Peptide Cross-Links in Bacterial Cell Wall Peptidoglycans Studied with Specific Endopeptidases from *Streptomyces albus* G*

Jean-Francois Petit,† Emilio Muñoz,‡ and Jean-Marie Ghuysen

ABSTRACT: Evidence is given for the existence of at least four types of peptide cross-linkages in lysine-containing peptidoglycans of cell walls from Gram-positive bacteria. Three kinds of such cross-linkages, by which the ε-amino group of the lysine residue of one peptide subunit is joined to the carboxyl group of the terminal D-alanine of a second, have been demonstrated, namely: bridging by tri-L-alanine (*Micrococcus roseus* Thr⁽⁻⁾), by tri-L-alanine-L-threonine (*M. roseus* R 27), and by pentaglycine (*Staphylococcus aureus* Copenhagen) residues. The lytic *Streptomyces* SA endopeptidase hydrolyzes D-alanylglycyl linkages in *S. aureus* and D-alanyl-L-alanine linkages in *M. roseus*, i.e., at the amino terminus of the peptide bridges and at the carboxyl terminus of the peptide subunits. The lytic *Streptomyces* MR endopeptidase hydrolyzes L-alanyl-L-threonine linkages within the peptide bridges in *M. roseus* R 27 and, with a much smaller rate, L-alanyl-L-alanine linkages within the peptide bridges in *M. roseus* Thr⁽⁻⁾. A fourth kind of cross-linkage, in which no additional amino acid is involved, can also occur in the form of a direct bonding between the C-terminal alanine residue of one peptide subunit and the ε-amino group of the lysine residue of a second (*Micrococcus lysodeikticus*). This latter alanyl-ε-lysine bond is sensitive to a third lytic *Streptomyces* enzyme, the ML endopeptidase. A structure for the peptide moiety of the peptidoglycan of *M. roseus* is proposed and compared to the peptidoglycan structure in *S. aureus*. The present studies lend support to recent proposals dealing with the mechanism of action of penicillin.

The rigid matrix of bacterial cell walls is a peptidoglycan. The glycan portion is made up of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid arranged in linear chains. Some, or all, of the carboxyl groups of the *N*-acetylmuramic acid units are involved in amide linkages to terminal L-alanine residues of the peptide moiety. A reasonable working hypothesis for the composition and structure of the peptide portion visualizes subunits of L-alanyl-γ-D-glutamyl-L-lysyl- (or -diaminopimelyl-)D-alanine, cross-linked by the glycan and by peptide bonds in which additional amino acids may participate.

The peptidoglycan retains its shape and insolubility as long as both the glycan chains and the peptide cross-links remain intact. Thus it is well known that enzymic hydrolysis of glycosidic bonds brings about lysis of whole cells or solubilization of cell walls. The existence of cross-links between the peptide chains was first suggested by the paucity of terminal groups (Salton, 1961; Mandelstam and Strominger, 1961) and the isolation of dimers of peptide subunits (Ghuysen,

1961; Primosigh *et al.*, 1961). It was substantiated with the help of specific peptidases discovered in the last decade (Salton and Ghuysen, 1957; Mori *et al.*, 1960; Kato *et al.*, 1962; Pelzer, 1963; Ghuysen *et al.*, 1964; Browder *et al.*, 1965; Ghuysen *et al.*, 1965a,b; Ensign and Wolfe, 1966). The role of peptide cross-links in cell wall stability is well illustrated by the case of *Staphylococcus aureus* walls, where enzymic disruption of polyglycine bridges causes dissolution of the walls (Ghuysen *et al.*, 1964, 1965a,b) without cleavage of any glycosidic linkages.

According to recent reports (Martin, 1964a,b; Wise and Park, 1965; Tipper and Strominger, 1965; Tipper, 1966; Izaki *et al.*, 1966), the penicillin-induced defect in the cell wall peptidoglycan of *S. aureus* consists in failure to form the bond at the amino terminus of the polyglycine bridges, thereby leading to fragile non-viable cells. Similarly, it has been suggested that an alanyl-ε-lysine peptide bond would be involved in the action of penicillin upon cells of *Micrococcus lysodeikticus* (Wise and Park, 1966). The present report describes several types of cross-linkages in various bacteria as elucidated by means of specific peptidases, and provides further evidence for the dependency of cell wall integrity on intact peptide cross-linkages. Preliminary accounts of this research have appeared (Petit *et al.*, 1965; Ghuysen *et al.*, 1966b).

Material and Methods

Analytical Methods. Identification and measurement

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of N-terminal, C-terminal, and free amino acids and the enzymatic determination of D- and L-alanine are described elsewhere (Ghuysen et al., 1965b, 1966a). Threonine was distinguised from allothreonine by paper chromatography in 1-butanol–methyl ethyl ketone–water–NH$_4$OH (Wheat et al., 1962). It was measured in the form of the dinitrophenyl derivative after thin layer chromatography on silica gel, using the previously described solvents 1 and 2 (Ghuysen et al., 1965b). The L-threonine was characterized and measured with the help of L-threonine dehydrase and then, of lactic dehydrogenase in presence of DPNH.[1] The same fluorometric method as for D- and L-Ala determination was applied (Ghuysen et al., 1966a). Amino acid analyses of the bacterial cell walls and of the disaccharide peptide fractions (after hydrolysis in 6 N HCl, 15 hr, 100°) were carried out in a Technicon autoanalyzer, resin chromobeads, type B (17 μ).

Cell Walls. Bacterial cell walls were prepared by differential centrifugation after disruption of the cells with glass beads for 1 hr at 4° using an Omnimixer Servall. A 25% (w/v) suspension of cells (wet weight) in water was used to which its weight of glass beads (0.12 mm) was added. The cell walls were purified by treatment at 37°, for 3 hr, with trypsin (0.1%; Difco 1:250; control 474214) in 0.05 M phosphate buffer, pH 7.5, washed, and freeze-dried. Cell walls from the following bacteria were prepared: *S. aureus* strain Copenhagen, *M. lysodeikticus* strain NCTC 2665, *Micrococcus roseus* strain R 27 (Pasteur Institute, Paris), *Micrococcus citreus* strain R 266 (Pasteur Institute), *Sarcina lutea* strain R 262 (Pasteur Institute), *Bacillus megaterium* strain KM. Other bacterial cell walls were also used: cell walls of *Micrococcus roseus*, a strain which has no threonine in the walls and which will be designated as *M. roseus* Thr$^{(-)}$, were provided by Dr. M. R. J. Salton, New York University; cell walls of *Corynebacterium anaerobicum* strains Prevot 3103 and 3471, of *Corynebacterium fermentans* strain 3211 (Pasteur Institute), of *Propionibacterium technicum* strain NCIB 5965, and of *Clostriduim histolyticum* strain G 54 (Pasteur Institute) were provided by Dr. R. Tinelli (Pasteur Institute, Paris); cell walls of *Propionibacterium petersonii* NCTC 5962 and of *Propionibacterium rubrum* NCIB 8901 were provided by Dr. E. Work, Twyford Laboratory, London; cell walls of *Streptococcus pyogenes* group A type 12 were provided by Dr. S. S. Barkulis and H. Heymann, Ciba Pharmaceutical Co., Summit, N. J., and those of *Micrococcus radiodurans* by Dr. Chr. Dean, Chester Beatty Institute, London.

For the four following strains, intact cells were used: *Bacillus subtilis* strain A33 (Pasteur Institute), *Micrococcus flavus* strain 53160 (Pasteur Institute), *Staphylococcus albus* strain 531246 (Pasteur Institute), and *Gaffkya tetragena* strain R 258 (Pasteur Institute).

Preparation of the Soluble Peptidoglycans GP$_1$ and GP$_2$ from Cell Walls of S. aureus. The procedure has been previously described (Ghuysen et al., 1965a). The cell walls were digested with an endo-N-acetyl-muramidase and the soluble peptidoglycan GP$_1$ was separated from the teichoic acid–peptidoglycan complex by chromatography on Ecteola-cellulose. Some of the polyglycine bridges in GP$_1$ were then opened by treatment with the lytic peptidase 1, yielding the peptidoglycan GP$_2$.

