The Peptide Subunit N^{α}-(L-Alanyl-D-isoglutaminyl)-L-lysyl-D-alanine in Cell Wall Peptidoglycans of *Staphylococcus aureus* Strain Copenhagen, *Micrococcus roseus* R 27, and *Sreptococcus pyogenes* Group A, Type 14*

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ABSTRACT: The tetrapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine has been isolated from cell walls of *Staphylococcus aureus* and of *Micrococcus roseus* in yields warranting the conclusion that it is the basal subunit of both cell wall peptidoglycans. The amidated tetrapeptides are cross-linked through the previously described peptide bridges to form oligomers. The tripeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysine was also isolated from cell walls of *M. roseus*. This amidated tripeptide can be visualized as located at the C terminus of peptide tetramers. The pentapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanyl-D-alanine is present in cell walls of *S. aureus* but its occurrence is low. It has been isolated from walls prepared from *S. aureus* cells grown in the presence of penicillin according to the technique of Tipper and Strominger. The tetrapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine has also been isolated from cell walls of *Streptococcus pyogenes*, although in low yield, probably owing to peculiarities of the glycan portion of the peptidoglycan. Peptide oligomers of relatively small size in which tetrapeptides are interlinked through L-alanyl-L-alanyl bridges are, however, representative to a large part of this cell wall peptidoglycan. Pentapeptide, tetrapeptides, and tripeptide were characterized by various means including enzymatic degradation, chemical degradation, and comparison with synthetic peptides.

The structure common to all bacterial cell wall peptidoglycans is a network of poly-*N*-acetylhexosamine chains cross-linked through peptide bridges. During the past few years, important structural features of the peptidoglycans of *Staphylococcus aureus* strain Copenhagen and of *Micrococcus roseus* strain R 27 have been elucidated through controlled degradation of the relevant cell walls by means of purified enzymes (Ghuysen and Strominger, 1963a,b; Ghuysen *et al.*, 1964; Tipper *et al.*, 1965, 1967b; Ghuysen *et al.*, 1965a,b,c; Petit *et al.*, 1966; Muñoz *et al.*, 1966). Both the polysaccharide and peptide moieties isolated in an undegraded state are soluble in water; hence the insolubility of the intact peptidoglycans arises from the manner in which the moieties are combined. The polysaccharide chains consist of β-1,4-linked, alternating *N*-acetylglucosamine (GlcNAc[1]) and *N*-acetylmuramic acid (MurNAc) residues, some of these latter being in *S. aureus*, *O*-acetylated on C_6. The peptide moieties consist mainly of short peptide *subunits* composed of L-alanine, D-alanine, D-glutamic acid, and L-lysine and of peptide *bridges* composed of pentaglycine groupings in *S. aureus* and of L-alanyl-L-alanyl-L-alanyl-L-threonine groupings in *M. roseus* R 27. All of the MurNAc are engaged in amide linkages to N-terminal L-alanine of the peptide subunits. The peptide bridges extend from the C-terminal D-alanine of one peptide subunit to the ϵ-amino group of L-lysine of a second peptide subunit.

A hypothesis for the structure of the peptide subunit in *S. aureus* first envisaged the sequence N^{α}-(L-alanyl-γ-D-glutamyl)-L-lysyl-D-alanine, *i.e.*, that sequence which had been proposed, on the basis of its biosynthesis, for the cell wall peptidoglycan precursor uridinediphospho-*N*-acetylmuramyl-N^{α}-(L-alanyl-γ-D-glutamyl)-L-lysyl-D-alanyl-D-alanine (Ito and Strominger,

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[1] Abbreviations used: GlcNc, *N*-acetylglucosamine; MurNc, *N*-acetylmuramic acid; *exo*-β-GlcNAcase, *exo*-β-N-acetylglucosaminidase; FDNB, fluorodinitrobenzene.

TABLE I: Isomeric Di-, Tri, and Tetrapeptides Synthesized.

Name	Abbreviation[a]	R_F[b]
γ-L-Glutamyl-L-lysine	L-Glu | —L-Lys	0.04
L-Isoglutaminyl-L-lysine	L-Glu-NH_2 | —L-Lys	0.10
α-D-Glutamyl-L-lysine	D-Glu-L-Lys	0.05
L-Glutaminyl-L-lysine	L-Gln-L-Lys	0.11
L-Alanyl-D-isoglutamine	L-Ala-D-Glu-NH_2	0.26
γ-L-Glutamyl-L-lysyl-D-alanine	L-Glu | —L-Lys-D-Ala	0.05
L-Isoglutaminyl-L-lysyl-D-alanine	L-Glu-NH_2 | —L-Lys-D-Ala	0.12
α-D-Glutamyl-L-lysyl-D-alanine	D-Glu-L-Lys-D-Ala	0.11
L-Glutaminyl-L-lysyl-D-alanine	L-Gln-L-Lys-D-Ala	0.13
L-Alanyl-D-isoglutaminyl-L-lysyl-D-alanine	L-Ala-D-Glu-NH_2 | —L-Lys-D-Ala	0.06
L-Isoglutaminyl-L-lysyl-D-alanyl-D-alanine	L-Glu-NH_2 | —L-Lys-D-Ala-D-Ala	0.12
ε-Glycyl-L-lysine	L-Lys | Gly	

[a] The following abbreviations are also used: N^{α} for the α-lysine derivatives on peptides; N^{ϵ} for the ε-lysine derivatives on peptides. [b] Descending paper chromatography in solvent VI.

1964). In contrast to the precursor peptide, however, the carbohydrate-free peptide isolated from *S. aureus* cell walls had only 1 mole of D-alanine, and in addition contained 1 mole of NH_3. An Edman degradation carried out on this isolated peptide demonstrated that this ammonia was a substituent of the α-carboxyl group of the glutamic acid (Tipper and Strominger, 1965; Tipper *et al.*, 1967a).

The studies reported here provide evidence that the basal peptide subunit of the cell wall peptidoglycan of the three Gram-positive bacteria: *S. aureus* Copenhagen, *M. roseus* R 27, and *Streptococcus pyogenes* group A, type 14, has indeed the structure N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine.

Material and Methods

Analytical Methods. Identification and measurement of free amino acids and N-terminal groups (by the FDNB technique), of C-terminal groups (by the hydrazinolysis technique), and of D- and L-alanine (by the enzymatic procedure) have been described (Ghuysen *et al.*, 1966). Determination of reducing power using the Park–Johnson procedure and measurement of acetamido sugars using the Morgan–Elson reaction after 7 or 30 min of heating in 1% borate have also been described (Ghuysen *et al.*, 1966). Analysis of total organic phosphorus was performed according to Lowry *et al.* (1954). Rhamnose was estimated according to Dische and Shettles (1948). The isoglutaminyl or glutaminyl residues in the peptides were identified by adapting the dehydration and reduction procedure described by Ressler and Kashelikar (1966). Amino acid analyses were carried out with a Technicon autoanalyzer (resin chromo beads, type β, 17μ). Electrophoresis was carried out on 3MM Whatman paper (40 × 40 cm) at pH 5.0 (acetic acid–pyridine–water, 2:4:1000) at 20 v/cm, for 90 min, using the Electrorheophor Pleuger (Antwerp, Belgium) apparatus. The compounds were detected with ninhydrin spray (0.5% in isopropyl alcohol–water, 9:1).

Chromatography Solvents. Chromatography of dinitrophenyl (DNP) peptides was performed on thin layer plates of silica gel (silica gel according to Stahl, Merck) using the following solvents: (I) 1-butanol–0.15 N ammonia (1:1, upper phase), (II) chloroform–methanol–acetic acid (85:14:1) (at 0°), (III) benzyl alcohol–chloroform–methanol–water–15 N ammonia

(30:30:30:6:2), (IV) dioxane–methanol–toluene–isopropyl ether–acetic acid–water (25:12:15:60:2:2) (at 0°).

Paper chromatography of the disaccharide peptide compounds was carried out on Whatman No. 1 paper, using solvent V (isobutyric acid–0.5 N ammonia, (5:3)). The compounds were detected by fluorescence after the paper had been dipped in a solution of 0.5 N NaOH in ethanol–1-propanol (6:4) and heated for 10 min at 120° (Sharon, 1964). Paper chromatography of the synthetic peptides was carried out on Whatman No. 1 paper, using solvent VI (1-butanol–pyridine–acetic acid–water (30:20:6:20)).

Synthetic Peptides. The di-, tri-, and tetrapeptides reported in Table I were synthesized and used as markers in the process of the identification of the natural cell wall peptide fragments and as models for studies of the enzymatic reactions. The various isomeric peptides containing glutamic acid or one of its amides were prepared by unequivocal syntheses. Their R_F values are reported in Table I. The syntheses of these peptides will be reported elsewhere. The following compounds were also used: L-isoglutamine (A grade, Calbiochem), L-glutamine (Mann Research Laboratories), α- and γ-L-glutamyl-L-alanine, D-alanyl-L-alanine (Cyclo Co.), L-lysyl-D-alanine, and L-alanyl-D-alanyl-L-alanine (these two latter peptides were a generous gift from Dr. Bernard Erlanger).

