Structure of the Cell Walls of *Micrococcus lysodeikticus*. III. Isolation of a New Peptide Dimer, N^{α}-[L-Alanyl-γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanyl-N^{α}-[L-alanyl-γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanine*

Jean-Marie Ghuysen, Evanghelos Bricas,† Marvin Lache, and Mélina Leyh-Bouille

ABSTRACT: The pentapeptide monomer N^{α}-[L-alanyl-γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanine and two isomeric peptide dimers have been quantitatively isolated from walls of *Micrococcus lysodeikticus*. In one of the peptide dimers, referred as to peptide dimer (Ala→Lys), two pentapeptide monomers are linked through N^{ϵ}-(D-alanyl)-L-lysine linkages. This linkage is hydrolyzed by the *Streptomyces* ML endopeptidase but not by the *Myxobacter* AL I protease. In the second peptide dimer, referred as to peptide dimer (Ala→Ala), two pentapeptide monomers are linked through D-alanyl-L-alanine linkages. This linkage is hydrolyzed by the *Myxobacter* AL I protease but not by the *Streptomyces* ML endopeptidase. According to the type of enzymatic degradation used, the pentapeptide monomer has been obtained in the free form, or substituted at its *N*-L-alanine terminus either by a D-lactic acid residue or by an *N*-acetylmuramic acid residue. Similarly, the peptide dimer (Ala→Ala) has been obtained in the free form or as a lactyl derivative. The peptide dimer (Ala→Lys) has only been obtained in the free form. A comprehensive structure for a major part of the wall peptidoglycan is proposed. This structure takes into account the yields with which the peptide fragments are produced by the various enzymatic degradations. It provides explanation for the existence of a large number of peptide unsubstituted *N*-acetylmuramic acid residues in the glycan moiety. The structural peculiarity of the peptide moiety in *M. lysodeikticus* walls is the occurrence of large oligopeptides in which several pentapeptide monomers are linked through the aforementioned D-alanyl-L-alanine linkages. The isolation of the *N*-acetylmuramyl pentapeptide monomer involves, in one of the steps of the wall degradation, the use of a *Streptomyces* exo-*N*-acetylhexosaminidase active on both β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid and β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine disaccharides.

This is the first known enzyme acting as an exo-β-*N*-acetylmuramidase.

The cell walls of *Micrococcus lysodeikticus* are composed of a glucose- and 2-acetamido-2-deoxymannuronic acid containing complex (Perkins, 1963) which is probably linked, *via* phosphodiester bonds involving muramic acid 6-phosphate (Lui and Gotschlich, 1967), to an insoluble, rigid peptidoglycan. It has been well established that the glycan portion of the peptidoglycan (Salton, 1956; Salton and Ghuysen, 1960; Perkins, 1960; Sharon *et al.*, 1966; Leyh-Bouille *et al.*, 1966; Mirelman and Sharon, 1967) consists of linear strands of β-1,4-linked alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, and that the peptide portion of the peptidoglycan is built-up of subunits N^{α}-[L-alanyl-γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanine. Indeed, such subunits, linked at their *N*-L-alanine termini to β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides, have been isolated (Ghuysen, 1961) though in very small amounts, and their structures fully established (Mirelman and Sharon, 1967). Moreover, a peptide fraction, representative of a substantial part of the peptide moiety, was isolated after solubilization of the walls with *Myxobacter* AL-1 enzyme. Sequential degradation of this peptide with phenyl isothyocyanate (Tipper and Strominger, 1965; Tipper *et al.*, 1967a) revealed L-alanine and D-glutamic acid to be the first and the second amino acid, respectively, in the sequence and a substituent glycine residue on the α-carboxyl group of the glutamic acid residue. Finally it is known that UDP[1]-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala are the nucleotide precursors of the linear peptidoglycan strands in *M. lysodeikticus* (Anderson *et al.*, 1966) and that the glycine residue is added at the later stage of the

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[1] Abbreviations are as listed in *Biochemistry* 5, 1445 (1966).

lipid intermediates (Katz *et al.*, 1967). However, if one considers the macromolecular structure of the peptide moiety in the walls, several major problems present themselves which stem from the following facts. Contrary to what is observed with many other bacterial walls, the solubilization of the walls of *M. lysodeikticus* with glycosidases results in the release of di- and tetrasaccharide fragments which are unsubstituted by peptide. Recent quantitations (Leyh-Bouille *et al.*, 1966; Muñoz *et al.*, 1966) revealed that at least 50% of the *N*-acetylmuramic acid residues have free carboxyl groups. In native walls, one can distinguish between *N*-acetylmuramic acid residues with unsubstituted carboxyl groups and those with peptide substituents by esterifying the carboxyl groups of intact walls, followed by reduction of the esters with $LiBH_4$ (Salton, 1961). The procedure converts into alcohol, the carboxyl groups of the unsubstituted *N*-acetylmuramic acid residues. Since, however, the number of pentapeptide subunits in *M. lysodeikticus* walls is roughly equivalent to the number of disaccharide units, it is evident that the peptide subunits must form polymers in order to preserve the observed molar ratios between the peptidoglycan constituents and to accommodate the unsubstituted glycan fragments (Salton, 1961). The paucity of free N- and C-terminal alanine in native walls was an indication that the polymerization of the pentapeptide subunits into larger peptides might be mediated through D-alanyl-L-alanyl linkages, involving the C terminus of one subunit and the N terminus of another (Ghuysen *et al.*, 1966a). This type of polymerization was also proposed by Pickering (1966) from data compiled from various sources. Recently, Schleifer and Kandler (1967) isolated and characterized the dipeptide D-alanyl-L-alanine from partial acid hydrolysates of *M. lysodeikticus* walls, thus giving strong support to this hypothesis. It should be emphasized, however, that the branching of the peptide units on some of the *N*-acetylmuramic acid residues of the glycan strands cannot give rise to an insoluble network structure. Peptide cross-linking is therefore necessary. It is known to occur through N^{ϵ}-(D-alanyl)-L-lysine linkages. The characterization of this type of linkage which involves, at the most, half of the total lysine residues, resulted from the following three pieces of evidence. (1) A bisdisaccharide peptide dimer in which two pentapeptide units are linked through N^{ϵ}-(D-alanyl)-L-lysine linkages was isolated (Ghuysen, 1961) and characterized (Mirelman and Sharon, 1967); (2) the hydrolysis of such linkages by the *Streptomyces* ML endopeptidase causes the solubilization of the cell walls (Petit *et al.*, 1966); (3) the N^{ϵ}-(D-alanyl)-L-lysine dipeptide and the N^{ϵ}-(D-alanyl)-L-lysyl-D-alanine tripeptide have also been isolated from partial acid hydrolysates of the walls and fully characterized (Schleifer and Kandler, 1967). The possibility still exists, however, that other types of peptide cross-linkages might also occur. Tsai *et al.* (1965) observed that the solubilization of *M. lysodeikticus* walls by *Sorangium* proteases is accompanied by increases in not only C-terminal alanine, N^{ϵ}-terminal lysine, and N-terminal alanine groups but also in C-terminal glycine groups. This suggests that the glycine residues at the C terminal of some of the peptide units are also involved in peptide cross-linkages.