Masking of the Free Amino Groups and Free Carboxyl Groups in Bacterial Cell Walls. Cell walls of *M. lysodeikticus* have a high intrinsic content of terminal amino groups (400 mμmoles/mg of ϵ-amino groups of lysine) and of terminal carboxyl groups (450 mμ-moles/mg of glycine and 100 mμmoles/mg of alanine). These groups could mask the liberation of a smaller amount of new amino and carboxyl groups liberated by digestion of the cell walls with an appropriate peptidase. The number of native amino groups was reduced to 20–25% of its initial value by hydroxy-methylation of the cell walls with ethylene oxide (Ghuysen et al., 1966a). The number of native carboxyl groups was greatly decreased by esterification and transformation into hydroxamates (Herman-Boussier et al., 1963); cell walls were washed three times with methanol and then suspended in methanol (10 mg/ml); 8.5 μl of 12 N HCl was added for each milliliter of the suspension; the suspension was gently stirred for 24 hr at room temperature and then centrifuged; the pellet was resuspended in a solution (pH 8) of 2 M hydroxylamine HCl neutralyzed with 3 N KOH–0.3 M NaHCO$_3$, 2:1 (v/v); the suspension was gently stirred for 24 hr at room temperature and then centrifuged. The modified cell walls were washed three times with water. Hydrazinolysis showed that the terminal carboxyl groups of glycine had been reduced to 65 mμmoles/mg. No terminal carboxyl group of alanine was detected. The product was still sensitive to the ML endopeptidase.

Enzymes. Most of the enzymes used in the present studies were obtained from culture filtrates of *S. albus* G: (a) the F$_1$ endo-N-acetylmuramidase (Salton and Ghuysen, 1960; Dierickx and Ghuysen, 1962); (b) the N-acetylmuramyl-L-alanine amidase (Ghuysen et al., 1962); (c) three bacteriolytic endopeptidases: the SA endopeptidase active on cell walls of *S. aureus* and *M. roseus*, the ML endopeptidase active on cell walls of *M. lysodeikticus*, and the MR endopeptidase active on cell walls of *M. roseus*; (d) nonlytic amino-peptidase(s) capable of degrading synthetic peptides or peptide chains having terminal amino groups in cell wall peptidoglycans. The endopeptidases and aminopeptidase(s) have been previously described (Ghuysen et al., 1965a,b) except the MR endopeptidase. The exo-β-N-acetylglucosaminidase was prepared from pig epididymis according to Sanderson et al. (1962).

Isolation of the Crude Streptomyces Enzymes. The selected strain of *Streptomyces* was grown in tanks of 500 l. in conditions previously described (Ghuysen et al., 1965a). When the maximal lytic activity was reached, the culture was centrifuged.

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[1] Abbreviations used: DPNH, reduced diphosphopyridine nucleotide; DAP, diaminopimelic acid.
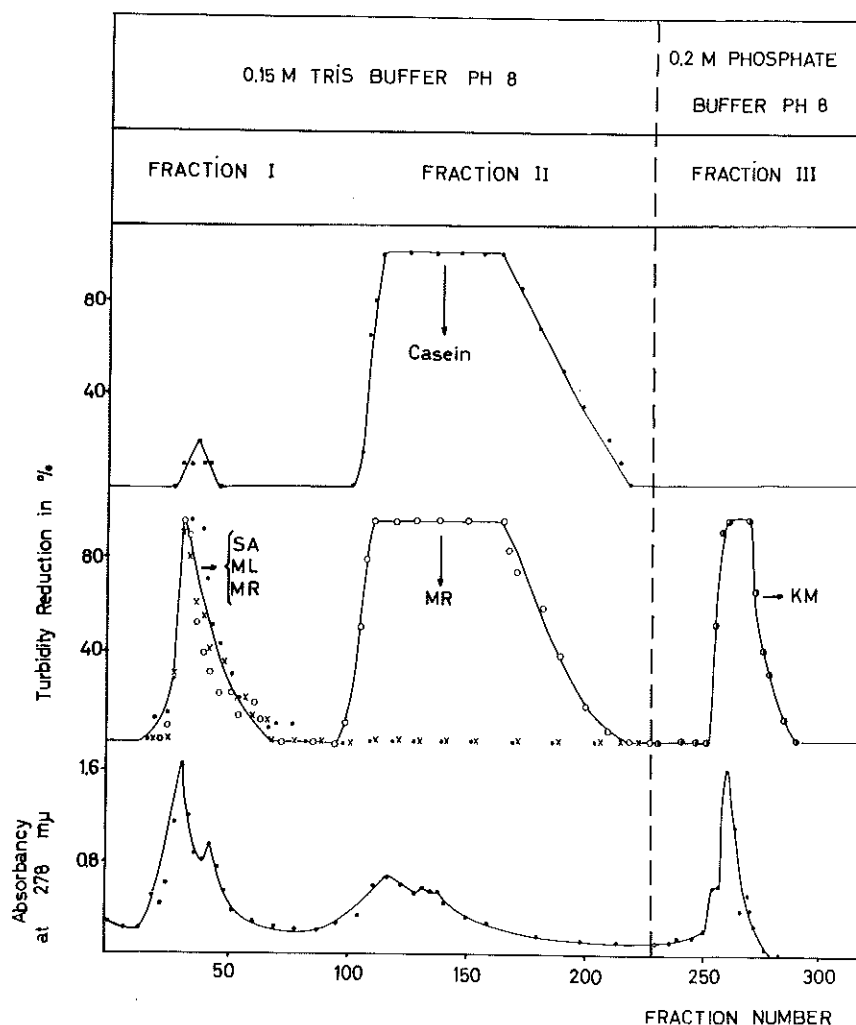
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FIGURE 1: Isolation of the crude *Streptomyces* enzymes. The crude extract corresponding to 16.5 l. of *Streptomyces* culture filtrate was adsorbed on a CM-cellulose column, 250-ml volume, in 0.01 M Tris–HCl buffer, pH 8.0. Elution was carried out with 0.15 M Tris–HCl buffer, pH 8.0, and then with 0.2 M K₂HPO₄ buffer, pH 8.0. The volume of the fractions was 8 ml. *Bacteriolytic activities:* aliquots of 25 μl of each fraction were incubated at 37° for 2 hr with 475 μl of an aqueous suspension of bacterial cell walls (1%, w/v). SA: *S. aureus* Copenhagen; ML: *M. lysodeikticus;* MR: *M. roseus* R 27; KM: *B. megaterium* KM. Turbidity was determined measuring the optical density at 550 mμ. *Caseinolytic activity:* 25 μl of each fraction was incubated at 37° for 20 min with 500 μl of a casein solution (0.2% in 0.05 M phosphate, pH 8). The incubation was stopped by addition of 4.5 ml of 1% trichloracetic acid solution. The turbidity was measured with a Pulfrich nephelometer.

Amberlite IRC 50 (XE64) H⁺ (2.5 kg) form was added to the supernatant and the mixture was adjusted to pH 5.0. The resin with the enzymes adsorbed on it was collected by decantation, washed with distilled water, freeze dried, and kept at −20° until used.

For the preparation of the enzymes, 100 g of resin, to which the enzymes from 16.5 l. of culture filtrate had been adsorbed, was suspended in a minimum volume of cold 10% K₂HPO₄ buffer, pH 8.0; to the suspension was added slowly cold 15 N NH₄OH until a pH of 8–8.5 was reached. The resin was filtered off. All subsequent operations were performed at 4°. The filtrate was dialyzed for 20 hr against an 0.01 M Tris–HCl buffer, pH 8.0. The slightly turbid solution

was centrifuged and the supernatant (800 ml having a total absorbancy of 8700 at 278 mμ) was applied to a 250-ml column of carboxymethylcellulose (CMC) equilibrated with 0.01 M Tris–HCl buffer, pH 8.0. After adsorption, the column was washed with the same buffer until the effluent was virtually free of protein. The 32 *endo-N*-acetylmuramidase (Ghuysen *et al.*, 1962; Ghuysen and Strominger, 1963a), the *N*-acetylmuramyl-L-alanine amidase (Ghuysen *et al.*, 1962; Ghuysen and Strominger, 1963a), and the *exo-β-N*-acetylhexosaminidase (Dierickx and Ghuysen, 1962) came through the column in the Tris buffer. The F₁ *endo-N*-acetylmuramidase and the peptidases were retained on the resin. Elution of the CMC column

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with cold 0.15 M Tris buffer, pH 8.4, yielded two fractions (Figure 1). Fraction I contained the amino-peptidase activities (not shown in Figure 1); it was lytic on cell walls of *S. aureus*, *M. lysodeikticus*, and *M. roseus;* it had no action on cell walls of *B. megaterium* and was weakly caseinolytic. Fraction II lysed cell walls of *M. roseus* only and was powerfully caseinolytic. This protease has been designated in previous publications as Caseinase C and has been shown to destroy parts of the envelope of certain animal viruses (Reginster, 1965, 1966; Osterrieth, 1965). Subsequent elution of the CMC column with 0.2 M phosphate buffer, pH 8.0 (Figure 1), yielded a third fraction capable of digesting all of the bacterial cell walls which had been prepared. This fraction III was nearly pure F_1 endo-N-acetylmuramidase. Fractions I–III contained respectively 1.5, 2, and 0.75% of the materials absorbing at 278 mμ which were present in the dialyzed preparation of crude enzymes. Each fraction was concentrated by dialysis on solid Carbowax 4000 BDH and then purified as described below.