Enzymes. SA endopeptidase (a lytic enzyme hydrolyzing peptide bridges in the cell wall peptidoglycans at their amino terminus), an aminopeptidase (present in the nonlytic peptidase 2 preparation), the *N*-acetylmuramyl-L-alanine amidase (a nonlytic enzyme hydrolyzing the linkages between the *N*-acetylmuramic acid residues and the peptide subunits), and the F_1 *endo-N*-acetylmuramidase (a lytic glycosidase hydrolyzing linkages of MurNAc to GlcNAc in the glycan portion of the peptidoglycans) were purified from *Streptomyces albus* G culture filtrates. These enzymes have been recently described (Petit *et al.*, 1966). An *exo*-β-*N*-acetylglucosaminidase (*exo*-β-GlcNAcase) was prepared from pig epididymis according to Sanderson *et al.* (1962). Protein concentrations are expressed in terms of lysozyme milligram equivalents at the wavelength of 278 mμ.

Cell Walls. Cell walls were prepared according to standard procedures (Petit *et al.*, 1966). After isolation by differential centrifugation, the cell walls of *S. aureus* and of *M. roseus* were purified by treatment at 37°, for 3 hr, with Difco trypsin (0.1%) in 0.05 M phosphate buffer, pH 7.5. The ester-linked D-alanine residues of the teichoic acid and the *O*-acetyl groups were removed from cell walls of *S. aureus* by treatment with 9 N NH_4OH for 20 min at room temperature. The amino acid composition of the cell walls of *M. roseus* and of the NH_3-treated cell walls of *S. aureus* have been reported (Petit *et al.*, 1966). Cell walls (1 mg) of *S. aureus* and of *M. roseus* contain 470 and 300 mμmoles, respectively, of peptidoglycan unit (*i.e.*, one disaccharide plus one peptide subunit plus one peptide bridge). The cell walls of *S. pyogenes* were prepared and protease treated as described earlier (Heymann *et al.*, 1963). The amino acid composition is, in millimicromoles per milligram: Glu, 512; Lys, 476; Ala, 1630. Other amino acids, identified on the basis of the elution volumes from the chromo beads resin column, occur in minor amounts: Asp, 66; Thr, 47; Ser, 54; Pro, 50; Gly, 101; Val, 25; Leu, 73; His, 14; Arg, 28; Ile, 15; Orn, 23; OH-Lys, 46. Most of them are believed to be residues of the protein components left after protease treatment of the cell walls. Hydroxylysine, however, appears to be a genuine component of the cell wall peptidoglycan (*vide infra*). The further identification of hydroxylysine rests upon the following evidence. Cell wall hydrolysates yield a peak in the position at which the unresolved mixture of DL-*allo*- and DL-hydroxylysine emerges from a 20 × 0.9 cm column of Bio-Rad A-4 resin, with 0.38 M sodium citrate buffer, pH 4.15, at 50°. Such a column is almost equivalent in resolving power to the 50-cm column of the Spackman *et al.* (1958) system. Preparative isolation of the eluate in the position referred to followed by desalting on Dowex 50-H^+ yielded an amino acid that was indistinguishable from DL-*allo*- and DL-hydroxylysine on paper chromatograms; further the unknown as well as the standard hydroxylysine spots gave a positive reaction for formaldehyde when treated with periodate, and then 1,3-pentanedione in the presence of ammonia (Nash reaction; Speck, 1962). Moreover, the ε-DNP-lysine appearing on characterization of *S. pyogenes* cell wall lysates was accompanied by a substance possessing chromatographic characteristics of ε-DNP-hydroxylysine. The possible significance of the substitution of about 10% of hydroxylysine for lysine will be dealt with elsewhere.

Experimental Section

Sequential Degradation of the Cell Walls. GENERAL SCHEME. Six steps were used to first dismantle the peptidoglycan network into free amino acids, liberated from the disrupted peptide bridges, and β-1,4-GlcNAc-MurNAc peptide units, and then further to degrade the latter to free GlcNAc, free MurNAc, free L-alanine from the N-terminal end of the peptide subunit, and a residual short peptide. Figure 1 illustrates this sequential degradation as applied to cell walls of *S. aureus*. Similar results were obtained using cell walls of *M. roseus* and of *S. pyogenes* in which the bridges consist of L-alanyl-L-alanyl-L-alanyl-L-threonine and of L-alanyl-L-alanine, respectively, as will be detailed further on. Complications arose, however, with cell walls of *S. pyogenes*, due to incomplete splitting of the glycan portion into disaccharide units through the action of the F_1 *endo-N*-acetylmuramidase (Muñoz *et al.*, 1966). The resulting lysate was thus a more complex mixture.

Through the actions of the SA endopeptidase which solubilizes the cell walls by hydrolyzing linkages at the amino terminus of the peptide bridges (hydrolysis of linkage 1, Figure 1), and next of the *Streptomyces* aminopeptidase (hydrolysis of linkage 2, Figure 1)

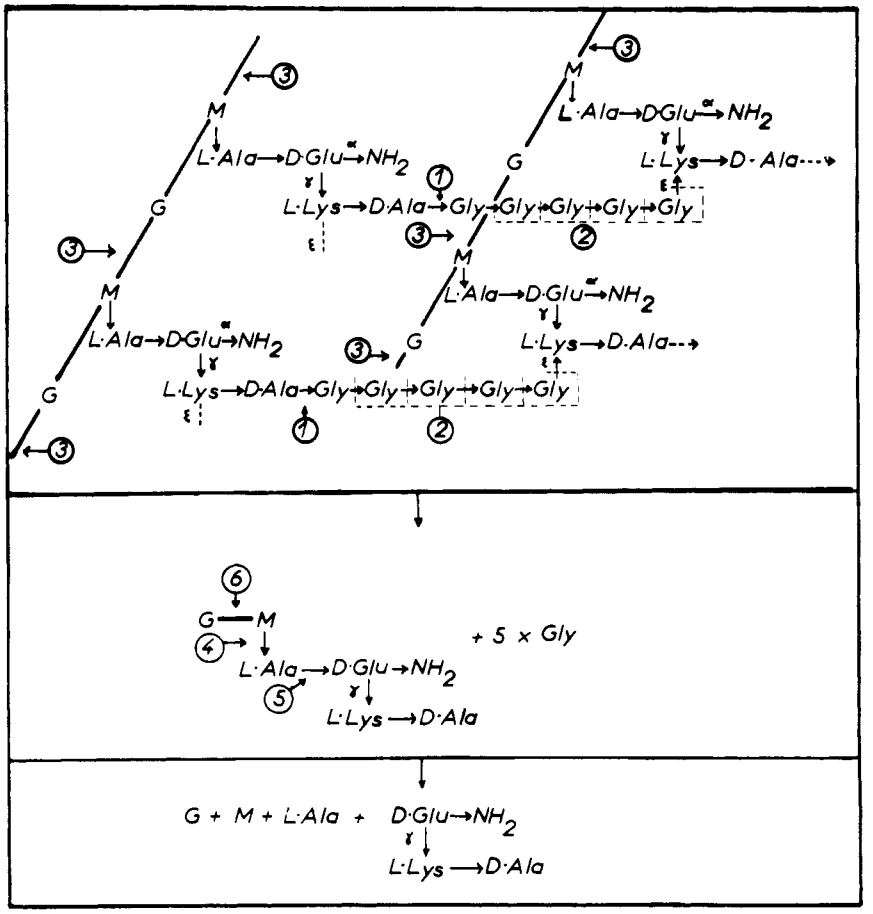

FIGURE 1: Sequential degradation of cell wall peptidoglycan of *S. aureus* Copenhagen. (A) Preparation of the disaccharide peptide subunit. (1) Site of action of *Streptomyces* SA endopeptidase. (2) Degradation of the opened peptide bridges with *Streptomyces* aminopeptidase. (3) Site of action of *Streptomyces* F_1 *endo-N*-acetylmuramidase. (B) Further degradation of the isolated disaccharide peptide. (4) Site of action of *Streptomyces N*-acetylmuramyl-L-alanine amidase. (5) Site of action of *Streptomyces* aminopeptidase. (6) Site of action of *exo-β-N*-acetylglucosaminidase (from pig epididymis).

