Propionibacterium* sp., Schleifer *et al.*, 1968a). Since the peptide with LL-diaminopimelic acid at the R_3 position is not as good a substrate of the DD carboxypeptidase as the peptide with the *meso* isomer, it is likely that peptide bridging is mainly ensured through glycine incorporation followed by transpeptidation reactions involving L-alanyl-D-isoglutaminyl-(L_1); glycyl-(L_2); -LL-diaminopimelyl-D-alanyl-D-alanine as donor and acceptor peptides.

(v) The observed similarity between the requirements of the *Streptomyces* DD carboxypeptidase and those deduced for the endogenous transpeptidases for the donor peptide strongly suggests that the two enzymes are connected. It may be envisaged that in bacterial cells, a single enzyme is, in fact, involved in both the carboxypeptidase and the transpeptidase activities, *i.e.*, that a soluble DD carboxypeptidase could act as a transpeptidase when it is integrated within the cell membrane (or *vice versa* that a membrane-bound transpeptidase could act as a carboxypeptidase when it is in a soluble form), and that the difference in effective function of the enzyme could be, at least partially, a question of the availability of water. In an aqueous environment, attack of the tetrapeptide–enzyme complex, after elimination of the terminal D-alanine, by [OH^-] would lead to simple hydrolysis (carboxypeptidase activity). In the hydrophobic environment of the membrane where [OH^-] is low, attack by a recognizable $-NH_2$ would lead to transpeptidation (*i.e.*, cross-linking):

R-D-Ala-D-Ala + enzyme (E) ⟶ R-D-Ala-D-Ala-E
R-D-Ala-D-Ala-E ⟶ R-D-Ala-E + D-Ala
R-D-Ala-E + OH^- ⟶ R-D-AlaOH + E (carboxypeptidase activity)
R-D-Ala-E + R′-NH_2 ⟶ R-D-Ala-NH-R′ + E (transpeptidase activity)

The functioning of the transpeptidase–carboxypeptidase system could vary depending upon the bacterial species. It appears that many bacteria perform both transpeptidase and carboxypeptidase activities. In most of them the carboxypeptidase remains cell bound whereas in others (*Streptomyces* sp.) it can be secreted in the external medium. Few bacteria (*S. aureus* and *L. acidophilus* 63 AM Gasser) do not perform any detectable carboxypeptidase activity, that is that the enzyme system is not bifunctional.

(vi) In peptidoglycans of chemotype IV (Ghuysen, 1968), the amino group involved in the transpeptidation reaction is located at the R_5 position, R_5 being a diamino acid or a diamino acid containing peptide. The influence exerted

N-acetylmuramyl-L-seryl-γ-[N^6-(D-glutamyl)-D-ornithine]-L-ornithyl-D-alanyl-D-alanine in *B. rettgeri;* (d) two amino groups at the R_3 and R_4 positions, respectively; L-alanyl-γ-(D-glutamylglycine)-L-lysyl-D-alanyl-D-alanine in *M. lysodeikticus*.

on the DD carboxypeptidase by an amino group located at such a position, is not yet known. Depending upon the bacterial species, the R_3 residue is either a neutral amino acid (L-homoserine in *C. poinsettiae*), or a diamino acid too such as, for example, L-diaminobutyric acid (in *C. insidiosum*), L-ornithine (in *B. rettgeri*), or L-lysine (in *M. lacticum*, Schleifer *et al.*, 1968b) (Chart I, in Ghuysen *et al.*, 1970). Although in many instances the amino groups at the R_3 position are free in the nucleotide precursors and in the completed wall peptidoglycans, in *C. insidiosum* at least the group is already acetylated in the nucleotide precursor (Perkins, 1968). Thus, this blocking either could facilitate the transpeptidase action of the donor peptide (by enhancing the capability of the enzyme of recognizing it) or may be needed to confer specificity on the acceptor peptide (by directing the enzyme action to the amino group located at the R_5 position). It may be that bacteria of this group possess a mechanism through which the amino groups at the R_3 position are blocked (through N^{ω} acetylation for example) before the transpeptidation occurs and may then be exposed again when peptide bridging is complete. In some instances, as in *C. insidiosum* and *C. sepedonicum*, there are very few amino groups in the final wall (Perkins, 1968).

In the peptidoglycans of chemotype III (Ghuysen, 1968) (*i.e.*, in *M. lysodeikticus* and related *Micrococcaceae*), N^{α}-[L-alanyl-γ-(D-glutamylglycine)]-L-lysyl-D-alanine peptides are interlinked through D-alanyl-L-alanine and N^{ϵ}-(D-alanyl)-L-lysine linkages. Two kinds of amino group, *i.e.*, the L-alanine at the R_4 position and the N^{ϵ}-L-lysine at the R_3 position, can act as acceptor. In both cases, the donor peptide would have free ε-amino groups unless it is also temporarily blocked by acetylation for example. The study of the biosynthesis of the wall peptidoglycan of chemotypes III and IV is thus of prime importance with regard to the idea that, as suggested by the present studies, all the bacterial transpeptidases would have the same restriction for free amino group in the donor peptide.

(vii) Finally, the postulated identity between the DD carboxypeptidase and the transpeptidase is consistent with the observation that the *E. coli* carboxypeptidase is sensitive to penicillin and so, too, is the transpeptidase of this organism (Izaki *et al.*, 1968), whereas, as it will be shown in the next paper of this series, the *Streptomyces albus* G carboxypeptidase is very resistant to it and so, too, is the strain that produces the enzyme.[2]

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[2] According to a short report of Strominger *et al.* (1969), the "cell membrane fraction" of *B. subtilis* exhibits a D-alanine carboxypeptidase activity. The enzyme is irreversibly inactivated by penicillin. It has been proposed that penicillin is bound as the thio ester of penicilloic acid and an SH group in the enzyme and that this enzyme might be the "uncoupled" transpeptidase.