The present work describes new types of sequential degradations of *M. lysodeikticus* walls, and the quantitative isolation and the characterization of two isomeric peptides: the peptide dimer (Ala→Lys) and the peptide dimer (Ala→Ala) (Figure 1). It also proposes a comprehensive structure for a major part of the wall peptide moiety.

Materials and Method

Analytical Methods. Identification and measurement of free amino acids (fluorodinitrobenzene technique), of reducing groups (Park-Johnson technique), of acetamido sugars (Morgan–Elson reaction), and of amino sugars (chemical acetylation followed by Morgan–Elson reaction) have been previously described (Ghuysen *et al.*, 1966b). D-Glucose was determined on acid hydrolysates using the glucostat obtained from Worthington Biochemical Corp., Freehold, N. J. Free D-lactic acid was determined using D-lactic acid dehydrogenase (Dennis, 1962) as described by Tipper (1968). The D-lactic acid dehydrogenase was a gift from Dr. D. J. Tipper. N- and C-terminal groups were measured by a modification of the method described by Ghuysen *et al.* (1966b). For N-terminal determinations, the peptide (0.1 μmole) was dissolved in 50 μl of water. The solution was mixed with 8 μl of a 10% (v/v) ethanolic triethylamine solution and with 25 μl of 0.1 M fluorodinitrobenzene solution, also in ethanol (Jarvis and Strominger, 1967). The mixture was heated at 60° for 30 min and evaporated to dryness, and the residue was dissolved in 50 μl of 6 N HCl. Free dinitrophenylamino acids, if present, were removed by ether extraction. The dinitrophenyl peptide (aqueous phase) was hydrolyzed by heating at 100° for 18 hr, in sealed tubes. The dinitrophenylamino acids were then analyzed and quantitated as previously described (Ghuysen *et al.*, 1966b). Hydrazinolysis was carried out using (for 0.2 μmole of peptide) 50 μl of freshly redistilled hydrazine prepared according to the method of Smith and Howard (1944). Extreme care was taken to prevent contamination by water at all stages. The tubes were sealed and maintained for 24 hr at 80°. Hydrazine was removed by evaporation under vacuum over H_2SO_4. The residue was dissolved in 150 μl of water and 75 μl of freshly redistilled benzaldehyde was added. The tubes were stoppered and vigorously shaken for 1 hr, using a rotary Evapo-Mix Buchler Instruments Inc. (Fort Lee, N. J.). After centrifugation, the aqueous phase was collected and treated again with 70 μl of benzaldehyde as described above. The aqueous phase was extracted with ether and an aliquot (usually 75 μl) was used for the estimation of free amino acids. Glycine, alanine, and tripeptides glycylglycylalanine and serylglycylglycine, treated under exactly the same conditions, were used as controls. With the glycine and alanine controls, recovery was 65–80% of the theoretical values. Relative to these recoveries, the estimation of the C-terminal group in both tripeptides was 100% of the theoretical values.

Paper Electrophoresis. Electrophoresis was carried

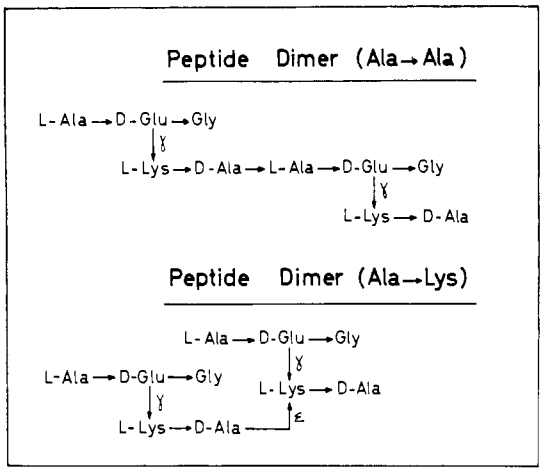

FIGURE 1: Structure of two isomeric peptide dimers isolated form cell walls of *M. lysodeikticus*.

out on Whatman No. 3MM paper in 0.1 M formic acid (pH 2) using either a high-voltage electrophorator Model D (Gilson Medical Electronics, Middleton, Wis.) apparatus (40 V/cm; paper sheet 30 × 100 cm) or an Electrorheophor (Pleuger, Antwerp, Belgium) apparatus (20 V/cm; paper sheet 40 × 40 cm).

Thin-Layer Chromatography. Chromatography was carried out on thin-layer plates of Stahl's silica gel G (Merck). Dinitrophenylamino acids were separated by sequential chromatography, in the same direction, first in solvent I (butanol–0.15 N ammonia, 1:1, upper phase) and then in solvent II (chloroform–methanol–acetic acid, 85:14:1, at 0°) (Ghuysen *et al.*, 1966b). The ε-monodinitrophenyllysine was separated using solvent III (benzyl alcohol–chloroform–methanol–water–concentrated ammonia, 30:30:30:6:2) (Ghuysen *et al.*, 1966a,b). Dinitrophenyl peptides were separated using solvent IV (*t*-amyl alcohol–chloroform–methyl alcohol–water–acetic acid, 30:30:30:20:3) (Katz and Strominger, 1967). Peptides were separated using solvent V (isobutyric acid–water–triethylamine, 150:86:13:9) (A. Worth, personal communication). The plates were developed for 6–7 hr, dried, and then developed again in the same solvent. The peptides were detected with ninhydrin spray.

Sephadex Column Chromatography. Separations of wall peptide fragments were carried out by gel filtration, in 0.1 M LiCl, on two columns: Sephadex G-50 fine (particle size 20–80 μ) and Sephadex G-25 fine (particle size 20–80 μ), connected in series, with a flow rate of about 30 ml/hr at room temperature. Although the size of the columns varied in the course of the work, the use of two identical columns of about 120 × 2.5 cm is quite satisfactory. The gel filtration properties of the compounds were expressed in terms of distribution coefficient $K_D = (V_e - V_i)/V_0$, where V_e is the elution volume of the compound, V_i is the volume of the solvent imbibed by the gel particules, and V_0 is the void volume of the column.

Cell Walls. The cell walls prepared by Petit *et al.* (1966) were the same preparation utilized in previous studies (Petit *et al.*, 1966; Leyh-Bouille *et al.*, 1966; Katz and Strominger, 1967). This preparation contained, per mg, approximately 480 mμequiv of disaccharide pentapeptide subunits traces of N-terminal alanine, 100 mμmoles of C-terminal alanine, 260 mμmoles of N^{ϵ}-terminal lysine, 260 mμmoles of D-glucose, and an undetermined amount of 2-acetamido-2-deoxymannuronic acid. Virtually all of the glycine residues are C terminal.

Wall Peptide. We are indebted to Dr. N. Sharon for a generous supply of disaccharide pentapeptide monomer and of bisdisaccharide peptide dimer (Ala→Lys) (Figure 1), both obtained after lysozyme degradation of *M. lysodeikticus* walls (Mirelman and Sharon, 1967), and to Dr. J. L. Strominger and Dr. W. Katz for a sample of mixture of *M. lysodeikticus* free peptides isolated after degradation of the walls with *Myxobacter* enzyme (Katz and Strominger, 1967).