all of the peptide bridges were completely degraded with liberation of free amino acids (Petit *et al.*, 1966). This degradation yielded, per mole of glutamic acid, 5 moles of glycine in the case of *S. aureus*, successively 3 moles of L-Ala and 1 mole of L-Thr in the case of *M. roseus*, and about 2–3 moles of L-Ala in the case of *S. pyogenes*. At the end of the reaction, virtually all of the ε-amino groups of lysine had been liberated. Subsequent treatment of the digest with F_1 *endo-N*-acetylmuramidase (hydrolysis of linkage 3, Figure 1) yielded the disaccharide peptide, quantitatively in the cases of *S. aureus* and *M. roseus* and partially in the case of *S. pyogenes*. The disaccharide peptide was then isolated and purified by appropriate means. The purified disaccharide peptide was cleaved into its two portions with the aid of *N*-acetylmuramyl-L-alanine amidase (hydrolysis of linkage 4, Figure 1). The N-terminal L-alanine which appeared as a consequence of the liberation of the disaccharide unit was itself liberated as free L-alanine by further treatment with the *Streptomyces* aminopeptidase (hydrolysis of linkage 5, Figure 1). Residual peptide subunits, containing N-terminal D-glutamic acid and L-lysine residues with free ε-NH_2 groups, were isolated and characterized as their didinitrophenyl derivatives. Hydrolysis of linkage 6, Figure 1, with the *exo-β*-GlcNAcase is not required for obtaining the peptide subunits. It can be carried out either on the free disaccharide after step 4 or 5 yielded free GlcNAc and MurNAc, or on intact disaccharide peptide after step 3 yielding free GlcNAc and MurNAc peptide. With the exception of the *exo-β*-GlcNAcase treatment, the other enzymatic treatments must follow one another in the sequence given, in order to obtain good yields.

Isolation of the Disaccharide Peptide from Cell Walls of S. aureus and of M. roseus. Steps 1 and 2 (Figure 1) of the degradation have been described in detail (Petit *et al.*, 1966). Cell walls (500 mg) were treated at 37°

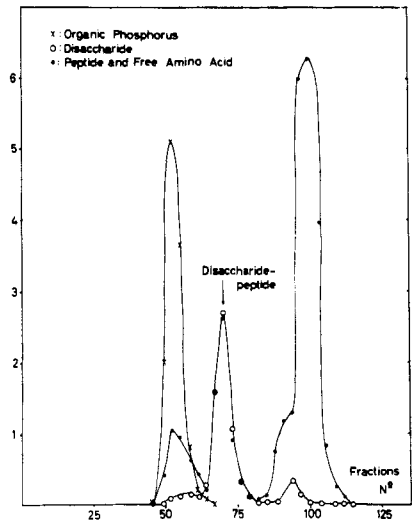

FIGURE 2: Isolation of disaccharide peptide from cell wall peptidoglycan of *S. aureus* Copenhagen. Solubilized cell walls (500 mg), after step 3 of their sequential degradation (see Figure 1), were filtered in water on an 800-ml column of Sephadex G-25 (bead form); 19.7-μl aliquots of fractions were used for estimation of total organic phosphorus, amino groups (N-terminal groups and free amino acids) relative to alanine, and β-1,4-GlcNAc-MurNAc disaccharides (using the Morgan–Elson reaction, after 30 min of heating in 1% borate and employing an ϵ_{585} of 7500 which was found characteristic of a disaccharide peptide unit). Results are expressed in micromoles per milliliter of effluent. The fractions were of 8.0-ml volume.

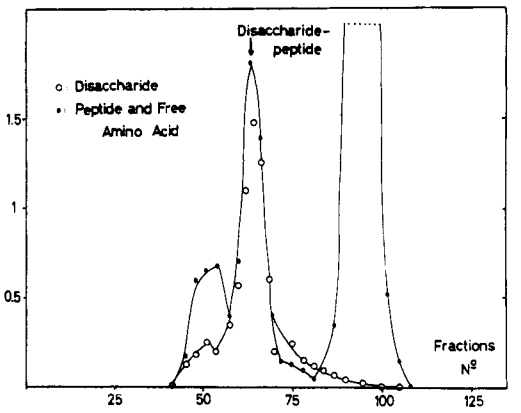

FIGURE 3: Isolation of disaccharide peptide from cell wall peptidoglycan of *M. roseus* R 27. Degradation of cell walls, filtration on Sephadex, estimation of amino groups, and disaccharide units (see Figure 2). Freeze-drying of fractions 42–57 yielded 300 mg of an unidentified nonpeptidoglycan product.

with SA endopeptidase (3.3 mg was used for *S. aureus;* 6.6 mg for *M. roseus*) in 50 ml of 0.004 M Veronal buffer, pH 8.5. Solubilization of the cell walls occurred within 6–8 hr. The incubations were prolonged up to 20 hr. The mixtures were then heated at 100° for 10 min and adjusted to pH 8. K_2HPO_4 buffer (0.5 ml of 1 M), pH 8, and aminopeptidase (27 mg in case of *S. aureus;* 9 mg in case of *M. roseus*) were added. After 10 hr of incubation at 37°, complete degradation of the peptide bridges had occurred with *M. roseus* only. Fresh aminopeptidase (10.7 mg) was added to the *S. aureus* solution and the incubation was prolonged for 9 hr. The two mixtures were again heated 10 min at 100° and adjusted with glacial acetic acid to pH 4 in the case of *S. aureus;* to pH 5 in the case of *M. roseus*. F_1 *endo-N*-acetylmuramidase (5 mg) was added and the solutions were incubated for 6 hr at 37°, yielding a complete degradation of the glycan.

The degraded *S. aureus* cell wall lysate contained 2000 and 450 mμmoles/mg of cell walls, respectively, of free glycine and β-1,4-GlcNAc-MurNAc units. Similarly, the *M. roseus* lysate contained 900 mμmoles of free L-alanine, 280 mμmoles of free L-threonine, and 290 mμmoles of disaccharide units. In both cases, all of the lysine residues had free ϵ-NH_2 groups. The degraded cell wall lysates were fractionated in water on a 800-ml column (68 × 4 cm) of Sephadex G-25 (bead form). As shown in Figures 2 and 3, the disaccharide peptides were obtained well separated from free amino acids and from gel-excluded materials, *i.e.*, the teichoic acid in the case of *S. aureus* and an unidentified polymer in the case of *M. roseus*. A last filtration on the same Sephadex column yielded, irrespective of the components used for the estimation, 122.2 μmoles of disaccharide peptide in the case of *S. aureus* and 87 μmoles in the case of *M. roseus*. These values represent 52 and 58% of the theoretical yields from *S. aureus* and *M. roseus*, respectively. As will be shown further, the *S. aureus* disaccharide peptide preparation is mainly composed of disaccharide tetrapeptide (Ala-Glu-Lys-Ala) and the *M. roseus* disaccharide peptide preparation is a mixture of the same disaccharide tetrapeptide and of the disaccharide tripeptide (Ala-Glu-Lys).

Isolation of the Disaccharide Peptide from Cell Walls of S. pyogenes. PILOT EXPERIMENTS. Earlier work had shown that these lysine-containing cell walls were susceptible to lysis using either the SA endopeptidase (Petit *et al.*, 1966) or the F_1 *endo-N*-acetylmuramidase (Muñoz *et al.*, 1966). Despite the fact that this latter enzyme was not capable of hydrolyzing all of the *N*-acetylmuramic acid linkages in the peptidoglycan, attempts were undertaken to study the structure of those peptidoglycan portions which could be degraded and isolated as well-defined disaccharide peptide units.

At completion of the action of the F_1 *endo-N*-acetylmuramidase (for optimal conditions and specific

activity; Muñoz *et al.*, 1966) 1 mg of solubilized cell walls developed a coloration of 2000 OD at 585 mμ in the Morgan–Elson reaction, performed with 30 min of heating in 1% borate and in a final volume of 600 μl. This digest also contained 250 mμmoles of total amino groups. These groups, native to the cell walls, were almost completely accounted for as 110 mμmoles of N-terminal alanine and an equivalent number of N^{ϵ}-terminal dibasic amino acids (mainly N^{ϵ}-terminal lysine and traces of N^{ϵ}-terminal hydroxylysine).

At completion of the degradation by the SA endopeptidase as described for the degradation of cell walls of *S. aureus*, 1 mg of solubilized cell walls gave a negative Morgan–Elson test. The digest contained the same number of N^{ϵ}-terminal dibasic amino acids as found after F_1 *endo-N*-acetylmuramidase treatment, but about 350 mμmoles of N-terminal alanine. Further treatment with aminopeptidase produced a collection of amino compounds which could be transformed into ether-extractable DNP derivatives. Most of these compounds probably arose from hydrolysis of some of the protein fragments left in the cell walls after protease treatment (though the aminopeptidase has no effect on intact cell walls) and were not further characterized. However, the liberation of free alanine and the exposure of new N^{ϵ}-terminal lysine and of new N^{ϵ}-terminal OH-lysine was striking. At completion of the reaction, all N-terminal alanine had disappeared, about 900 mμmoles of L-alanine was liberated, and virtually all the lysine and OH-lysine ε-amino groups were liberated.