Purification of the Streptomyces F_1 endo-N-Acetylmuramidase. The purification of the F_1 enzyme from fraction III (Figure 1) will be described elsewhere. The preparation finally obtained was three times more active than egg-white lysozyme in terms of milligrams of cell walls of *M. lysodeikticus* digested per milligram of protein (specific activity of the F_1 enzyme = 700 mg of cell walls/mg hr). The F_1 enzyme has a lytic spectrum broader than that of lysozyme.

Preparation of the Lytic MR Endopeptidase. Fraction II (Figure 1) was dialyzed against 0.01 M Tris–HCl buffer, pH 8.4, and readsorbed on a 175-ml column of CMC which had previously been equilibrated with the same buffer. Elution was then carried out with a gradient of increasing concentration of Tris–HCl buffer, pH 8.4, obtained by adding 0.20 M buffer to a flask containing 2000 ml of 0.01 M buffer. The volume of the solution in the mixing flask was kept constant. Both MR endopeptidase and Caseinase C activities were eluted in the range of 0.07–0.08 M. The active fractions were concentrated on solid Carbowax, dialyzed against water, and centrifuged to remove a slight turbidity appearing during concentration.

Preparation of the Lytic SA Endopeptidase and of the Lytic ML Endopeptidase. Fraction I (Figure 1) was subjected to gradient chromatography on CMC under the same conditions as those used for preparing the MR endopeptidase. Aliquots of the fractions which were collected were analyzed for lytic activity on cell walls of *S. aureus*, *M. roseus*, and *M. lysodeikticus*, and for hydrolytic activity upon tetraglycine, L-Ala-L-Ala-Gly, and the soluble *S. aureus* peptidoglycan GP$_2$ (Figure 2). Fractions E and G were lytic on cell walls of both *S. aureus* and *M. roseus*. Fraction D and F were lytic on cell walls of *M. lysodeikticus*. The four fractions were concentrated separately by dialysis on Carbowax and the lytic actions of the preparations were investigated on the relevant sensitive cell walls. Fractions E and G were found to be indistinguishable with regard to the nature and to the number of peptide bonds
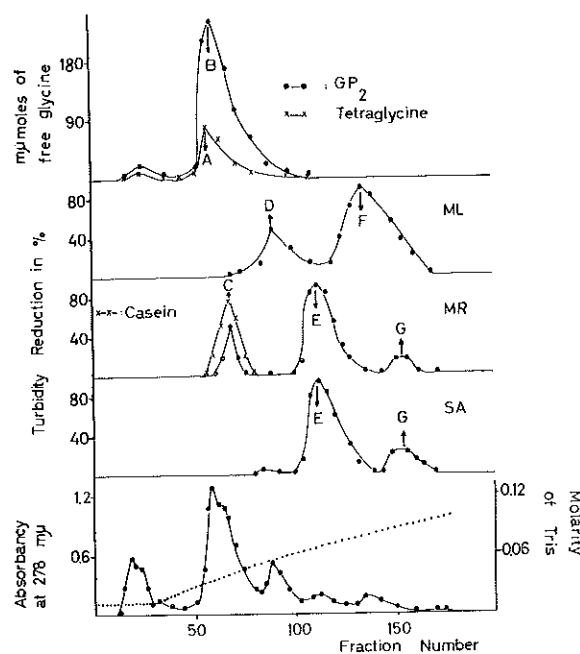

FIGURE 2: Preparation of SA endopeptidase, of ML endopeptidase, and of peptidase 2. Fractionation on CM-cellulose of the mixture of enzymes present in fraction I of Figure 1. For conditions, see text. Gradient of increasing Tris–HCl buffer concentration (pH 8.4) was started at fraction 30. The volume of the fractions was 8 ml. *Bacteriolytic activities:* 500 μg of cell walls (SA, MR, ML; see Figure 1) were incubated at 37° with 100 μl of each fraction for 1 hr (SA and MR) or for 20 min (ML) in a final volume of 500 μl. If necessary the mixtures were supplemented with concentrated Tris–HCl buffers, pH 8.4, in order to reach a final concentration of 0.01 M. *Caseinolytic activity:* 50 μl of each fraction was incubated at 37° with 500 μl of the casein solution (see Figure 1) for 20 min. *Hydrolytic activities on tetraglycine and on Staphylococcus peptidoglycan GP$_2$:* 8.96 μl of each fraction was added with 10 μl of 0.03 M phosphate buffer, pH 8.0, and with 20 μg of tetraglycine (80 mμmoles) or with 150 μg of peptidoglycan GP$_2$ in a final volume of 40 μl. After 18 hr of incubation at 37°, free glycine residues were estimated by thin layer chromatography as previously described (Ghuysen *et al.*, 1965b). Results are expressed as mμmoles of free glycine present in the incubated mixtures.

hydrolyzed in cell walls of *S. aureus* and *M. roseus;* they are two different forms of the same peptidase: the SA endopeptidase. A similar observation was made with the ML endopeptidase of fraction D and the ML endopeptidase of fraction F, with regard to their lytic activities on cell walls of *M. lysodeikticus*. Similarly, fraction C, which is capable of digesting casein and cell walls of *M. roseus*, was identified as a second form of the MR endopeptidase of fraction II (Figure 1). Rechromatography on CMC of fraction

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F, *i.e.*, the main form of the ML endopeptidase, and fraction E, *i.e.*, the main form of the SA endopeptidase, did not induce any further dissociation into two active forms, but yielded[2] the *ML endopeptidase* and the *SA endopeptidase.*

The rechromatographed ML endopeptidase and SA endopeptidase were the enzymes used in subsequent studies, unless otherwise stated. The study of the minor form of the endopeptidases has not been carried further.

Preparation of Peptidase 2. Fraction A (Figure 2) was capable of degrading the tetrapeptide Gly-Gly-Gly-Gly and the tripeptide L-Ala-Gly-Gly into free amino acids. The kinetics of the degradation of this latter tripeptide implicated an aminopeptidase. When the soluble peptidoglycan GP₂ was used as substrate, the opened pentaglycine chains having free terminal amino groups were also degraded into free glycine residues (Figure 2) and new ε-amino groups of lysine appeared (not shown in Figure 2). However with GP₂ as substrate, the spectrum of activity was slightly shifted, the activity being now represented by curve B. This shift was repeatedly obtained and seems to be significant. It might be due to the presence of the nonlytic endopeptidase, the existence of which has been postulated in earlier work (Ghuysen *et al.*, 1964, 1965b) and which would be able to open glycine bridges left intact by the SA endopeptidase. The nonlytic endopeptidase would be eluted at slightly higher concentrations than the aminopeptidase so that the maximum release of glycine residues from GP₂ would then be observed with the fraction (maximum of curve B) which contains the most adequate proportion of aminopeptidase and of nonlytic endopeptidase. Fractions A and B were pooled and concentrated by dialysis on Carbowax. The enzymes were readsorbed on a column of CMC equilibrated with 0.01 M Tris–HCl buffer, pH 8.8, and the elution was carried out with a gradient of increasing concentration of Tris–HCl buffer, pH 8.8. At this pH, the aminopeptidase(s) active on tetraglycine and on *Staphylococcus* peptidoglycan GP₂ were still eluted at a low concentration (about 0.025 M) but they were well separated from the contaminating MR endopeptidase (fraction C of Figure 2) which was now eluted at 0.07 M. The fractions active on tetraglycine and on the *Staphylococcus* peptidoglycan were pooled, concentrated by dialysis on Carbowax, dialyzed against water, and centrifuged. The clear supernatant yielded a preparation, designated peptidase 2.