Enzymes. *Streptomyces* F_1 endo-*N*-acetylmuramidase (Muñoz *et al.*, 1966), *Streptomyces* ML endopeptidase (Petit *et al.*, 1966), and *Streptomyces N*-acetylmuramyl-L-alanine amidase (Ghuysen *et al.*, 1962) have been described. *Myxobacter* ALI enzyme (a preparation containing 7260 units/ml; Ensign and Wolfe, 1965, 1966) was a generous gift from Dr. Ensign and Dr. D. J. Tipper. An exo-*N*-acetylhexosaminidase was prepared from a culture filtrate of *Streptomyces*. Unlike the pig epididymis enzyme which is active on the β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide but not on the β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine disaccharide, the *Streptomyces* enzyme hydrolyzes the two former isomeric disaccharides at identical rates. Optimum pH ranges from 5.5 to 8.5 using 0.02–0.1 M phosphate or Veronal buffers. No exo-*N*-acetylhexosaminidase preparation has yet been obtained free of lytic F_1 endo-*N*-acetylmuramidase, since the two enzymes were inseparable during the course of fractionation of the *Streptomyces* enzymes until the last filtration on Sephadex, in water. This procedure selectively denatures or irreversibly adsorbs the exo enzyme. The exo enzyme used in the present work was dissolved in 0.2 M phosphate buffer (pH 7).

Experimental Section

Degradation of the Cell walls of M. lysodeikticus GENERAL PROCEDURES. The cell walls were submitted to six different types of degradation (Table I). In a first series (procedures A–B–C), the N^{ϵ}-(D-alanyl)-L-lysine linkages were first hydrolyzed by ML endopeptidase (Petit *et al.*, 1966). The glycan was then degraded using either egg white lysozyme or the F_1 endo-*N*-acetylmuramidase. It must be emphasized that some of the glycosidic linkages from *N*-acetylmuramic acid to *N*-acetylglucosamine remain intact at completion of the reaction. The peptide units were then isolated either in the form of *N*-acetylmuramyl peptides after further treatment with the *Streptomyces* exo-*N*-acetylhexosaminidase (procedure A), or in the form of free peptides after further treatment with the *N*-acetylmuramyl-L-alanine amidase (procedure B), or in the form of D-lactyl peptides after β elimination carried out by alkali treatment (Ghuysen *et al.*, 1967; Tipper, 1968) (procedure C). In a

TABLE 1: Degradation of Cell Walls of *M. lysodeikticus*.

Procedure	Enzymatic Treatment	Degraded Products Isolated
A (Figure 3)	ML endopeptidase + lysozyme + exo-*N*-acetylhexosaminidase	*N*-Acetylmuramyl pentapeptide monomer
B (Figure 4)	ML endopeptidase + F_1 endo-*N*-acetylmuramidase + *N*-acetylmuramyl-L-alanine amidase	Pentapeptide monomer, peptide dimer (Ala→Ala), oligopeptide
C (Figure 5)	ML endopeptidase + F_1 endo-*N*-acetylmuramidase + NaOH	Lactyl pentapeptide monomer, monolactyl peptide dimer (Ala→Ala)
D (Figure 7)	*Myxobacter* ALI protease	Pentapeptide monomer, peptide dimer (Ala→Lys)
E (Figure 8)	Lysozyme	Disaccharide pentapeptide monomer
F (Figure 9)	F_1 endo-*N*-acetylmuramidase + NaOH	Traces of lactyl pentapeptide monomer and lactyl peptide dimer likely occur

second series of degradations (procedure D), the walls were solubilized with *Myxobacter* ALI protease working alone. It has been reported by Katz and Strominger (1967) that this enzyme acts on *M. lysodeikticus* walls as a lytic amidase only. We observed, however, that the wall degradation was accompanied by liberation of not only N-terminal alanine groups, but also of C-terminal alanine groups. The kinetics were measured as follows. Walls (20 mg) were suspended in a solution containing 1 ml of 0.05 M Veronal buffer (pH 9), 0.8 ml of water, and 0.2 ml of *Myxobacter* enzyme. Aliquots of 100 μl of the suspension were withdrawn after various times of incubation at 37°. Each aliquot was immediately mixed with 25 μl of a 0.2 M acetate buffer (pH 5) and maintained in a 100° water bath for 5 min. After cooling, 60 μg of lysozyme (6 μl of a 10-μg/μl solution) was added and the aliquots were incubated at 37° for 5 min. The lysozyme treatment completely clarified those aliquots which were removed before *Myxobacter* enzyme had solubilized the walls. As shown in Figure 2, a rapid release of about 200 mμmoles (per milligram of walls) of both N- and C-terminal alanine groups parallels the solubilization of the walls by *Myxobacter* enzyme. On prolonged incubation, no additional C-terminal alanine groups were liberated but N-terminal alanine groups continued to appear at a slow rate. After 20 hr, 380 mμmoles/mg of walls of N-terminal alanine was released. No liberation of any N^{ϵ}-terminal lysine groups was ever observed. The solubilization of the walls and the rapid coincident release of C- and N-terminal alanine groups are interpreted as being the result of the hydrolysis of the proposed D-alanyl-L-alanine interpeptide linkages. The subsequent slower release of N-terminal alanine can be explained as the result of the slow hydrolysis of *N*-acetylmuramyl-L-alanine amide linkages. The *Myxobacter* enzyme has been shown to have both endopeptidase and amidase activities on walls of *Staphylococcus aureus* (Tipper *et al.*, 1967b) and walls of *Arthrobacter cristallopoietes* (Krulwich *et al.*, 1967). In fact, with cell walls of *Arthobacter*, evidence was obtained that the enzyme catalyzed cleavage of a D-alanyl-L-alanyl linkage, although in a different position than that to be reported in this paper. In a third series of degradations (procedures E and F), walls were treated by glycosidases only. In one case, wall solubilization was followed by β elimination of the lactylpeptides. These degradations were carried out in order to estimate the occurrence of peptide monomers and peptide oligomers of small size in the native walls.

Preparation of the N-Acetylmuramyl Pentapeptide Monomer. PROCEDURE A. Walls (200 mg) were suspended in 10 ml of ML endopeptidase solution made up in 0.01 M phosphate buffer (pH 8.2). After 6 hr at 37°, complete clarification occurred and all the lysine residues had their ε-amino groups free. Lysozyme (5 mg) and *Streptomyces* exo-*N*-acetylhexosaminidase (60 ml in 0.2 M phosphate buffer, pH 7) were added. After 15 hr at 37°, maximum release of *N*-acetylhexosamine residues was obtained. Analyses indicated that 70% of the peptidoglycan *N*-acetylhexosamine residues was in the form of monomers. It should be noted that after degradation of the walls by lysozyme alone, only 30–40% of the peptidoglycan *N*-acetylhexosamine residues is in the form of *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides. The combined use of lysozyme and *Streptomyces* exo-*N*-acetylhexosaminidase thus results in further degradation of those oligosaccharide

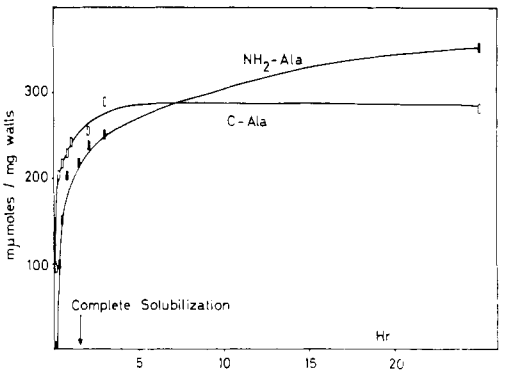

FIGURE 2: Kinetics of degradation of walls of *M. lysodeikticus* with *Myxobacter* ALI enzyme.