From the foregoing and owing to the known mechanisms of action of the SA endopeptidase and of the aminopeptidase (Petit *et al.*, 1966), an hypothesis for the structure of the peptide moiety of *S. pyogenes* cell wall peptidoglycan envisages small oligomers of peptide subunits bridged through L-alanyl-L-alanine groupings (data do not exclude, however, the presence of some tri-L-Ala bridges) extending from the C-terminal D-Ala of one peptide subunit to the N^{ϵ}-terminal L-Lys of a second peptide subunit. About 40% of the hypothetical peptide subunits, however, have N^{ϵ}-terminal lysine residues either unsubstituted or substituted by uncross-linked di-L-Ala chains, thus indicating a low degree of peptide cross-linking.

PREPARATIVE RUN. Cell walls (600 mg) were treated at 37° with 60 mg of a crude *Streptomyces* peptidase preparation (*i.e.*, a mixture of several endopeptidases and of aminopeptidase) (Petit *et al.*, 1966) in 60 ml of 0.004 M Veronal buffer, pH 8.8. Solubilization was complete after 80 min. The incubation was continued up to 5 hr at which time some N-terminal alanine was still present. The mixture was adjusted to pH 8.0 and 0.6 ml of 1 M K_2HPO_4 buffer, pH 8, and 18 mg of purified aminopeptidase was added. After 3 hr of incubation at 37°, all N-terminal alanine had disappeared. The solution was heated 10 min at 100°. F_1 *endo-N*-acetylmuramidase (15 mg) was added and the mixture was incubated at 37° for 10 hr. For *S. pyogenes* walls, the pH optimum of the F_1 enzyme

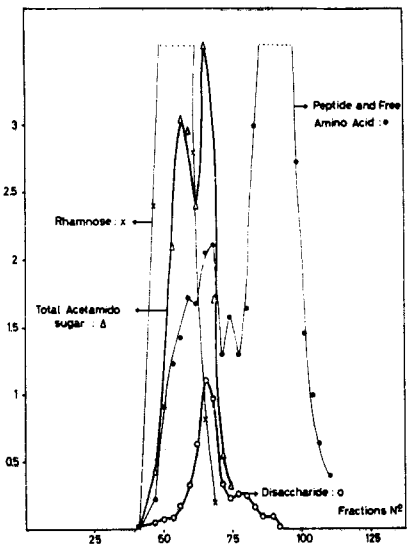

FIGURE 4: Isolation of disaccharide peptide from cell wall peptidoglycan of *S. pyogenes* (group A, type 14). Degradation of cell walls, filtration on Sephadex, and estimation of amino groups and disaccharide units were performed as indicated in the legend of Figure 2, on 19.7-μl aliquots. Rhamnose and total hexosamines were estimated on aliquots of 14.1 and 36.7 μl, respectively. In order to demonstrate the distribution of hexosamine residues indigenous to peptidoglycan fragments, the values for total hexosamine were corrected for the GlcNAc residues contained in the C polysaccharide on the basis of 0.4 GlNAc for 1 rhamnose (Heymann *et al.*, 1963). Results are expressed in micromoles per milliliter of effluent. The fractions were of 8-ml volume.

lies at pH 8.0. Analysis indicated that all of the enzymatic reactions involved in the degradation had reached completion. Isolation of the disaccharide peptide through a one-step filtration on Sephadex G-25 (same column as above) was attempted. Figure 4 shows the complexity of the degradation products. Three main fractions, however, were detected. Fractions 46–66 were rich in rhamnose-containing polysaccharide C (*i.e.*, the nonpeptidoglycan component of the *S. pyogenes* cell walls); fractions 55–74 were rich in disaccharide peptide; fractions 74–110 were rich in free amino acids. Partially degraded peptidoglycan products, as expected from a partial splitting of the glycan, were present throughout fractions 46–75. The disaccharide peptide enriched fractions (61–74) were pooled and freeze dried. The residual material was divided into four aliquots and each of them was successively filtered on a column (118 × 1.5 cm) of Sephadex G-25 (bead form) employing water as the eluent. The disaccharide peptide fraction so obtained had more terminal amino groups than expected on the basis of its hexosamine content. Hence, it was further purified by fractionation on a 800-ml column of carboxymethylcellulose (H^+

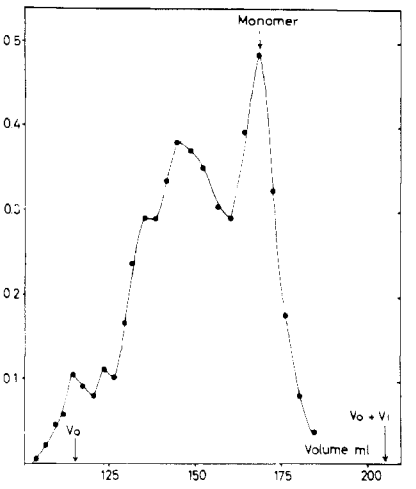

FIGURE 5: Isolation of disaccharide peptide (N^{ϵ}-pentaglycine) from cell wall peptidoglycan of penicillin-treated *S. aureus* Copenhagen. Cell walls (80 mg) were thoroughly digested with F_1 *endo-N*-acetylmuramidase. The soluble products not adsorbed on Ecteola-cellulose were filtered in water on two columns of Sephadex G-50 and Sephadex G-25 connected in series (for details, see text). Disaccharide units were estimated as indicated in the legend of Figure 2. The monomer was further purified from the pooled fractions collected between 160 and 180 ml. Details of this procedure utilizing two Sephadex columns in series were provided by Tipper (1966; D. J. Tipper and J. L. Strominger, in preparation).

form) (volume 650 ml). Under these conditions, disaccharide peptide was retained on the column. Purified disaccharide peptide could be eluted either by prolonged washing with water or by employing a gradient of LiCl from 0 to 0.2 M. In the experiment reported here, water was used as eluent and the compound emerged between 1000 and 1300 ml. The yield was 48 μmoles of purified disaccharide peptide, irrespective of the components used for the estimation. This represents an actual yield of 16% based on the assumption that there are 500 mμequiv of peptidoglycan subunits/mg of cell walls, and of about 30% when expressed in terms of color obtained from a Morgan–Elson reaction performed on an aliquot of the nonfractionated lysate.

Slightly different procedures of fractionation were applied to three other samples of degraded cell walls, which yielded the same amounts of purified disaccharide peptide (75–85 mμmoles/mg of cell walls). As will be shown below, the *S. pyogenes* disaccharide peptide preparation is composed mainly of disaccharide tetrapeptide (Ala-Glu-Lys-Ala).

Isolation of a Disaccharide Pentapeptide N^{ϵ}-Pentaglycine from Walls of Penicillin-Treated S. aureus Cells. According to recent reports, the loss of the C-terminal D-alanine of the precursor uridinediphosphomuramyl pentapeptide (L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) takes place during one of the last steps of the biosynthesis of the *S. aureus* peptidoglycan. In this step, a transpeptidation is effected in which the terminal D-Ala of a peptidoglycan subunit chain is exchanged for the N-terminal Gly residue of a pentaglycine chain attached to the ϵ-NH_2 group of the lysine residue in another peptidoglycan subunit (Tipper and Strominger, 1965; Strominger and Tipper, 1965; Wise and Park, 1965). A partial inhibition of this transpeptidation resulted when *S. aureus* was grown in the presence of sublethal doses of penicillin. As a consequence, the "weakened" walls which were prepared from these cells had a high proportion of pentapeptide monomers, substituted by uncross-linked N-terminal pentaglycine chains. This pentapeptide pentaglycine monomer was prepared and isolated in the form of disaccharide peptide, essentially according to Tipper and Strominger (1965).

S. aureus strain Copenhagen was grown at 37°, on a mechanical shaker in 1-l. flasks containing 200 ml of medium, at pH 7, composed as follows: Bactopeptone (1 g), yeast extract (1 g), K_2HPO_4 (0.2 g), and glucose (0.4 g) (pH 7). The medium was inoculated with 2 ml of a *S. aureus* culture in exponential phase (generation time is 25 min). After 90 min, 0.1 unit/ml of penicillin G was added and the cells were harvested 90 min later. Cell walls were prepared and treated with trypsin and ammonia as described in Methods. Cell walls (80 mg) were obtained from 2 l. of culture. They were treated with 400 μg of F_1 *endo-N*-acetylmuramidase in 8 ml of 0.02 M citrate buffer, pH 5.4, at 37° for 3 hr, yielding 37 μequiv of soluble reducing groups relative to GlcNAc. The degraded products were filtered in water through a column (30 × 1 cm) of Ecteola-cellulose (OH^- form). The teichoic acid–glycopeptide complexes were retained and 75% of the reducing groups emerged in the water eluate. This material was freeze dried and filtered in water on two columns connected in series, first of Sephadex G-50 (bead form) (37 × 2 cm) and next of Sephadex G-25 (bead form) (same size) (Figure 5). The material eluted between 160 and 175 ml was freeze dried and its aqueous solution was refractionated on a column (118 × 1.5 cm) of Sephadex G-25 (bead form). The compound eluted between 98 and 123 ml contained 6.6 μmoles of reducing groups relative to GlcNAc, *i.e.*, 18% of the content of the solubilized cell walls. As will be shown further, this compound was identified as a β-1,4-GlcNAc-MurNAc pentapeptide substituted on the ϵ-NH_2 group of the lysine residue by an uncross-linked pentaglycine chain, *i.e.*, β-1,4-GlcNAc-MurNAc-Ala-Glu-Lys-(N^{ϵ}-Gly_5)-Ala-Ala.