Preparation of Highly Purified MR Endopeptidase, ML Endopeptidase, and SA Endopeptidase. With the use of the procedure which has just been described, the three lytic endopeptidases retained minor amounts of aminopeptidase activity. This contamination was hardly detectable when tetraglycine or *Staphylococcus*

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[2] The order of elution of the ML endopeptidase and of the SA endopeptidase from the CMC column using Tris–HCl buffers of increasing molarities varied according to the pH. At pH 7, a poor dissociation was obtained. At pH 8.8, the ML endopeptidase was first eluted, followed by the SA endopeptidase.

peptidoglycan GP₂ were used as substrates and therefore in many cases these enzyme preparations could be used as such. However, poly-L-alanine chains having free terminal amino groups, such as are produced in cell walls of *M. roseus* by treatment with SA endopeptidase (see further), are extremely sensitive to contaminating amino peptidase. For this reason, these compounds were also used as substrates in the fractionation assay represented in Figure 2. The maximum release of free alanine was still found associated with fractions A–B, but aminopeptidase activity could be detected throughout, up to fraction 200.

Highly purified MR endopeptidase, ML endopeptidase, and SA endopeptidase have been obtained, although in a very poor yield. The crude *Streptomyces* enzyme complex was adsorbed on a CMC column as described above. Elution was carried out with 0.2 M Tris–HCl buffer, pH 8.0, instead of 0.15 M buffer. Now, all the peptidases, including the MR endopeptidase, were eluted together. The F₁ *endo-N*-acetylmuramidase was still retained on the resin and was eluted with the 0.2 M phosphate buffer, pH 8.0. The crude peptidase preparation in Tris–HCl buffer was precipi-

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TABLE I: Lytic Spectrum of the *Streptomyces* Endopeptidases.

Substrate	ML Endopeptidase	SA Endopeptidase	MR Endopeptidase
M. lysodeikticus, walls	a	b	b
M. citreus, walls	a	b	b
M. flavus, cells	a	b	b
S. lutea, walls	a	b	b
S. aureus, walls	b	a	b
S. albus, cells	b	a	b
G. tetragena, cells	b	a	b
S. pyogenes A, walls	b	a	b
M. radiodurans, walls	b	a	b
M. roseus R 27, walls	b	a	a
M. roseus Thr⁽⁻⁾, walls	b	a	a

[a] A complete clarification of a suspension of the substrate occurs in 0.01 M Tris or Veronal buffer, pH 8.8, when the incubation is carried out with the appropriate enzyme. [b] No clarification occurs. The cell walls from the bacteria reported in the table contain L-lysine or, in the case of *M. radiodurans*, L-ornithine. Cell walls from the following bacteria, all of which contain DAP rather than lysine, were not lysed by any of the three enzymes: *M. varians, B. megaterium, B. subtilis* (for this species, intact cells were used), *C. histolyticum, P. technicum, C. fermentans, C. anaerobicum, P. rubrum* and *P. petersonii.* It should be noted that the DAP in *C. anaerobicum* (R. Tinelli, personal communication) and in *P. rubrum* and *P. petersonii* (Allsop and Work, 1963) is in the LL form.

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TABLE II: Nature of the Bonds Attacked by the Lytic Endopept'dases.

Endopeptidase	Bacterial Cell Wall	Sensitive L nkage
SA Endopeptidase	*S. aureus* Copenhagen	D-Alanyl-glycyl
	M. roseus R 27 or Thr(⁻)	D-Alanyl-L-alanyl
MR Endopeptidase	*M. roseus* R 27	L-Alanyl-L-threonyl
	M. roseus Thr(⁻)	L-A'anyl-L-alanyl
ML Endopeptidase	*M. lysodeikticus*	Alanyl-ε-lysyl

tated at 0° by the addition of solid ammonium sulfate to 0.8 saturation. Most of the exopeptidase activities remained in the supernatant (from which they could not be recovered). The three endopeptidases present in the pellet were rechromatographed several times on CMC at pH 8.8 using gradients of increasing Tris–HCl buffer concentration, as already indicated, until each of them was virtually free from each other and of the last detectable trace of aminopeptidase.

Results

Enzymatic Specificity of the Lytic Endopeptidases. Each of the three lytic endopeptidases is active within a narrow range, as seen on incubation with whole Gram-positive cells or their walls in 0.01 M Tris–HCl or Veronal–HCl buffer at pH 8.8. None of them acts on cell walls containing DAP, and the lysine-containing cell walls which do serve as substrates fall into three groups (Table I), respectively typified by *M. lysodeikticus*, *S. aureus*, and *M. roseus*. L-Ornithine-containing cell walls of *M. radiodurans* (Work, 1964) behave as cell walls of the *S. aureus* group.

Peptide bonds undergo hydrolysis when cell walls of *S. aureus*, *M. roseus*, and *M. lysodeikticus* are digested by the appropriate endopeptidases. No glycosidase is involved in the phenomenon since the lysis is not accompanied by the appearance of reducing groups or of *N*-acetylamino sugars. By comparing the C- and N-terminal residues present in lysates of cell walls obtained with an *endo-N*-acetylmuramidase with those obtained using highly purified endopeptidases, the sensitive linkages have been identified (see Table II). The stereochemical configuration of the C-terminal alanine was enzymatically determined after isolation of the free alanine residues from the products of hydrazinolysis by Dowex 50 chromatography (Ghuysen *et al.*, 1966a). The stereochemical configuration of the N-terminal alanine and threonine were also determined enzymatically after they had been released by the aminopeptidase of peptidase 2. The determination was made either directly on the digest or after isolation of the free alanine or free threonine by Sephadex G-25 chromatography. As noted earlier, native cell walls of *M. lysodeikticus* have a high content of free N- and C-terminal residues which was greatly reduced by the masking procedures described earlier. Thus, the

ε-amino groups of lysine which appeared as a consequence of the treatment with the ML endopeptidase were measured in hydroxymethylated cell walls and the carboxyl groups of alanine which had also appeared, were measured in cell walls previously treated with methanol followed by hydroxylamine (see Methods).

The Peptidoglycan of Cell Walls of S. aureus Strain Copenhagen. Part of the cell wall of *S. aureus* is a teichoic acid polymer having ester-linked D-alanine residues. Since difficulties in interpreting the mechanism of action of the peptidases in the presence of these labile groups were anticipated, they were first removed by treatment of the cell walls with a 9 N NH₄OH solution (20 min at room temperature). This treatment also removed the *O*-acetyl groups which substitute some of the *N*-acetylmuramic acid residues of the peptidoglycan (Ghuysen and Strominger, 1963b; Tipper *et al.*, 1965). The amino acid composition of the NH₃-treated cell walls is, in millimicromoles per milligram: Ala 1000 (L-Ala 470; D-Ala 525; when measured by the enzymatic procedure described under methods); Glu 470; Lys 470; Gly 2170.

Earlier work (Ghuysen *et al.*, 1964, 1965a,b) have shown that 1 mg of cell walls contains 470 mμmoles of disaccharide peptide units cross-linked by pentaglycine bridges (Figure 3), and that the pentaglycine bridges are linked to the ε-amino group of lysine residues. In this series of experiments, cell walls of *S. aureus* had been first digested by an *endo-N*-acetylmuramidase, then further degraded with a crude preparation of SA endopeptidase (formerly, peptidase 1) and finally with peptidase 2. When the enzymes were used in this order, only about 60% of the polyglycine bridges were opened and eventually degraded. It was found afterwards that the action of SA endopeptidase is much depressed when the cell walls have been first digested with an *endo-N*-acetylmuramidase. Consequently, in the present series of experiments enzymes were used in a different order: first the SA endopeptidase, then peptidase 2, and finally the F₁ *endo-N*-acetylmuramidase, with as a result, the disruption of virtually all of the pentaglycine bridges.