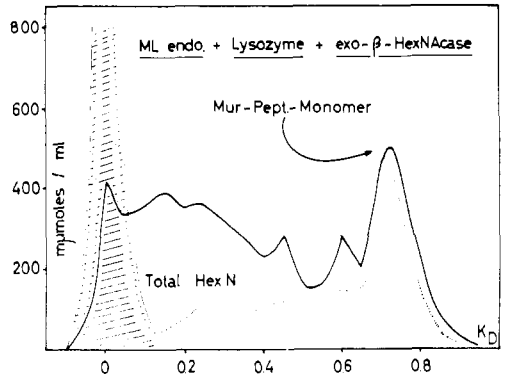

FIGURE 3: Sephadex filtration in LiCl of *M. lysodeikticus* cell walls degraded according to procedure A. For conditions, see text. Solid line: terminal amino groups. Dotted line: total *N*-acetylhexosamine residues. Shaded area: D-glucose.

fragments liberated by lysozyme treatment alone. The final solution was freeze dried and desalted by filtration in water on a Sephadex G-25 column (4.5 × 80 cm). Free *N*-acetylglucosamine (about 300 mμmoles/mg of walls) and free *N*-acetylmuramic acid (about 250 mμmoles/mg of walls) were found with the salts in the low molecular weight fraction. The high molecular weight fraction was freeze dried and filtered, in 0.1 M LiCl, on two Sephadex G-50 and Sephadex G-25 columns connected in series ($V_0 + V_i$ for the whole system = 360 ml). The fraction with K_D = 0.75 (Figure 3) contained one *N*-acetylhexosamine residue per one terminal amino group. It was further purified and desalted by filtration on Sephadex G-25 in water. The material was characterized as *N*-acetylmuramyl pentapeptide monomer (see below).

Preparation of Free Pentapeptide Monomer, Free Peptide Dimer (Ala→Ala), and Free Peptide Oligomer. PROCEDURE B. Walls were treated by the ML endopeptidase as described above. The pH of the solution was then adjusted to 4.5 with glacial acetic acid. A new incubation was carried out at 37°, for 15 hr, in the presence of F_1 endo-*N*-acetylmuramidase (10 μg of enzyme/mg of walls). At completion of the reaction, 360 mμmoles of reducing groups (per milligram of walls) was liberated. The solution was finally incubated at 37°, for 5

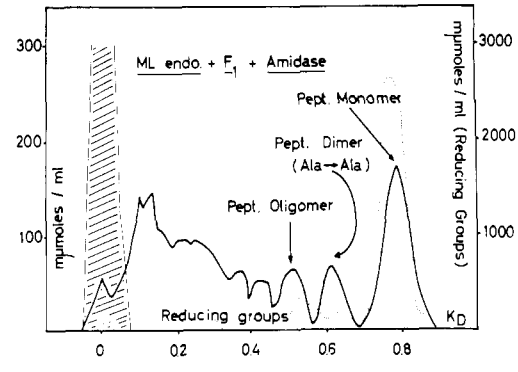

FIGURE 4: Sephadex filtration in LiCl or *M. lysodeikticus* cell walls degraded according to procedure B. For conditions, see text. Solid line: terminal amino groups (left ordinate). Dotted lines: reducing groups (right ordinate). Shaded area: D-glucose (left ordinate).

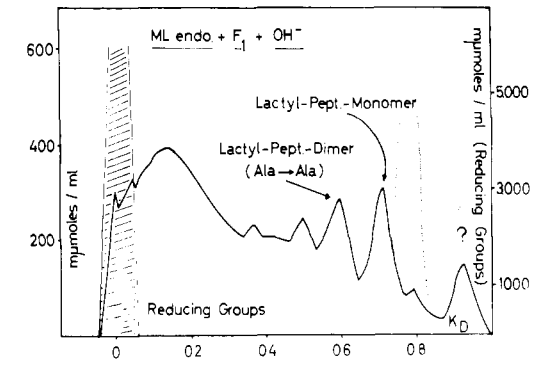

FIGURE 5: Sephadex filtration in LiCl or *M. lysodeikticus* cell walls degraded according to procedure C. For conditions, see text. For legend, see Figure 4.

hr, with *N*-acetylmuramyl-L-alanine amidase (50 μl of enzyme preparation/mg of walls) in the presence of 0.025 M acetate buffer (pH 5.5). A total of 120 mμmoles of N-terminal alanine/mg of walls was ultimately liberated. The solution was freeze dried. Aliquots corresponding to 30 mg of original walls were filtered, in 0.1 M LiCl, on connected Sephadex G-50 and Sephadex G-25 columns ($V_0 + V_i$ for the whole system = 800 ml) (Figure 4). The three fractions with K_D = 0.5, 0.6, and 0.78 were separately purified by refiltrations in 0.1 M LiCl, on the same system, and then by filtration, in 0.1 M LiCl, on Sephadex G-25 alone. Fractions K_D = 0.78 and 0.6 were desalted on Sephadex G-25 in water. The materials were characterized as, respectively, the pentapeptide monomer and the peptide dimer (Ala→Ala) (*vide infra*). Fraction K_D = 0.5 was retarded on Sephadex by filtration in water and only a partial desalting was achieved. The fraction was submitted to preparative paper electrophoresis in 0.1 M formic acid. After elution from the paper, the peptide was again passed through a Sephadex G-25 column, in water. It was tentatively characterized as a peptide oligomer, most likely a trimer (*vide infra*).

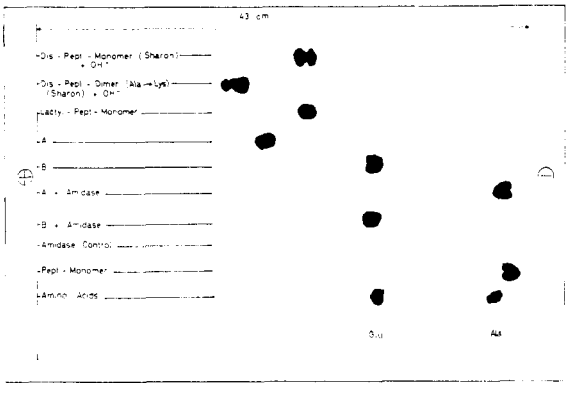

FIGURE 6: Paper electrophoresis in 0.1 M formic acid of various fragments of *M. lysodeikticus* walls. Electrophoresis was carried out using the Electrophorator Model G under 40 V/cm for 2 hr. Compounds were spotted at the anode side of the paper sheet. Detection was made with ninhydrin. The peptide monomer is that obtained according to procedure B. The lactyl peptide monomer is that obtained according to procedure C.