Enzymatic Degradation of the Disaccharide Peptide Compounds. TREATMENT WITH *N*-ACETYLMURAMYL-L-ALANINE AMIDASE (hydrolysis of linkage 4, Figure 1). Disaccharide peptide (1 μmole) was treated with 150 μg of amidase in 1 ml of 0.03 M acetate buffer, pH 5.4, for 2 hr at 37°. Disaccharide (1 μmole) was liberated and 1 μmole of N-terminal L-alanine appeared.

TREATMENT WITH *exo*-β-GLCNACASE (hydrolysis of linkage 6, Figure 1). Disaccharide peptide (1 μmole) or free disaccharide (1 μmole) was treated with

TABLE II: Analysis of the Disaccharide Peptide Compounds Prepared from Cell Walls of *S. aureus*, *M. roseus* R 27, and *S. pyogenes*.[a]

	S. aureus (penicillin-treated cells)	*S. aureus* (normal cells)	*M. roseus*	*S. pyogenes*[b]
A. Total Amino Acids				
Glutamic acid	1000	1000	1000	1000
Lysine	1078	1005	1020	843
Alanine	3005	2165	1603	2063
D-Alanine	1887	1116	513	860
L-Alanine	880	888	910	830
Glycine	4650	136	82	96
Hydroxylysine	0	0	0	108
NH_3	+	+	+	+
B. Amino-Terminal Groups				
ε-Lysine	0	927	981	850
Glycine	432	0	0	0
C. Disaccharide Moiety				
Total disaccharide after amidase action	—	970	1130	1030
Total *N*-acetylhexosamine after *exo*-β-GlcNAcase	1970	1630	1875	1660
Total hexosamine after HCl hydrolysis	1650	1675	1842	1800

[a] Data expressed as moles/1000 moles of glutamic acid. [b] The disaccharide peptide fraction prepared from cell walls of *S. pyogenes* contains additional amino acids tentatively identified as: threonine 12, ornithine 60, and serine 30.

60 μl of the enzyme preparation in a final volume of 3 ml of 0.01 M citrate buffer, pH 4.4, for 4 hr at 37°. The hydrolysis was followed by means of the Morgan–Elson reaction performed with 1% borate and 7 min of heating; the pertinent molar absorbancy is ε 18,000 for free *N*-acetylhexosamines.

TREATMENT WITH AMINOPEPTIDASE (hydrolysis of linkage 5, Figure 1). Aminopeptidase (200 μg) and 400 μl of 1 M K_2HPO_4 buffer, pH 8.0, were added to 1 μmole of the amidase-treated disaccharide peptide in a final volume of 2 ml. The mixture was incubated for 6 hr at 37°. One L-Ala was liberated and no N-terminal Ala remained. One N-terminal Glu appeared. The bridge-bearing disaccharide pentapeptide subunit, GlNAc-MurNAc-Ala-Glu-Lys-(N^{ϵ}-Gly_5)-Ala-Ala, also reacted with the amidase as expected. Subsequent action of the aminopeptidase not only hydrolyzed linkage 5 (Figure 1) but also partially degraded N-terminal glycine bridges to free glycine. Since the ε-Gly-Lys linkage is hydrolyzed at a low rate, large amounts of enzyme are needed for complete liberation of all of the glycine and of the ε-amino groups of lysine. To this end, 800 μg of aminopeptidase was used for 1 μmole of amidase-treated compound, in the same conditions as above.

Isolation of the Di-DNP Peptides Remaining after Degradation as Described Previously. A general procedure will be given here. For details, the reader is referred to Ghuysen *et al.* (1966). $K_2B_4O_7$ solution (500 μl of 5%) and 300 μl of a 0.1 M FDNB solution in alcohol were added to the 2-ml solution containing 1 μmole of the amidase- and aminopeptidase-treated disaccharide peptides. Similarly, 2 ml of water, 1 ml of 5% $K_2B_4O_7$, and 500 μl of 0.1 M FDNB solution were added to the 2-ml solution containing 1 μmole of amidase- and aminopeptidase-treated disaccharide pentapeptide N^{ϵ}-pentaglycine. The mixtures were mixed with a Vortex mixer, heated for 30 min at 60°, and then cooled to room temperature. HCl (1.25 ml of 12 N) was added to give a final normality of about 4 N, and the mixtures were extracted three times with 1 ml of ether. The residues of the pooled ether extracts were chromatographed on thin layer plates of silica gel, first in solvent I, then in solvent II, both solvents ascending in the same direction. The remaining aqueous phases were diluted to a final normality of 2 N and extracted three times with water-saturated 1-butanol. The residues of the pooled butanol extracts were dissolved in absolute methanol and dried *in vacuo*. This process was repeated three times. The final residues were chromatographed on thin layer silica gel with solvent III. The DNP peptide spots from the ether extracts as well as from the butanol extracts were eluted from the gel by vigorous agitation with ethanol–water–15 N NH_3 (1000:1000:5.4). The extracts were evaporated *in vacuo*. The purified DNP peptides were then studied.

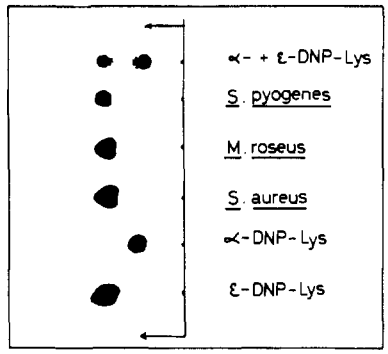

FIGURE 6: Characterization of the N-terminal lysine in disaccharide peptides. The dinitrophenylated disaccharide peptides were hydrolyzed in 6 N HCl at 100° for 15 hr. After ether extraction of the hydrolysates, mono-DNP-lysine was extracted with l-butanol from the aqueous phases and compared by thin layer chromatography on silica gel in solvent IV with authentic α-DNP-lysine and ε-DNP-lysine.

Dehydration–Reduction of the Disaccharide Peptide Compounds. Each of the disaccharide peptide preparations obtained from normal cell walls were submitted to dehydration and reduction according to the technique of Ressler and Kashelikar (1966) except that the dehydration was prolonged up to 20 hr. Disaccharide peptide (4 μmoles) was suspended in 400 μl of triethyl phosphite. Ethylene chlorophosphite (50 μl) was added and the mixture was heated at 100° for 20 hr. The solution was cooled to room temperature and 400 μl of ethanol was added. After 15 min, the solution was distilled at 70°, for 1 hr, *in vacuo* (10^{-2} mm). Methanol (100 μl) was added. The mixture was cooled to −40° and a few milliliters of liquid NH_3 was added. The solution was treated with solid sodium until the blue coloration was present for about 1 min. After freeze drying, the residue was dissolved in 2 ml of water and the solution was adjusted to pH 2 with 6 N HCl. The solution was passed through a Dowex 50-X2 column (14 × 0.6 cm). After washing with water, elution was carried out with ~20 ml of 3 N NH_4OH solution. The eluate was freeze dried, the residue was hydrolyzed in 6 N HCl at 120° for 20 hr, and the amino acids were determined with the Technicon autoanalyzer.

Results

The Sequences L-*Ala*-D-*Glu*-L-*Lys*, L-*Ala*-D-*Glu*-L-*Lys*-D-*Ala*, *and* L-*Ala*-D-*Glu*-L-*Lys*-D-*Ala*-D-*Ala in the Disaccharide Peptide Compounds.*[2] CHEMICAL COMPOSITION (Table II). After hydrolysis with 6 N HCl for 15 hr at 100°, individual amino acids were estimated using the autoanalyzer, and L- and D-alanine were estimated enzymatically. It is known that glutamic acid and lysine in cell wall peptidoglycans are exclusively in the D and in the L form, respectively (Ikawa and Snell, 1956; Salton, 1964). The stereochemical configuration of these two amino acids was not reinvestigated during the present studies. The disaccharide moiety was estimated by determination of (1) total hexosamine, after hydrolysis for 3 hr at 100° with 3 N HCl; (2) the *N*-acetylglucosamine liberated by *exo*-β-GlcNAcase with the aid of the Morgan–Elson reaction performed with 7 min of heating in 1% borate; and (3) the β-1,4-GlcNAc-MurNAc liberated by *N*-acetylmuramyl amidase, by the Morgan–Elson reaction performed with 30 min of heating, conditions under which a molar absorbancy of ε 9500 is obtained for the disaccharide.[3] As shown in Table II, all four disaccharide peptide preparations contain virtually equivalent amounts of disaccharide, L-alanine, L-lysine, and D-glutamic acid. When expressed per mole of glutamic acid, the disaccharide peptide of *M. roseus* contains 0.56 mole of D-alanine; the disaccharide peptides from *S. pyogenes* and normal cell walls of *S. aureus* both contain 1 mole of D-alanine, while the disaccharide peptide from penicillin-treated cell walls of *S. aureus* contains 2 moles of D-alanine and 5 moles of glycine.