A. THE PEPTIDE BRIDGES IN *S. aureus* CELL WALL. When SA endopeptidase directly lyses cell walls of *S. aureus*, it liberates N-terminal glycine and C-terminal D-alanine, therefore defining the point of attachment of the N-terminal end of the bridges (Figure 3). This
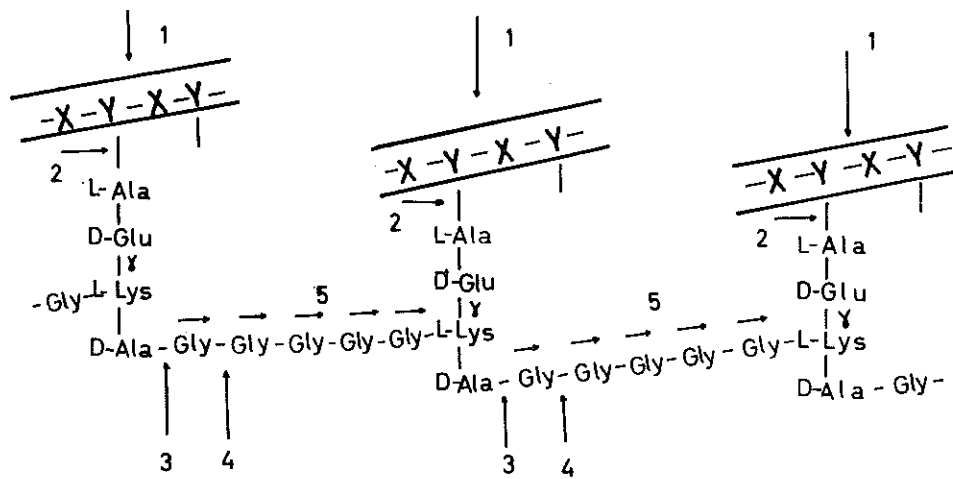
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FIGURE 3: Structural schemes of the peptidoglycan of cell walls of *S. aureus* Copenhagen and sites of action of the enzymes used throughout their degradation. X, *N*-acetylglucosamine; Y, *N*-acetylmuramic acid. (1) Hydrolysis of the glycan with F_1 *endo-N*-acetylmuramidase. (2) Action of the *N*-acetylmuramyl-L-alanine amidase. (3) Opening of peptide bridges with SA endopeptidase. (5) Degradation of the opened chains with the aminopeptidase. Experiments (for full discussion, see text) suggest that the SA endopeptidase only opens 50–60% of the polyglycine bridges. The opening of all of the peptide bridges would then be achieved by a nonlytic endopeptidase of peptidase 2. The site of action of the postulated nonlytic endopeptidase would be in 3 or perhaps in 4.

finding is in agreement with the fact that in cell walls of *S. aureus*, the α-carboxyl groups of glutamic acid exists in the form of a carboxamide (Tipper and Strominger, 1965). Also, a similar conclusion has been reached from studies of the staphylolytic enzyme from *Flavobacterium* (K. Kato and J. L. Strominger, in preparation).

Cell walls (500 mg) of *S. aureus* (deprived of the ester-linked D-Ala residues) were digested with 3.3 mg, estimated as protein, of SA endopeptidase in 50 ml of 0.01 M Veronal–HCl buffer, pH 8.8. After 6 hr of incubation at 37°, complete solubilization had occurred; 250 mμmoles of N-terminal glycine and an equivalent number of C-terminal D-alanine had appeared per milligram of cell walls. This number was not modified during a prolonged incubation. After 20 hr, the soluble digest was heated at 100° for 10 min and adjusted to pH 8.0 with HCl, and 0.5 ml of 1 M K_2HPO_4 solution was added. Aliquots containing 500 μg of digested cell walls were then treated with 27 μg, estimated as protein, of peptidase 2 and were analyzed after increasing times of incubation. As shown in Figure 4, addition of peptidase 2 induced the appearance of new terminal amino groups of glycine, liberated increasing amounts of free glycine residues up to about 2000 mμmoles/mg, which is at least 90% of the total glycine content of the cell walls, and eventually exposed 470 mμmoles/mg of ϵ-amino group of lysine, equivalent to the total lysine content of the cell walls. At the end of the process, terminal amino groups of glycine had virtually disappeared.

B. ISOLATION OF DISACCHARIDE PEPTIDE SUBUNITS FROM *S. aureus* CELL WALLS. After exhaustive digestion of 500 mg of cell walls with SA endopeptidase and peptidase 2, the presumed tetrapeptide chains (Ala-Glu-Lys-Ala) had been disconnected from each other but were still attached to an intact glycan polymer. The latter was now completely degraded into disaccharide units by means of the F_1 *endo-N*-acetylmuramidase. The final digest was fractionated by filtration in water, on a column of Sephadex G 25, bead form (4 × 70 cm), yielding a teichoic acid polymer, a disaccharide peptide fraction, and finally free glycine. In terms of lysine residues (246 mμmoles of lysine from 1 mg of cell walls), the actual yield of the disaccharide peptide was 52% of the theoretical value. This figure does not take into account the glycopeptide fragments which occur in the teichoic acid fraction (about 20% in terms of total ϵ-amino groups of lysine present in the enzymatic digest before Sephadex filtration). The analytical data for the disaccharide peptide fraction, together with its degradation by the pig epididymis *exo-β-N*-acetylglucosaminidase and by the *N*-acetylmuramyl-L-alanine amidase, indicate that one 4-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid unit is connected by an amide linkage to an alanyl peptide of the composition: Glu 1; Lys 1; Ala 2.16 (L-Ala 1; D-ala 1.16; employing the enzymatic procedure); Gly 0.13. All of the ϵ-amino groups of lysine are free. The C-terminal residues are those of D-alanine and glycine. Furthermore, treatment of the peptide moiety with peptidase 2, after the disaccharide had been freed through the action of the *N*-acetylmuramyl-L-alanine amidase, liberated, per lysine, one L-Ala residue and unmasked one N-terminal Glu. A more complete study of this disaccharide peptide fraction is in progress and a detailed report will be forthcoming. However, the results briefly reported here support the structure GlcNAc-(β-1-4)-MurNAc-L-Ala-Glu-Lys-D-Ala for at

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least 70% of the disaccharide peptide fragments which have been isolated. Small amounts of disaccharide-Ala-Glu-Lys-Ala-Gly and disaccharide-Ala-Glu-Lys-Ala-Ala are also likely to be present. The disaccharide-Ala-Glu-Lys-Ala-Ala fragments must belong to a nascent uncross-linked peptidoglycan (Tipper and Strominger, 1965). The persistence of such pentapeptide residues indicates that the preparations, SA endopeptidase and peptidase 2, are devoid of the D-alanine carboxypeptidase activity which had been detected in earlier preparations (Ghuysen *et al.*, 1964, 1965a,b).

The Peptidoglycan of Cell Walls of M. roseus Strain R 27. The amino acid composition of the cell walls is, in millimicromoles per milligram: Ala 1730 (L-Ala 1180; D-Ala 460; employing the enzymatic procedure); Glu 400; Lys 290; L-Thr 300; Gly 86; Ser 44; Asp 40; Val, Leu: traces. The results obtained by analysis of the products of several sequences of enzymatic degradation described below lead us to propose the structure shown in Figure 5 for the peptidoglycan of the cell walls of *M. roseus*. About 75 mμmoles of this statistical unit (or 300 mμmoles of the peptide subunits) are present in 1 mg of cell walls. This unit consists of three disaccharide tetrapeptides and of one disaccharide tripeptide, bridged by three L-alanyl-L-alanyl-L-alanyl-L-threonyl chains which link the C-terminal D-Ala of one disaccharide peptide to the ε-amino group of lysine of a second disaccharide peptide. In each unit exists also a (L-Ala)₃-L-Thr chain in which the N-terminal end is free.

A. THE GLYCAN PORTION OF *M. roseus* CELL WALLS. The cell walls were digested with the F₁ *endo-N*-acetylmuramidase. At completion of the reaction, 300 mμmoles/mg of disaccharide 4β-N-acetylglucosaminyl-N-acetylmuramic acid had appeared. Treatment with the N-acetylmuramyl-L-alanine amidase then led to about 280 mμmoles of new terminal amino groups being exposed. A more detailed description of this degradation will be forthcoming.