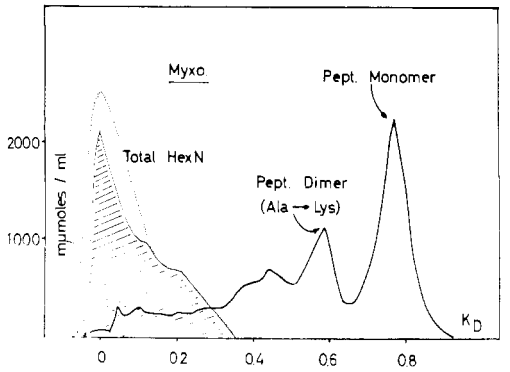

FIGURE 7: Sephadex filtration in LiCl of *M. lysodeikticus* cell walls degraded according to procedure D. For conditions, see text. For legend, see Figure 3.

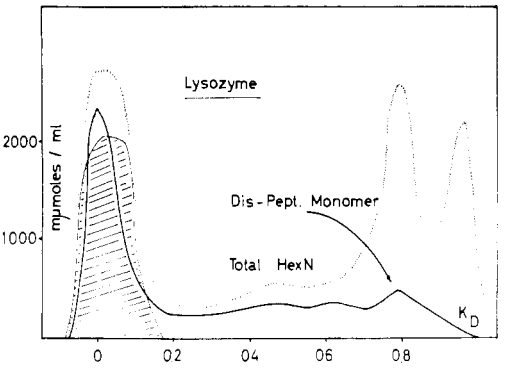

FIGURE 8: Sephadex filtration in LiCl of *M. Lysodeikticus* cell walls degraded according to procedure E. For conditions, see text. For legend, see Figure 3.

Preparation of Lactyl Pentapeptide Monomer and Lactyl Peptide Dimer (Ala→Ala). PROCEDURE C. After treatment of the walls with ML endopeptidase and with F_1 *endo-N*-acetylmuramidase as in the previous degradation, the solution was freeze dried. The residue was treated, for 1 hr at 37° with 0.05 N NaOH, using a ratio of 5 μmoles of NaOH/mg of original walls. After neutralization, aliquots corresponding to 100 mg of walls were filtered in 0.1 M LiCl on the Sephadex G-50 and Sephadex G-25 columns described in procedure B. The results are shown in Figure 5. Fractions K_D = 0.72 and 0.60 were refiltered in 0.1 M LiCl on the same column system and were finally desalted on Sephadex G-25 in water. The material K_D = 0.72 was characterized as the lactyl pentapeptide monomer (*vide infra*). It should be pointed out here, that this compound has the same electrophoretic mobility in 0.1 M formic acid as the lactyl pentapeptide monomer obtained after β elimination of the disaccharide pentapeptide monomer of Mirelman and Sharon (1967) (Figure 6). Under the same electrophoresis conditions, the material with the aforementioned K_D = 0.60 gave rise to two products, A and B (Figure 6), both having mobilities different from that of the lactyl pentapeptide monomer and from that of the bislactyl peptide dimer (Ala →Lys) obtained after β elimination of the bisdisaccharide peptide dimer (Ala→Lys) isolated by Mirelman and Sharon (1967). The lactyl pentapeptide monomer, compound A, and compound B were separately treated with the *N*-acetylmuramyl-L-alanine amidase under identical conditions. The lactyl pentapeptide monomer and compound B (Figure 6) were completely resistant to the amidase, while compound A was quantitatively degraded into free peptide. Compound A is, thus, believed to be an oligosaccharide-peptide complex which escaped the β elimination. Compound B, isolated by preparative paper electrophoresis, was purified by filtration in water on Sephadex G-10. It was characterized as the monolactyl peptide dimer (Ala→Ala) (*vide infra*).

Preparation of Free Pentapeptide Monomer and Free Peptide Dimer (Ala→Lys). PROCEDURE D. This degradation was similar to that previously described by Katz and Strominger (1967). Walls (200 mg) were treated at 37° for 20 hr with 2 ml of the *Myxobacter* enzyme in a final volume of 20 ml of 0.025 M Veronal buffer (pH 9). (The kinetics of the degradation have been presented; see I and Figure 2.) After freeze drying, the degraded products were filtered in 0.1 M LiCl on connected Sephadex G-50 and Sephadex G-25 columns ($V_0 + V_i$ for the whole system = 550 ml). Materials with K_D = 0.8 and 0.6 (Figure 7) were filtered again on the same system and were finally desalted on Sephadex G-25 in water. The two compounds were characterized, respectively, as pentapeptide monomer and peptide dimer (Ala→Lys) (*vide infra*).

Occurrence of Peptides of Small Size in the Native Walls. PROCEDURES E and F. In the following experiments, the walls were degraded by an endo-*N*-acetylmuramidase (lysozyme or F_1 enzyme) without prior treatment with endopeptidase. Figure 8 shows the fractionation profile of a lysozyme digest (50 mg of walls) obtained by filtration in 0.1 M LiCl on a Sephadex G-50 column ($V_0 + V_i$ = 190 ml) (procedure E). Fraction K_D = 0.90 is free β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide. Fraction K_D = 0.80 is a mixture of free tetrasaccharide and of disaccharide pentapeptide monomer. Figure 9 shows the filtration profile, in 0.1 M LiCl, on connected Sephadex G-50 and Sephadex G-25 columns ($V_0 + V_i$ for the whole system = 800 ml), of a F_1 endo-*N*-acetylmuramidase digest of the walls which has been submitted to β elimination (procedure F). A very small proportion of the peptide

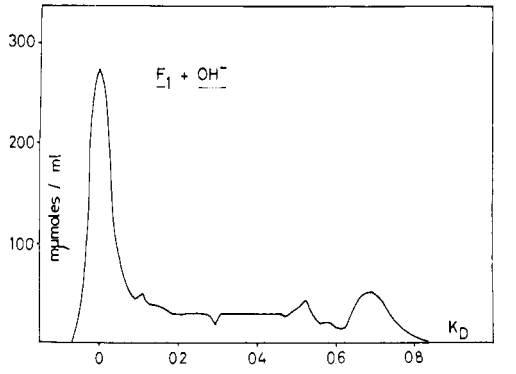

FIGURE 9: Sephadex filtration in LiCl of *M. lysodeikticus* cell walls degraded according to procedure F. For conditions, see text. Solid line: terminal amino groups.

TABLE II: Characterization of the Peptides Isolated from the Walls of *M. lysodeikticus* (chemical data).