N-TERMINAL GROUPS (Table II). The three disaccharide peptides obtained from normal cell walls contain per mole of glutamic acid essentially 1 mole of N^{ϵ}-terminal lysine. Mono-DNP-lysine was measured after dinitrophenylation of the compounds and hydrolysis at 100° for 15 hr with 6 N HCl and characterized as ε-DNP-lysine by thin layer chromatography in solvent IV (Figure 6). In the case of the disaccharide peptide pentaglycine from penicillin-treated *S. aureus* cell walls, glycine is found at the amino terminus. After dinitrophenylation and HCl hydrolysis only 0.43 mole of DNP-glycine/glutamic acid was detected. This low value is the result of an incomplete labeling of the terminal amino group of the pentaglycine chains. Indeed it has been observed that the extent of dinitrophenylation, which is complete for glycine, is drastically reduced for polyglycine oligomers of chain lengths higher than 3. For example, the yield of DNP-tetraglycine obtained using the described conditions of dinitrophenylation was 40% of the theoretical values. It should be noted that paper chromatography in solvent V of the glycine-containing subunit is consistent with a monomeric composition. The observed R_F 0.54, identical with that reported by Tipper and Strominger (1965) for such a monomer, is very similar to that of the disaccharide peptide compounds obtained from normal cell walls, whose content of N^{ϵ}-

[2] The first paragraph of this section will be restricted to the study of the sequence of the constitutive amino acids of the peptide subunits. The symbol Glu as used in this paragraph should not be taken to imply either the presence or the absence of an amide group. The second paragraph of this section will be concerned with the positions of the amide substituent on the glutamic acid residue.

[3] As observed earlier (Muñoz *et al.*, 1966), peptide substitution of the disaccharide depresses its color development. Disaccharide units interlinked through complex peptides (as they occur in F_1 *endo-N*-acetylmuramidase digests of cell walls of *S. aureus* and *M. roseus*) have an ϵ_{585} of 5700. The three disaccharide peptide preparations from normal cells have a higher ϵ_{585} of 7500–8000.

TABLE III: Enzymatic Degradation of the Disaccharide Peptide Compounds Prepared from Cell Walls of *S. aureus*, *M. roseus*, and *S. pyogenes*.[a,b]

Disaccharide Peptide	After Step	N-Terminal Amino Groups				Free Amino Acids	
		N-L-Ala	*N*-Gly	*N*-Glu[c]	N^{ϵ}-Lys	L-Ala	Gly
S. aureus (penicillin-treated cells)	4	920	432	0	0	0	0
	5	0	0	1000	970	1005	4925
S. aureus (normal cells)	4	831	0	0	930	0	0
	5	0	0	1002	930	994	0
M. roseus	4	921	0	0	980	0	0
	5	0	0	1013	980	1070	0
S. pyogenes	4	826	0	0	850	0	0
	5	0	0	937	920	1120	0

[a] Data are expressed as moles/1000 moles of glutamic acid. [b] Changes in terminal amino groups of Gly, L-Ala, Glu, and N^{ϵ}-Lys, and in free amino acids Gly and L-Ala after sequential degradation with *N*-acetylmuramyl-L-alanine amidase (step 4, Figure 1) and next with aminopeptidase (step 5, Figure 1). [c] Measured on purified di-DNP derivatives. Labeling of the N-terminal glutamic acid of the diamino peptides with FDNB was about 75% of the theoretical (see text).

terminal lysine excludes the possibility of an oligomeric structure. No N-terminal alanine was detected in any of the four disaccharide peptide compounds examined.

C-TERMINAL GROUPS. All four disaccharide peptide compounds have C-terminal D-alanine. Traces of C-terminal glycine are also present in the preparation obtained from normal *S. aureus* cell walls. C-Terminal D-alanine and C-terminal L-lysine occur in the ratio 6:4 in the *M. roseus* disaccharide peptide preparation. The actual yields for the estimation of these C-terminal groups by hydrazinolysis were only 25–30% of the theoretical values, if one assumes that the content of C-terminal D-alanine or, in the case of *M. roseus*, the sum of the amounts of C-terminal D-alanine and C-terminal L-lysine, is equivalent to the content in glutamic acid. However, similar yields were obtained when the same procedure was applied to the synthetic peptides, L-isoglutaminyl-L-lysine (25%), L-glutaminyl-L-lysine (24%), L-isoglutaminyl-L-lysyl-D-alanine (26%), L-glutaminyl-L-lysyl-D-alanine (30%), and L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (28%).

THE SEQUENCE DISACCHARIDE-L-ALA-D-GLU. Hydrolysis of each of the four disaccharide peptides studied with *N*-acetylmuramyl-L-alanine amidase, followed by aminopeptidase treatment, established (Table III) that the L-alanine is located at the amino terminus of the peptide and is bound in amidic linkage to the carboxyl of the disaccharide and in peptide linkage to the amino group of the glutamic acid residue. Thus, amidase treatment of a disaccharide peptide resulted in the appearance of about 1 mole of N-terminal alanine/glutamic acid residue. Treatment of this product with aminopeptidase resulted in the liberation of about 1 mole of free alanine, shown enzymatically to be in L form, and in the concomitant appearance of about 1 mole of N-terminal glutamic residue. The final peptide bearing N-terminal glutamic acid could not be degraded further by enzymatic means. In the disaccharide peptide pentaglycine from penicillin-treated cell walls, the same linkages as above were hydrolyzed. Moreover, five glycines were liberated and one N^{ϵ}-terminal lysine was exposed (Table III).

THE SEQUENCE D-GLU-L-LYS, D-GLU-L-LYS-D-ALA, AND D-GLU-L-LYS-D-ALA-D-ALA. From the foregoing, it can be anticipated that, at the end of the sequential enzymatic degradation of the disaccharide peptide compounds, the main residual peptides must be: (1) the tetrapeptide D-Glu-L-Lys-D-Ala-D-Ala in the case of *S. aureus* (penicillin-treated cells); (2) the tripeptide D-Glu-L-Lys-D-Ala in the case of *S. aureus* (normal cells) and of *S. pyogenes;* (3) a mixture of the latter tripeptide and the dipeptide D-Glu-L-Lys in the case of *M. roseus.*

Each of the degradation mixtures was dinitrophenylated. Ether extraction removed in all cases DNP-alanine and, in the case of *M. roseus* only, an additional compound (compound A in Figure 7). This compound was purified by chromatography in solvents I + II ($R_{DNP\text{-}Ala}$ 0.56) and was characterized as the di-DNP derivative of the dipeptide N^{α}-Glu-Lys, as described below. Butanol extraction of the aqueous phases followed by chromatography of the extracted materials in solvent III yielded the di-DNP derivative of the tripeptide N^{α}-Glu-Lys-Ala in the cases of *M. roseus*, *S. pyogenes*, and *S. aureus* (compound B in Figure 7). In the case of the disaccharide peptide pentapeptide from penicillin-treated *S. aureus*, ether extraction removed DNP-alanine and DNP-glycine.