B. THE NATIVE UNCROSS-LINKED PEPTIDE CHAINS IN *M. roseus* CELL WALLS. An F₁ *endo-N*-acetylmuramidase digest of the cell walls contains 50–70 mμmoles/mg of native N-terminal alanine residues. Acting on such a digest, the aminopeptidase of peptidase 2 degrades only the native uncross-linked chains (L-Ala)₃-L-Thr. Cell walls (10 mg) were solubilized in 30 min, at 37°, with 50 μg of F₁ *endo-N*-acetylmuramidase in 1 ml of 0.015 M citrate buffer, pH 5.5. To aliquots containing 500 μg of digested cell walls, 11 μl of 0.1 M K₂HPO₄ buffer, pH 8.0, and 15 μg, measured as protein, of peptidase 2 were added. As shown in Figure 6, lower part and solid lines of upper part, the amino peptidase in peptidase 2 removed *seriatim*, 210 mμmoles/mg of free alanine and 70 mμmoles/mg of free threonine residues which were identified as L-alanine and L-threonine by the enzymatic procedure described under Methods. Eventually, a number of ε-amino groups of lysine, equivalent to the number of L-threonine residues which had been liberated, were unmasked (70 mμmoles/mg). In another experiment the velocity of the enzymatic degradation was reduced by adding

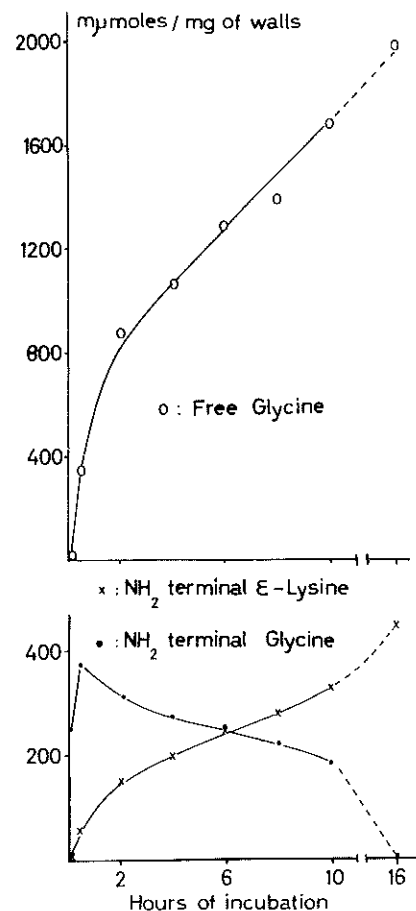

FIGURE 4: Changes in free glycine, amino-terminal glycine, and free ε-amino group of lysine during the degradation by peptidase 2 of a SA endopeptidase 1 digest of *S. aureus* cell walls. Cell walls of *S. aureus* were digested with SA endopeptidase as indicated in the text. Aliquots containing 500 μg of digested cell walls were incubated at 37° with 27 μg of peptidase 2. After 10 hr of incubation, a new addition of fresh peptidase 2 (27 μg) was made.

only 3 μg of peptidase 2 to aliquots containing 500 μg of F₁-digested cell walls. As shown in Figure 6, upper part, dashed lines, free threonine residues began to appear only when 80% of that number of alanine residues which are eventually produced were already liberated. This supports the proposed sequence L-Ala-L-Ala-L-Ala-L-Thr which, attached by its C terminus to the ε-amino group of lysine, constitutes the native uncross-linked peptide chains.

C. THE PEPTIDE BRIDGES IN *M. roseus* CELL WALLS. Cell walls (500 mg) of *M. roseus* were digested with 6.6 mg, measured as protein, of SA endopeptidase in 50 ml of 0.005 M Veronal–HCl buffer, pH 8.8. After 8 hr of incubation, complete solubilization had occurred but the incubation was prolonged for 18 hr. Analysis of the digest showed that SA endopeptidase had hydrolyzed D-alanyl-L-alanyl linkages. About 180–200
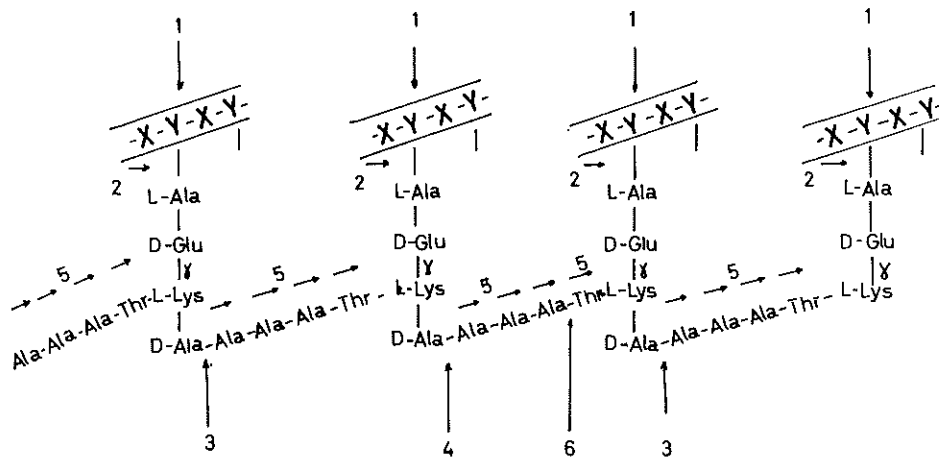
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FIGURE 5: Structural schemes of the peptidoglycan of cell walls of *M. roseus* (R 27) and sites of action of the enzymes used throughout their degradation. Alanine and threonine residues in peptide bridges are in the L form. X, Y, 1, 2, 3, 4, 5: see Figure 3; 6, opening of tri-L-Ala-L-Thr bridges with MR endopeptidase. Experiments suggest that two kinds of bridges could occur in almost equivalent amounts. They would be respectively sensitive to SA endopeptidase (in 3) or to MR endopeptidase (in 6). It is also suggested that those bridges untouched by the SA endopeptidase would then be sensitive to the postulated nonlytic endopeptidase of peptidase 2, acting on linkage 4.
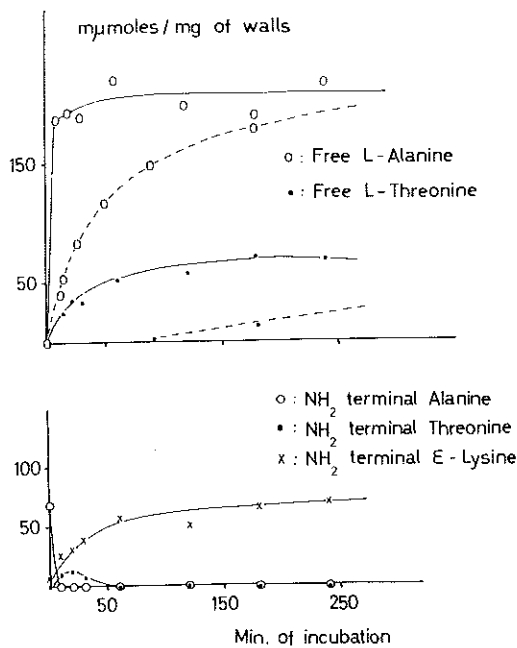

FIGURE 6: Degradation of the native uncross-linked tri-L-Ala-L-Thr chains in cell walls of *M. Roseus* R 27. Changes in free L-Ala and L-Thr, amino-terminal Ala and Thr, and free, ε-amino group of lysine during the degradation, by peptidase 2, of an F_1 endo-N-acetylmuramidase digest of the cell walls. For conditions: see text. The curves drawn in solid lines represent the kinetics of the degradation when 15 μg of peptidase 2 were added to 500 μg of digested cell walls. The curves in dashed lines refer to the case where only 3 μg of peptidase 2 were added.

mμmoles of terminal amino groups of alanine was now present in the digest. The digest was heated at 100° for 10 min and adjusted to pH 8.0, and 0.5 ml of a 1 M K_2HPO_4 solution was added. Subsequent treatment with 9 mg, measured as protein, of peptidase 2 liberated first about 900 mμmoles/mg of alanine, enzymatically identified as L-alanine (which represents all the L-alanine residues available for cross bridging), and then 300 mμmoles/mg of threonine (which represents the total threonine content of the cell walls). Parallel to the appearance of free threonine, ε-amino groups of lysine were exposed and, at the end of the process, they were equivalent to the total lysine content of the cell walls.

Employing adequate amounts of peptidase 2, it has been possible to liberate from an SA endopeptidase digest of the cell walls all of the removable L-alanine residues without liberating appreciable amounts of threonine. In one experiment, the incubation was stopped after the alanine residues had been liberated. The lyophilized digest was subjected to gel filtration in water, on a column of Sephadex G-25, bead form, and a high molecular weight compound was recovered free of L-alanine. This material was treated once more with a larger amount of peptidase 2 which now induced the liberation of the threonine residues. These residues were isolated by a further Sephadex filtration and found to possess the L configuration by the procedure described under Methods.