Peptides (with ref to the procedure of isolation)	Amino Acid Composition				Terminal Groups before and after Subsequent Treatment with Peptidases				
	Ala	Glu	Gly	Lys	Peptidase Used	N^{ϵ}-Lys	NH_2-Ala	C-Ala	C-Gly
					Free Peptides				
Monomer (procedure B)	2	1.22	1.05	1	None	0.86	1	1.13	1.26
					ML endo[c]	1	0.89		
					Myxobacter[d]	0.86	0.89		
Monomer (procedure D)	2.02	1.06	1.08	1	None	1	1.03	0.95	1.14
					ML endo[c]	0.91	1		
					Myxobacter[d]	1	0.96		
Dimer (Ala→Ala) (procedure B)	2.10	1.04	0.94	1	None	1.04	0.46	0.59	0.90
					ML endo[c]	0.97	0.54	0.51	0.92
					Myxobacter[d]	1	0.98	0.93	1.01
Dimer (Ala→Lys) (procedure D)	1.78	1.18	1.10	1	None	0.57	0.80	0.52	1.07
					ML endo[c]	0.98	0.80	0.96	1.15
					Myxobacter[d]	0.56	0.86	0.56	0.88
Trimer (procedure B)	1.88	1.27	1.09	1	None	0.98	0.32	0.65	0.77
					ML endo[c]	1.13	0.36		
					Myxobacter[d]	1	0.80	1	1.09
Lactyl peptide[a] monomer (procedure C)	1.97	1.21	1.18	1	None	1.02	0	1.03	1.03
					ML endo[c]	0.89	0		
					Myxobacter[d]	1.02	0.09		
Dimer (Ala→Ala) (procedure C)	2.23	1.07	0.86	1	None	0.97	0	0.60	1.11
					Myxobacter[d]	0.97	0.67	0.85	1.11
Muramyl peptide[b] monomer (procedure A)	2.02	0.90	0.85	1	None	1.20	0	0.98	0.87

[a] The lactyl peptide monomer contains 1.05 D-lactic acid/lysine. [b] The muramyl peptide monomer contains 1.05 *N*-acetylmuramic acid/lysine. [c] *Streptomyces* ML endopeptidases. [d] *Myxobacter* AL I protease.

material is found in the low molecular weight fractions, particularly at K_D = 0.70 and 0.59. These products were not further studied.

Characterization and Properties of the Pentapeptide Monomers, Peptide Dimer (Ala→Ala), Peptide Dimer (Ala→Lys), Peptide Oligomer, Lactyl Pentapeptide Monomer, Monolactyl Peptide Dimer (Ala→Ala), and N-Acetylmuramyl Pentapeptide Monomer. Table II presents the composition of the purified compounds, determination of N- and C-terminal groups, as well as the results of subsequent treatment by ML endopeptidase or by *Myxobacter* enzyme.[2] Compounds (350 mμequiv in total lysine) were treated for 20 hr at 37° with either 75 μl of the ML endopeptidase in a final volume of 150 μl of 0.02 M phosphate buffer (pH 8.2), or with 75 μl of *Myxobacter* enzyme in 100 μl of 0.025 M Veronal buffer (pH 9). Figure 10 presents the chromatographic properties on thin-layer silica gel using solvent V of the pentapeptide monomers, the peptide dimer (Ala→Lys), the peptide dimer (Ala→Ala), and the peptide oligomer, before and after treatment with each of the two endopeptidases. Although not shown in Figure 10, it should be noted that in this system, the lactyl pentapeptide monomer has almost the same R_F as that of the free pentapeptide monomer, and that the monolactyl peptide dimer (Ala→Ala) has almost the same R_F as that of the free peptide dimers. Figure 11 presents the paper electrophoretic properties in 0.1 M formic acid of the peptide monomer (used as control) and of both the lactyl pentapeptide monomer and the monolactyl peptide dimer (Ala→Ala) before and after treatment with either endopeptidases. It should be recalled here that the monolacty peptide dimer (Ala→Ala) is distinguishable, under similar conditions of electrophoresis, from the bislactyl peptide dimer (Ala→Lys) (Figure 6). Figure 12 presents the thin-layer chromatographic properties, in solvent IV, of the dinitrophenyl derivatives of

[2] Although not shown in Table II, C-terminal D- and L-alanines (in the products of hydrazinolysis) and N-terminal D- and L-alanines (by the decrease in D- and L-alanines occurring when the product is dinitrophenylated prior to hydrolysis) (Ghuysen *et al.*, 1966b) were determined on cell walls degraded by F1 endo-*N*-acetylmuramidase or by *Myxobacter* AL I protease, and on the lactyl peptide dimer (Ala→Ala) before and after treatment by *Myxobacter* AL I protease. Results confirmed that *Myxobacter* AL I enzyme cleaves D-alanyl-L-alanine linkages. These experiments were carried out by Dr. J. L. Strominger, Department of Pharmacology, University of Wisconsin.

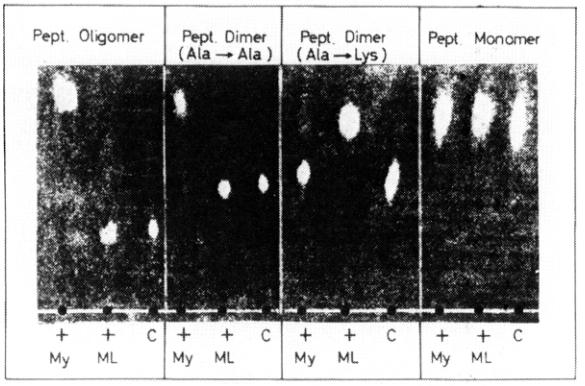

FIGURE 10: Thin-layer silica gel chromatography in solvent V. The two peptide monomers prepared according to procedure B or according to procedure D are indistinguishable. C = untreated compounds. +My = compounds after treatment with *Myxobacter* enzyme. +ML = compounds after treatment with ML endopeptidase. Detection was made with ninhydrin.

the pentapeptide monomers, the peptide dimer (Ala→Lys), and the *N*-acetylmuramyl pentapeptide monomer after treatment with *N*-acetylmuramyl-L-alanine amidase. These compounds are compared with: (1) an unresolved mixture of free peptides obtained by Katz and Strominger (1967) after *Myxobacter* degradation of *M. lysodeikticus* walls; (2) with the pentapeptide monomer and with the peptide dimer (Ala→Lys), respectively, obtained by amidase treatment of the corresponding disaccharide derivatives isolated by Mirelman and Sharon (1967).

Conclusions

(1) The two pentapeptide monomers obtained by degradations B and D, the peptide moiety of the *N*-acetylmuramyl pentapeptide monomer obtained by degradation A, and the peptide moiety of the disaccharide pentapeptide monomer isolated by Mirelman and Sharon (1967) are identical (Figure 12). Their composition (Table II) agrees with the sequence N^{α}-[L-alanyl-γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanine which has been assigned to the *M. lysodeikticus* peptide monomer (Tipper *et al.*, 1967; Mirelman and Sharon, 1967).

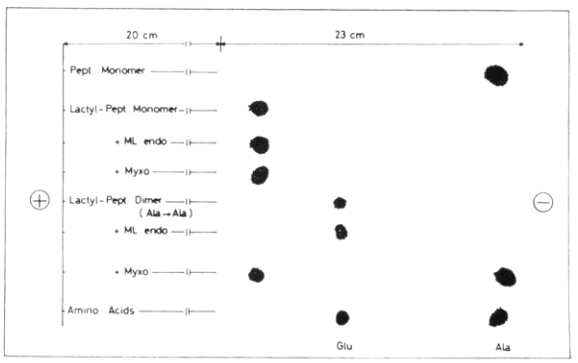

FIGURE 11: Paper electrophoresis in 0.1 M formic acid of various fragments of *M. lysodeikticus* walls. For conditions of electrophoresis, see text and legend of Figure 6.