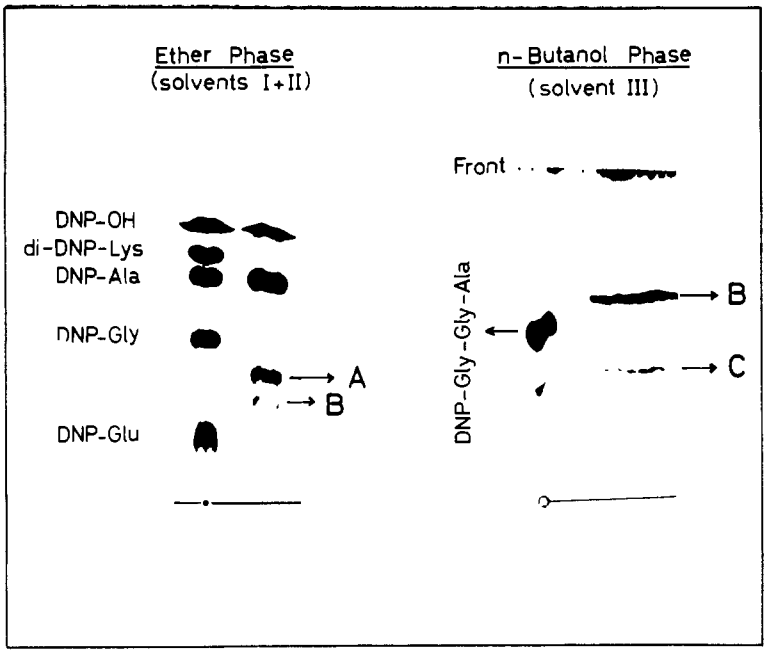

FIGURE 7: Purification of the di-DNP dipeptide (Glu-Lys) and of the di-DNP tripeptide (Glu-Lys-Ala) from degraded cell walls of *M. roseus* R 27. The degradation products of the disaccharide peptide (after step 5 of the sequential degradation, see Figure 1) were dinitrophenylated. DNP-alanine and di-DNP dipeptide (compound A) were extracted with ether (from 4 N HCl solution) and separated by sequential thin layer silica gel chromatography in solvents I and II. Traces of di-DNP tripeptide (compound B) were also extracted with ether. It migrates in solvents I and II with an R_F lower than that of di-DNP dipeptide. Di-DNP tripeptide was extracted with l-butanol from the aqueous phase and purified by thin layer silica gel chromatography in solvent III. It has an R_F higher than that of DNP-Gly-Gly-Ala. Disaccharide peptides from *S. aureus* (normal cells) and from *S. pyogenes* yielded, on similar treatment, DNP-Ala and di-DNP tripeptide only. Disaccharide peptide (N^{ϵ}-pentaglycine) from penicillin-treated *S. aureus* yielded DNP-Ala, DNP-Gly, and di-DNP tetrapeptide (Glu-Lys-Ala-Ala). The butanol extracts also contain a small amount of mono-ε-DNP peptides (compound C) due to an incomplete dinitrophenylation of the N-terminal glutamic acid residues.

Butanol extraction followed by chromatography in solvent III yielded the di-DNP derivative of the tetrapeptide N^{α}-Glu-Lys-Ala-Ala.

The purified di-DNP dipeptide was characterized as follows. After acid hydrolysis (6 N HCl, 100°, 15 hr) and freeze drying, the material was dissolved in 4 N HCl. DNP-glutamic acid was extracted by ether. The aqueous phase was diluted with an equal volume of water. ε-DNP-lysine was extracted by 1-butanol. ε-DNP-lysine and DNP-glutamic acid were found in a ratio of about 1:1. Another aliquot of the hydrolyzed material was submitted to a new dinitrophenylation. DNP-Glu and an equivalent amount of di-DNP-Lys, but no other DNP-amino acid, were extracted by ether. The purified di-DNP tripeptide and di-DNP tetrapeptide were similarly characterized. Results identical with those reported for the di-DNP dipeptide were obtained, except that in the present cases, dinitrophenylation of the hydrolyzed samples yielded not only DNP-glutamic acid and di-DNP-lysine as above, but also DNP-alanine. The amount of DNP-alanine was equivalent to that of DNP-glutamic acid in the case of the tripeptide, and was essentially twice that of the DNP-glutamic acid in the case of the tetrapeptide.

When expressed relative to the glutamic acid content of the respective undegraded disaccharide peptide preparations, the purified di-DNP peptides were obtained with the following yields. (1) The disaccharide peptide pentaglycine preparation from penicillin-treated *S. aureus* yielded 65% of di-DNP tetrapeptide (Glu-Lys-Ala-Ala). (2) The disaccharide peptide preparations from *S. pyogenes* and from normal cells of *S. aureus* yielded 72 and 82%, respectively, of di-DNP tripeptide (Glu-Lys-Ala). (3) The disaccharide peptide preparation from *M. roseus* yielded 42% of the di-DNP tripeptide and 35% of di-DNP dipeptide (Glu-Lys). It should be noted that the synthetic peptides L-isoglutaminyl-L-lysine, L-isoglutaminyl-L-lysyl-D-alanine, and L-isoglutaminyl-L-lysyl-D-alanyl-D-alanine were also transformed into di-DNP derivatives with yields of about 75%. In the above conditions of dinitrophenylation, about 25% of the original peptides were transformed into mono-ε-DNP-lysyl derivatives. These

TABLE IV: Characterization by Dehydration–Reduction of the α-Carboxamide of the Glutamic Acid in the Disaccharide Peptide Compounds. Transformation of the Isoglutaminyl Residue into γ-Aminobutyric Acid.[a]

Preparations	Before Treatment	After Treatment			Relative Transformation in %[c]	
	A	B	C	D	C/A × 100	D/A × 100
S. aureus (normal cells)	31.6	6.4	20.7	0	65	0
S. pyogenes[b]	33.1	6.3	18.2	0	55	0
M. roseus	38.1	11.5	18.1	0	48	0
L-Gln-L-Lys-D-Ala	50	1.86	0	39.8	0	80

[a] Results are expressed: (1) as moles of glutamic acid/100 moles of (alanine + lysine). Values for the untreated compounds are indicated in column A (see Table II), values for the treated compounds are shown in column B; (2) as moles of γ-aminobutyric acid (column C) or ornithine (column D)/100 moles of (alanine + lysine) in the treated compounds. [b] In this case, the sum (Ala + Lys + OH-Lys) has been taken as basis for the calculations. [c] Alanine and lysine do not give rise to ornithine or γ-aminobutyric acid. They may, however, be partially lost in the procedure, especially when they occur as C-terminal amino acids. This might explain the variations observed in the relative yields.

mono-DNP compounds were readily separated from the di-DNP compounds by thin layer chromatography in solvent III (see compound C of Figure 7).

Amide Substitution of the α-Carboxyl Group of Glutamic Acid. ELECTROPHORETIC MOBILITIES. On paper electrophoresis at pH 5.0, the undegraded disaccharide peptides are neutral compounds whereas the peptides obtained after each step of the sequential degradation with *N*-acetylmuramyl-L-alanine amidase and aminopeptidase are basic (Figure 8). The observations indicate that the carboxyl group of the glutamic acid residue, which is not engaged in peptide bond formation, does not contribute to the electrical charge of the disaccharide peptides. In agreement with this conclusion, the synthetic tripeptide γ-L-glutamyl-L-lysyl-D-alanine is uncharged and the synthetic peptides L-isoglutaminyl-L-lysyl-D-alanine or L-isoglutaminyl-L-lysyl-D-alanyl-D-alanine are cationic, under the same conditions of electrophoresis (Figure 8).

CHEMICAL DEGRADATION OF THE DISACCHARIDE PEPTIDES. Amino acid analysis of the disaccharide peptides showed the presence of NH_3. More accurate analysis carried out on the carbohydrate-free peptides obtained from cell walls of *S. aureus* (Tipper *et al.*, 1967a) and of *M. roseus* (J. F. Petit, unpublished) indicated the presence of about 1 mole of NH_3/glutamic acid residue. Through an Edman degradation carried out on the *S. aureus* polypeptide moiety (Tipper and Strominger, 1965; Tipper *et al.*, 1967a), first N-terminal alanine was removed and N-terminal glutamic acid appeared (thus confirming the sequence Ala-Glu of the peptide subunit). After the second cycle of the degradation, ammonia was liberated and N-terminal groups disappeared demonstrating that NH_3 was a substituent of the α-carboxyl group of glutamic acid. Further chemical identification of the isoglutaminyl residue in the *endo* position in the disaccharide peptides of *S. aureus*, *M. roseus*, and *S. pyogenes* was brought about by dehydration and reduction, followed by HCl hydrol-

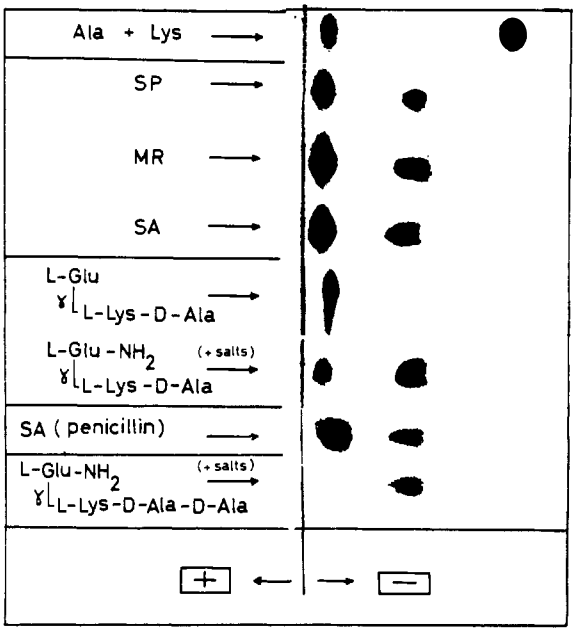

FIGURE 8: Paper electrophoresis of the degradation products after sequential treatment of the disaccharide peptides with *N*-acetylmuramyl-L-alanine amidase and with aminopeptidase. Electrophoresis was performed at pH 5.0 (for conditions, see Methods) after the compounds had been spotted at the center of the paper sheet. Natural peptides: from penicillin-treated *S. aureus* (SA penicillin), normal *S. aureus* (SA), *M. roseus* (MR), and *S. pyogenes* (SP). See Table I for synthetic peptides. The tri- and tetrapeptide amides were mixed with the enzyme and the electrolytes used for the enzymatic treatments. The neutral compound present in the degraded disaccharide peptides was characterized as free L-alanine. This L-alanine is contaminated by some materials from the enzymes preparations. These impurities are also separated by electrophoresis from the cationic amidated peptides.

ysis (Table IV) (see Experimental Section). The three natural compounds, the synthetic tripeptide L-isoglutaminyl-L-lysyl-D-alanine, and other peptides containing one isoglutaminyl residue in the endo position yielded γ-aminobutyric acid (from the isoglutaminyl residues). No ornithine, which would be indicative of the presence of glutaminyl residues, was detected (Table IV). Conversely, the synthetic peptide L-glutaminyl-L-lysyl-D-alanine yielded ornithine but no γ-aminobutyric acid under the same conditions (Table IV).