D. ISOLATION OF DISACCHARIDE PEPTIDE SUBUNITS FROM *M. roseus* CELL WALLS. After exhaustive digestion of 500 mg of cell walls of *M. roseus* with SA endopeptidase and peptidase 2, the glycan polymer was completely

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degraded into disaccharide units by the action of the F_1 endo-N-acetylmuramidase. Filtration of this latter digest in water, on a column of Sephadex G-25 bead form (4 × 70 cm), yielded three fractions: an as yet unidentified polymer still containing peptidoglycan debris (about 25% in terms of total ε-amino groups of lysine), a disaccharide peptide fraction, and a mixture of free L-alanine, free L-threonine, and, as has occurred with some enzyme preparations, free D-alanine.[3] In terms of lysine residues (174 mμmoles of lysine residues from 1 mg of walls), the actual yield of the disaccharide peptide was 58% of the theoretical value. Analytical data of the disaccharide peptide fraction show that the alanyl peptide is composed of Glu 1; Lys 1; Ala 1.60 (L-Ala 1; D-Ala 0.57; by the enzymatic procedure). All of the lysine residues have free ε-amino groups. D-Alanine and, to a smaller extent, lysine are both C terminal. Acting on the peptide chains obtained after digestion of the disaccharide peptide fraction with the N-acetylmuramyl-L-alanine amidase, peptidase 2 liberated, per lysine, one L-Ala residue and unmasked one N-terminal Glu. Here also, a more complete study of the disaccharide peptide fraction is in progress. However, the present results suggest that disaccharide-L-Ala-Glu-Lys-D-Ala and disaccharide-L-Ala-Glu-Lys must both occur in the fraction. The disaccharide tripeptide must be a genuine constituent of the cell walls of $M.$ roseus as they are prepared, and not an artifact resulting from the enzymic digestion since no such defective peptide is found when the cell walls of $S.$ aureus are degraded with the aid of the same enzyme preparations.

The Possible Occurrence of Two Kinds of Peptide Bridges in Cell Walls of S. aureus Copenhagen and M. roseus R 27. After successive treatments with SA endopeptidase and peptidase 2, all of the peptide bridges in cell walls of $S.$ aureus and of $M.$ roseus are completely degraded into free amino acids; indeed, the number of ε-amino groups of lysine finally unmasked is equal to the total lysine content of the cell walls. However, this number always exceeds the number of N-terminal glycine residues, in the case of $S.$ aureus, or the number of N-terminal L-alanine residues, in the case of $M.$ roseus, resulting from the action of SA endopeptidase, i.e., from the original bridge-opening at the N-terminal point of attachment; NH₂-glycine (250 mμmoles) and 200 mμmoles of NH₂-L-alanine were found in the SA endopeptidase digests of the relevant cell walls, whereas at the end of the degrada-

tion with peptidase 2, 470 and 300 mμmoles of ε-NH₂-lysine, respectively, were present. The C-terminal D-alanine which appeared as a consequence of the SA endopeptidase treatment and which was measured after hydrazinolysis of the cell walls digests also indicates that about 60% of the peptide bridges have been opened. Considerable caution, however, must be used in the interpretation of these data and it is possible that all of the peptide bridges in the cell walls are opened at their amino terminus by the SA endopeptidase but that only part of the newly formed C- and N-terminal groups are actually measured. However, if, as the experimental data show, the SA endopeptidase is, in fact, only capable of opening about 60% of the peptide bridges in cell walls of $S.$ aureus and of $M.$ roseus, then peptidase 2 must contain a nonlytic endopeptidase capable of opening those bridges left untouched by SA endopeptidase. The affinities of SA endopeptidase and of the postulated nonlytic endopeptidase contained in peptidase 2 might be respectively directed toward highly and little polymerized peptide substrates. In such a case, the nonlytic endopeptidase would begin to act when SA endopeptidase had degraded the substrate to such an extent that it would no longer act itself. Also, the affinities of SA endopeptidase and of the presumed nonlytic endopeptidase might be directed toward two kinds of peptide bridges, as it has been previously suggested for cell walls of $S.$ aureus (Ghuysen et al., 1965b).

The presence of two kinds of peptide bridges in cell walls of $M.$ roseus is suggested by the mechanism of action of the MR endopeptidase. The lytic MR endopeptidase hydrolyses about 150 mμmoles of L-alanyl-L-threonyl linkages in 1 mg of $M.$ roseus cell walls. Acting on such a digest, peptidase 2 then degrades those chains which were uncross-linked in the native cell walls (as it does when acting upon an F_1 endo-N-acetylmuramidase digest of the walls) and those bridges that have been opened by the MR endopeptidase. Indeed, peptidase 2 liberates 200 mμmoles of L-alanine and 200 mμmoles of L-threonine (i.e., 50–70 from the native uncross-linked chains and 150 from the N-terminal threonine produced by the MR endopeptidase treatment). Concomitant with the release of L-threonine, 200 mμmoles of ε-amino group of lysine appears. Therefore, it can be concluded with certainty that MR endopeptidase acts only on about 50% of the peptide bridges and that peptidase 2 is now unable to open those bridges left intact. Another experiment strongly suggests that the bridges untouched by the MR endopeptidase are sensitive to the SA endopeptidase. Indeed, the digestion of the cell walls of $M.$ roseus with a mixture of SA endopeptidase and of MR endopeptidase results in the appearance of about 100 mμmoles/mg of each of N-terminal Ala and N-terminal Thr, which, together, represents the opening of about 90% of the bridges. Further treatment with peptidase 2 (Figure 7) now liberates 300 mμmoles of free L-threonine but only about 630 mμmoles of free L-alanine instead of 900 mμmoles as was observed when, prior to the action of peptidase 2, the digestion

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[3] The rate of liberation of D-alanine is slow; the opening of all the peptide cross-bridges and their degradation are complete before any free D-Ala residue is detected. It is believed that 250 mμmoles/mg of D-Ala, together with 100 mμmoles of Glu, the various odd amino acids, and 90 mμmoles of organic phosphorus, are not in the peptidoglycan portion of the walls and form part of a nonglycopeptide polymer. The intact polysaccharide moiety of the peptidoglycan was isolated after digestion of the cell walls with the *Myxobacterium* bacteriolytic enzyme complex (Ensign and Wolfe, 1966). It was found to contain Glu and Ala residues (but virtually no Lys or Thr residues) in amounts compatible with the proposed hypothesis.
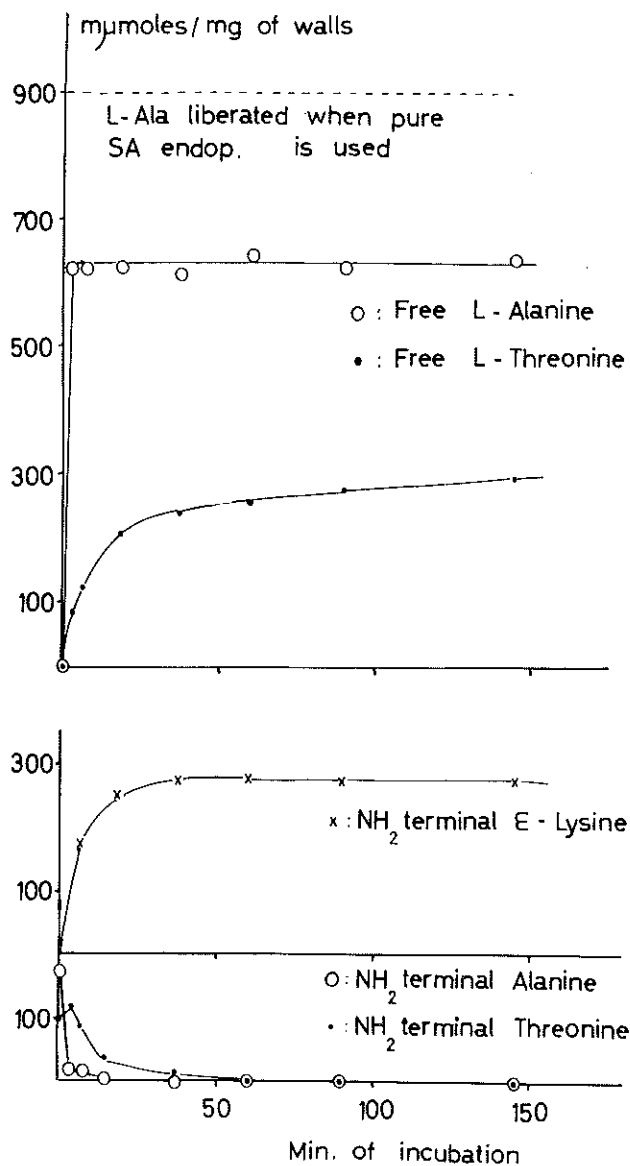
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FIGURE 7: Changes in free L-Ala and L-Thr, amino-terminal Ala and Thr, and free ε-amino group of lysine during the degradation by peptidase 2 of a SA endopeptidase and MR endopeptidase digest of cell walls of *M. roseus* R 27. Walls (10 mg) were digested in 2 hr at 37° in a final volume of 1 ml of 0.005 M Veronal HCl buffer, pH 8.8, with a mixture of the two lytic enzymes: SA endopeptidase which induced an increase in N-terminal Ala, per milligram of walls, from 50–70 to 170 mμmoles, and MR endopeptidase which induced the appearance of 100 mμmoles of N-terminal Thr. The digest was heated at 100° for 10 min, adjusted to pH 8 with HCl, and added with 10 μl of a 1 M K₂HPO₄ solution. Aliquots containing 200 μg of digested walls were added with 3 μg, in protein, of peptidase 2 (6 μl) and analyzed after increasing times of incubation.