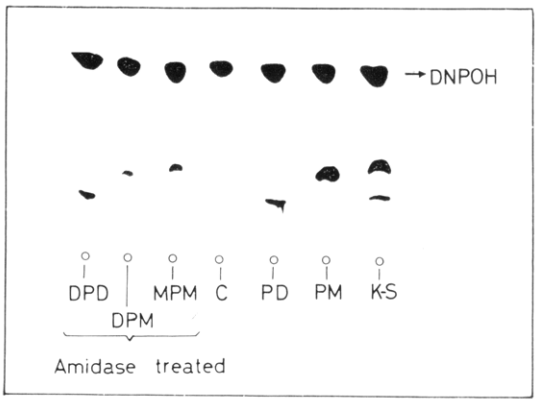

FIGURE 12: Thin-layer silica gel chromatography in solvent IV of dinitrophenyl peptides. DPD = disaccharide peptide dimer (Ala→Lys) from Mirelman and Sharon (1967). DPM = disaccharide pentapeptide monomer from Mirelman and Sharon (1967). MPM = *N*-acetylmuramyl pentapeptide monomer prepared according to procedure A. C = amidase control. PD = peptide dimer (Ala→Lys) prepared according to procedure B. PM = peptide monomer prepared according to procedure B or procedure D. KS = a mixture of peptides 1 and 2 prepared by Katz and Strominger (see Figure 4 in Katz and Strominger, 1967).

(2) The peptide dimer (Ala→Ala), isolated according to procedure B, and the peptide dimer (Ala→Lys), isolated according to procedure D, are indistinguishable by paper electrophoresis in 0.1 M formic acid (they have mobilities identical with that of free pentapeptide monomer) or by thin-layer chromatography in solvent V (Figure 10). However, the peptide dimer (Ala→Ala) has, per lysine residue, 0.5 C-terminal alanine, 0.5 N-terminal alanine, and 1 N^{ϵ}-terminal lysine (Table II). It is not a substrate for the ML endopeptidase but it is completely cleaved into monomers by the *Myxobacter* enzyme through the hydrolysis of alanylalanine linkages (Figure 10 and Table II). Conversely, the peptide dimer (Ala→Lys) has, per lysine residue, 0.5 C-terminal alanine, 1 N-terminal alanine, and 0.5 N^{ϵ}-terminal lysine (Table II). It is not a substrate for *Myxobacter* enzyme but it is completely cleaved into monomers by the ML endopeptidase through the hydrolysis of N^{ϵ}-(D-alanyl)-L-lysine linkages (Figure 10 and Table II). The structure shown in Figure 1 can thus be assigned to the two isomeric peptide dimers. (3) The peptides isolated by Katz and Strominger (1967) after *Myxobacter* treatment of the walls (spot K-S in Figure 12) are, respectively, a peptide monomer and a peptide dimer. It had been believed previously (Katz and Strominger, 1967) that these compounds were a dimer and a trimer rather than a monomer and a dimer. The N- and C-terminal amino acid estimations are subject to systematic errors. Repetition of the analyses and comparison with other isolated compounds has led to the conclusion that they are in fact monomer and dimer. (4) The lactyl pentapeptide monomer isolated according to procedure C is indistinguishable by electrophoresis from the lactyl pentapeptide monomer obtained by β elimination of the disaccharide pentapeptide monomer of Mirelman and Sharon (1967) (Figure 6). The chemical data (Table II) are compatible with the sequence N^{α}-[D-lactyl-L-alanyl-

TABLE III: Yields of Peptide Fragments Produced by Enzymatic Degradations of *M. lysodeikticus* Cell Walls.[a]

Enzyme Treatment		Procedure	Peptide Monomer		Peptide Dimer (Ala→Lys)		Peptide Dimer (Ala→Ala)	
			Actual Yield (%)	Theoretical Yield (%)	Actual Yield (%)	Theoretical Yield (%)	Actual Yield (%)	Theoretical Yield (%)
Myxobacter		D	42	50	22	34	0	0
ML endo + F_1 + amidase		B	20	24	0	0	9	10
ML endo + F_1 + OH^-		C[b]	25	24	0	0	6	10
ML endo + lysozyme + exo-β-HexNAcase	M	A[c]	23	24	0	0	?	10
Lysozyme		E[d]	5	5	Traces	4	?	0
F_1 + OH^-		F[b]	Traces	5	Traces	4	?	0
Lysozyme		Mirelman and Sharon (1967)[d]	4	5	3	4	?	0

[a] The actual yields are compared with the theroetical ones which would arise if the repartition of the peptide subunits in the walls into monomers, dimers, trimers, and hexamers were that indicated in Figure 13. Data are expressed in per cent of total pentapeptide subunits. [b] Peptides obtained as lactyl peptides. [c] Peptides obtained as *N*-acetylmuramyl peptides. [d] Peptides obtained as disaccharide peptides. Abbreviations: exo-β-HexNAcase, exo-β-*N*-acetylhexosaminidase.

γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanine. This lactyl peptide is not a substrate for the *Streptomyces N*-acetylmuramyl-L-alanine amidase and it is a very poor substrate for *Myxobacter* enzyme (9% of hydrolysis under the conditions used, Table II). (5) The monolactyl peptide dimer (Ala→Ala) isolated according to procedure C (compound B in Figure 6) is distinguishable by paper electrophoresis from the lactyl pentapeptide monomer and from the bislactyl peptide dimer (Ala→Lys) obtained by β elimination of the bisdisaccharide peptide dimer (Ala→Lys) of Mirelman and Sharon (1967). The lactyl peptide dimer (Ala→Ala) is not a substrate for the ML endopeptidase (Figure 11). It is cleaved by *Myxobacter* enzyme into lactyl pentapeptide monomer and free pentapeptide monomer through hydrolysis of alanylalanine linkages (Figure 11). This and the relevant analytical data (Table II) establish that the compound is indeed the peptide dimer (Ala→Ala) (Figure 1) substituted at its *N*-alanine terminus by a D-lactic acid. (6) The peptide oligomer, isolated according to procedure B, contains, per lysine residue, approximately one terminal N^{ϵ}-lysine and 0.33 N-terminal alanine. Thus, it would be a peptide trimer. Its migration on silica gel in solvent IV (Figure 10) and its K_D during Sephadex fractionation (Figure 4) are compatible with such a degree of polymerization. All the lysine residues present in the trimer have their ε-amino groups free. This trimer is, thus, different from that represented in Figure 13 (*vide infra*) since no lysine residue is involved in the polymerization. In agreement with this, the trimer presently isolated is completely resistant to the ML endopeptidase. It can be degraded into monomers by *Myxobacter* enzyme (Figure 10). Estimation of terminal groups before and after enzymatic degradation (Table II) suggests that this trimer would contain a pentapeptide monomer residue and a peptide dimer (Ala→Ala) residue, linked through a glycyl-L-alanine linkage. Although the occurrence of a very few glycyl-L-alanine interpeptide linkages in *M. lysodeikticus* peptidoglycan is compatible with previous observations made by Tsai *et al.* (1965), the structure proposed for the peptide trimer is not considered as being firmly established and must await further confirmation.

Discussion

Table III presents the actual yields of the peptide monomer and of the two isomeric peptide dimers (Ala→Lys and Ala→Ala) as they are obtained either in the free form or in the form of various derivatives. Such yields cannot arise from the structure proposed by Katz and Strominger (1967) and by Schleifer and Kandler (1967) for the peptide moiety of *M. lysodeikticus* walls. In the model of Katz and Strominger (1967) only one type of interpeptide bridges occurs, *i.e.*, those mediated through N^{ϵ}-(D-alanyl)-L-lysine linkages. Such a model does not provide any explanation for the production of pentapeptide monomers by *Myxobacter* enzyme action and of peptide dimer (Ala→Ala) by ML endopeptidase action. In the model of Schleifer and Kandler (1967), both N^{ϵ}-(D-alanyl)-L-lysine and D-alanyl-L-alanine linkages occur. Two-

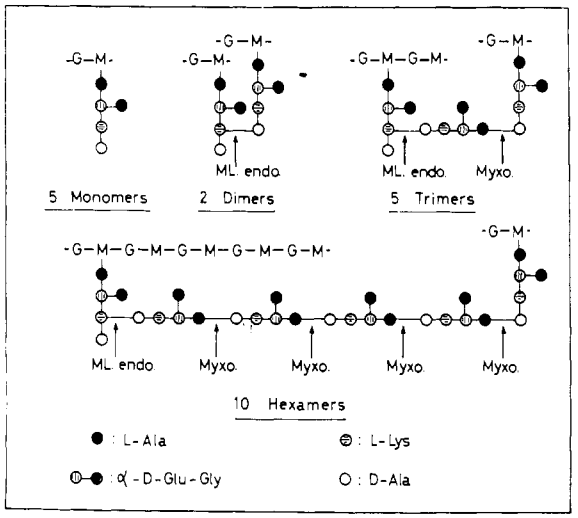

FIGURE 13: Proposed structure of the peptide moiety of the walls of *M. lysodeikticus*. Theoretical molar distribution of peptide monomers, dimers, trimers, and hexamers. The arrows indicate the peptide linkages specifically hydrolyzed either by ML endopeptidase or by *Myxobacter* protease. Although not represented in Figure 13, it should be understood that in the course of the walls degradation, the *N*-acetylmuramyl-L-alanine linkages are also hydrolyzed either with the *Streptomyces* amidase following the action of ML endopeptidase or as a result of the amidasic activity performed by the *Myxobacter* enzyme preparation itself. Note that the trimer is the structural unit proposed by Schleifer and Kandler (1967) for the *M. lysodeikticus* peptide moiety. G = *N*-acetylglucosamine. M = *N*-acetylmuramic acid.

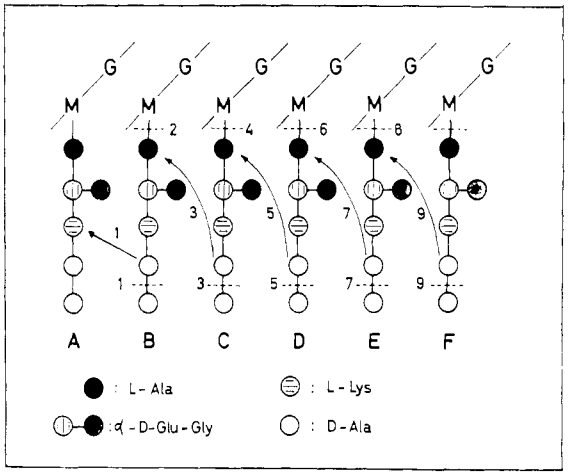

FIGURE 14: Proposed biosynthetic sequence of that peptide hexamer unit shown in Figure 13. Reactions 1, 3, 5, 7, and 9 are transpeptidations. Reactions 2, 4, 6, and 8 are amidasic hydrolyses. M = *N*-acetylmuramic acid. G = *N*-acetylglucosamine.

thirds of the pentapeptide subunits is branched on some of the *N*-acetylmuramic acid residues of the glycan strands, while the remainder form bridges between substituent peptides, through D-alanyl-L-alanine and N^{ϵ}-(D-alanyl)-L-lysine linkages. The degradation of such a peptide moiety using the ML endopeptidase or the *Myxobacter* enzyme, would produce mainly peptide dimers and a small amount of peptide monomers. A peptide pentamer, for example, in which three subunits are glycan substituents and two subunits serve as bridges, would give rise to 80% of the peptide subunits in the form of dimers and 20% in the form of monomers, irrespective of the endopeptidase used for its degradation. The fact that treatment of the walls with *Myxobacter* enzyme liberates nearly half of the peptide subunits as monomers requires the existence of peptide polymers consisting of several monomeric units joined by D-alanyl-L-alanine linkages. The yields of the peptide fragments obtained by the various degradation procedures (Table III) are consistent with a wall structure in which 5% of the pentapeptide subunits occur as monomers, 4% as dimers (Ala→Lys), 15% as trimers, and 60% as hexamers, *i.e.*, the molar distribution indicated in Figure 13. Moreover, according to the structure proposed in Figure 13: (1) large glycan fragments unsubstituted by peptides, demonstrated by Leyh-Bouille *et al.* (1966) and Muñoz *et al.* (1966), can occur and still accommodate the observed 1:1 ratio between the peptide and the disaccharide subunits; (2) theoretically, about 80% of the lysine residues should have their ϵ-amino groups free; actual data range from 60 to 80% (Petit *et al.*, 1966); and (3) there is one C-terminal D-alanine for four peptide subunits (actual data, 100 mμ-moles for 480 peptidoglycan units). The biosynthesis of the peptide hexamer unit represented in Figure 13 might occur through successive alternate transpeptidations and amidase actions, as suggested by Schleifer and Kandler (1967), following the sequence schematically shown in Figure 14. In reaction 1, a transpeptidation, the penultimate D-alanine residue of peptide B is transferred to the lysine residue of peptide A with the concomitant loss of the terminal D-alanine residue. Reaction 2 is an amidasic action in which the amide linkage at the *N*-alanine terminus of peptide B is hydrolyzed. Reaction 3, again a transpeptidation, results in the penultimate D-alanine residue of peptide C being bound to the N-terminal L-alanine residue of peptide B with the exclusion of one D-alanine residue. Reactions 4–9 would follow in the indicated order resulting ultimately in the formation of the peptide hexamer. It should be understood, however, that the peptide hexamers shown in Figure 13 represent a statistical average among oligomers having bridges of various sizes and accommodate only a major part of the wall peptide subunits. About 16% of the peptide subunits is not accounted for by the model of Figure 13. Some of the enzymatic reactions used in the sequential degradations may not have reached completion. Also, structural peculiarities, such as other types of interpeptide linkages (Tsai *et al.*, 1965), may occur in a small part of the peptide moiety. Finally it is understood that the conclusions presented in this paper are based on results obtained with a single preparation of walls. However, according to experiments presently in progress, the general structure proposed here appears to exist in a number of species in the family *Micrococcaceae*.

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