of the cell walls was carried out with SA endopeptidase alone. From the foregoing, it may be postulated that some bridges in cell walls of *M. roseus* are preferentially sensitive to the lytic SA endopeptidase and that others are preferentially sensitive to the lytic MR endopeptidase or, after previous treatment with SA endopeptidase, to the postulated nonlytic endopeptidase of peptidase 2. There are no data, however, indicating how these two kinds of bridges might be different.

Peptide Bridges in Cell Walls of M. roseus Strain Thr⁽⁻⁾. Another kind of peptide cross-linkage, in the form of tri-L-Ala bridges, occurs in a different strain of *M. roseus* (Salton and Pavlik, 1960). In this case, the amino acid ratio in the cell walls is Lys 1; Glu 1; Ala 5; Thr 0. The action of peptidase 2 on an F_1 *endo-N-*acetylmuramidase digest of the cell walls showed that one native uncross-linked chain $(L-Ala)_3$ must occur for four cross-linked tetrapeptide subunits. The successive treatments of the cell walls with SA endopeptidase and peptidase 2 liberated three L-alanine residues per lysine residue. At this stage, all the lysine residues had free ε-amino groups. Cell walls of *M. roseus* Thr⁽⁻⁾ are also digested by the MR endopeptidase which in this case hydrolyses L-Ala-L-Ala linkages. The rate of hydrolysis is about 10 times smaller than that observed with cell walls of *M. roseus* R 27 where L-Ala-L-Thr linkages are hydrolyzed.

Peptide Bridges in Walls of M. lysodeitkicus Strain NCTC 2665. The amino acid composition of the walls, in millimicromoles per milligram, is Glu 500; Lys 500; Ala 1000; and Gly 475. This cell wall contains 500 mμmoles/mg of glucosamine and muramic acid, as measured by the Morgan–Elson reaction (Ghuysen et al., 1966a). ML endopeptidase hydrolyzes 60–100 mμmoles of alanyl-ε-lysyl linkages in 1 mg of cell walls. The composition of the cell walls excludes the possibility of polyalanine bridges and the 475 mμmoles/mg of glycine are adequately accounted for as substituents on the glutamic acid residues (W. Katz, D. J. Tipper, and J. L. Strominger, personal communication). Thus, peptide units appear to be linked directly to one another without any intervening bridges. The ML endopeptidase hydrolyses a limited number of peptide bonds, indicating a low order of cross-linking and, in agreement with this, 75% of the lysine has free ε-amino groups in the native cell wall. However, only a few C-terminal Ala are detectable. Moreover, one-half of the muramic acid carboxyl groups are free (Leyh-Bouille, 1965). Since the ratio between muramic acid and Glu is one, one would expect to find about 250 mμmoles of N-terminal Ala. In fact, there is a pronounced deficit in these amino groups. An explanation of this deficit and also of the paucity of C-terminal Ala, requires further investigations.

Discussion

The nature of the diamino acid in bacterial cell wall peptidoglycan, *i.e.*, L-lysine or one or more of the isomers of diaminopimelic acid, is a characteristic of taxonomic importance. It is not surprising that the

specificity of lytic peptidases should depend on the nature of the diamino acid in the substrates. Thus none of the peptidases from *Streptomyces* cultures acts upon any of the DAP-containing cell walls examined, including these in which DAP has been shown to be in LL form, as in *Propionibacterium petersonii* and *rubrum* (Allsop and Work, 1963) and in *Corynebacterium anaerobicum* (R. Tinelli, personal communication). An unexpected result, however, was the observation that the various peptidases differ in their affinities for L-lysine-containing cell walls depending on the nature of the cross-linkages. Three kinds of such cross-linkages, by which the ε-amino group of the lysine residue of one peptide chain is joined to the carboxyl group of the terminal D-alanine of a second, have been demonstrated, namely: bridging by tri-L-alanine, by tri-L-alanyl-L-threonine, and by pentaglycine residues. Although peptide bridges of different kinds have not been demonstrated to occur in the same cell wall, the mechanisms of action of the endopeptidases suggest the existence in the cell wall peptidoglycans of both *S. aureus* and *M. roseus* of two types of peptide bridges. A fourth kind of cross-linkage in which no additional amino acid is involved can also occur in the form of a direct bonding between the C-terminal alanine residue of one peptide chain and the ε-amino group of the lysine residue of a second (*M. lysodeikticus*). In all cases, the integrity of the cross-linkages is essential for the rigidity of the bacterial cell walls. In light of the foregoing, the antimicrobial effect of penicillins is, at least in part, explained by the formation of noncross-linked peptidoglycan, since it has been shown (Wise and Park, 1965; Tipper and Strominger, 1965) that penicillin inhibits the bonding of the N terminus of polyglycine units in *S. aureus* and that, as a consequence, a nascent uncross-linked peptidoglycan accumulates in the inhibited cells (Tipper and Strominger, 1965). It is striking that the same linkage, *i.e.*, a D-alanyl-glycine peptide bond, is involved in both the action of penicillin upon growing cells of *S. aureus* and in the action of SA endopeptidase upon resting (or possibly even growing) cells of the same organism; in the first case, the synthesis of that linkage is inhibited and in the second it undergoes hydrolysis. Similarly, alanyl-ε-lysine linkages are hydrolyzed when cell walls of *M. lysodeikticus* are digested by ML endopeptidase and a same peptide bond would be involved in the action of penicillin upon cells of this organism (Wise and Park, 1966).

Among the DAP-containing cell walls, direct linkages from D-alanine to DAP have been postulated to exist in the peptidoglycan from *Escherichia coli* (Weidel and Pelzer, 1964) and have been demonstrated for cell walls of *Corynebacterium* (K. Kato and J. L. Strominger, in preparation). No cross-linking peptide bridges have yet been observed in the DAP group of bacterial cell walls.

In a few bacterial cell walls, there is no lysine or DAP. The dibasic amino acid used by these cells to build up the peptidoglycan is L-2,4-diaminobutyric acid (Perkins and Cummins, 1964) or L- or possibly D-ornithine (Work, 1964; Perkins and Cummins, 1964; Cummins, 1965) or 2,6-diamino-3-hydroxypimelic acid

(Perkins, 1965). The L-ornithine-containing cell walls of *M. radiodurans* have been found to be digested by the SA endopeptidase, and in this respect they behave like the L-lysine-containing cell walls of the *S. aureus* group.

A priori, glutamic acid residues could also be involved in cross-linking by serving as anchor point for the N-terminal end of the bridges. However, no experimental demonstration of this type of cross-linking has been forthcoming.

Acknowledgment

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CORRECTION

In the paper entitled "The Behavior of Horseradish Peroxidase at High Hydrogen Peroxide Concentrations," by Ira Weinryb, in Volume 5, June 1966, on p 2006, second column, bottom line, "catalytic" should read "catalatic."