CHROMATOGRAPHIC PROPERTIES. The di-DNP peptides obtained after enzymatic degradation of the disaccharide peptides were compared to synthetic di-DNP peptides by sequential chromatography on thin layer silica gel in solvent I followed by solvent II. DNP-glutamic acid, DNP-glutamine, and DNP-isoglutamine were also prepared. DNP-glutamic acid, the two DNP-dipeptides α- or γ-Glu-Lys, the two DNP-dipeptides glutaminyl- and isoglutaminyllysine, and the two DNP-tripeptides α- or γ-Glu-Lys-Ala are readily extracted with ether from solutions in 4 N HCl. DNP-glutamine, DNP-isoglutamine, and the DNP-tripeptide isoglutaminyllysylalanine, are very poorly ether soluble. Under the conditions described above, 10–20 mμmoles of these DNP derivatives was extracted from 4 N HCl solutions containing 220 mμmoles of compounds. As already pointed out (Figure 7), the DNP derivatives of the natural tripeptides have similarly low ether solubilities. The DNP tripeptide glutaminyllysylalanine and the DNP tetrapeptide isoglutaminyllysylalanylalanine are virtually insoluble in ether. All the ether-insoluble DNP compounds were readily extracted with 1-butanol.

In solvent 1, the di-DNP di- and tripeptides possessing a C-terminal glutamic acid have a very low mobility and are indistinguishable from DNP-glutamic acid (Figure 9, lower part). In contrast to this, the same di-DNP di- and tripeptides in which the C-terminal glutamic acid is amide substituted have higher R_F values, virtually identical with that of DNP-glutamine or DNP-isoglutamine. After one run in solvent 1, the plates were dried in a stream of cold air and were then developed several times in solvent II. After three such ascents (Figure 9, upper part), the di-DNP compounds can be classified into several groups depending on which of the carboxyl groups of the glutamic acid residue is substituted. Compounds **3**, **7**, and **11** in Figure 9 have, respectively, an amide, a lysine residue, and a lysylalanine dipeptide as α substituents of the glutamic acid, and exhibit R_F values higher than that of DNP-glutamic acid. Conversely, **2**, **4**, and **8** in Figure 9, which have the same substituents in the γ position, show R_F values lower than that of DNP-glutamic acid. Finally, compounds **5**, **6**, and **10** (Figure 9), in which both the α- and the γ-carboxyl groups of glutamic acid are substituted, have R_F values similar to that of DNP-glutamic acid. Only **9**, the DNP tripeptide from glutaminyllysylalanine, presents some abnormalities. It migrates in solvent I in the form of an elongated spot and in solvent II has an R_F higher than expected, *i.e.*, almost equivalent to that

TABLE V: Activity of *Streptomyces* Aminopeptidase on Synthetic Peptides.

Substrates	Degradation Products and % Hydrolysis after 1 and 6 Hr of Treatment[a]		
I. N-Terminal L-Glutamyl Compounds			
α substituted			
L-Glu-NH_2[b]	L-Glu + NH_3		100
L-Glu-L-Ala	L-Glu + L-Ala		100
γ substituted			
L-Gln	No action		
L-Glu └—L-Ala	No action		
L-Glu └—L-Lys	No action		
L-Glu └—L-Lys-D-Ala	No action		
α and γ substituted			
L-Gln-L-Lys	L-Gln + L-Lys	85	100
L-Gln-L-Lys-D-Ala	L-Gln + L-Lys-D-Ala		100
L-Glu-NH_2[b] └—L-Lys	L-Glu + NH_3 └—L-Lys	45	100
L-Glu-NH_2[b] └—L-Lys-D-Ala	L-Glu + NH_3 └—L-Lys-D-Ala	15	80
II. N-Terminal D-Glutamyl Compounds			
α substituted			
D-Glu-L-Lys	No action		
D-Glu-L-Lys-D-Ala	No action		
III. Miscellaneous			
N-Terminal L-amino acid			
L-Ala-L-Ala-L-Ala	Free alanine		100
L-Ala-D-Glu-NH_2	L-Ala + D-Glu-NH_2		100
L-Ala-D-Glu-NH_2 └—L-Lys-D-Ala	L-Ala + D-Glu-NH_2 └—L-Lys-D-Ala		100
L-Lys-D-Ala	L-Lys + D-Ala		5.7
L-Ala-D-Ala-L-Ala	L-Ala + D-Ala-L-Ala	12.5	25
N-Terminal D-amino acid			
D-Ala-L-Ala	No action		
N-Terminal glycyl peptides			
Gly-Gly-Gly-Gly	Free glycine		
N^{ϵ}-Gly-L-Lys	Gly + L-Lys	30	60

[a] For conditions, see text. [b] Note the progressive decrease in the rates of deamidation of L-isoglutamine, L-isoglutaminyl-L-lysine, and L-isoglutaminyl-L-lysine-D-alanine.

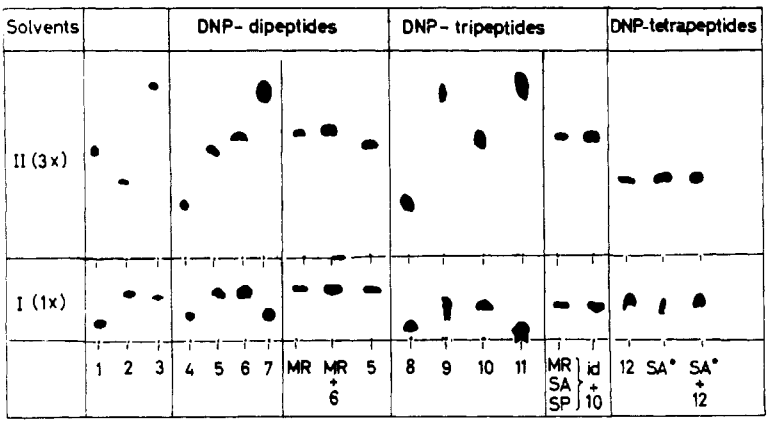

FIGURE 9: Sequential chromatography of DNP-glutamyl compounds on thin layer silica gel and in solvents I and II. (A) Synthetic compounds: (1) L-glutamic acid; (2) L-glutamine; (3) L-isoglutamine; (4) γ-L-glutamyl-L-lysine; (5) L-glutaminyl-L-lysine; (6) L-isoglutaminyl-L-lysine; (7) α-D-glutamyl-L-lysine; (8) γ-L-glutamyl-L-lysyl D-alanine; (9) L-glutamyl-L-lysyl-D-alanine; (10) L-isoglutaminyl-L-lysyl-D-alanine; (11) α-D-glutamyl-L-lysyl-D-alanine; (12) L-isoglutamyl-L-lysyl-D-alanyl-D-alanine. (B) Natural peptides: from *M. roseus* (MR), *S. aureus* (SA), *S. pyogenes* (SP), and penicillin-treated *S. aureus* (SA*). Ether solubility: all DNP compounds are soluble, in ether except **2, 3, 10,** and the DNP tripeptides MR, SA, and SP which are very slightly soluble, and **9,** and **12** and the DNP tetrapeptide SA* which are insoluble.

of the monosubstituted **11**, the DNP tripeptide from α-glutamyllysylalanine.

The chromatographic properties of the DNP compounds, as shown in Figure 9, were found highly reproducible with the exception of that shown by the DNP-tripeptides **9** and **11**. Even so, DNP-tripeptides **9** and **11** always migrated more rapidly than **10**, the DNP tripeptide from isoglutaminyllysylalanine.

The di-DNP dipeptide (from *M. roseus*) and the di-DNP tripeptides (from *M. roseus*, *S. aureus*, and *S. pyogenes*) migrated in solvents I and II like amidated glutamyl peptides. Chromatography with internal synthetic markers showed (Figure 9) that the di-DNP dipeptide was indistinguishable from the synthetic di-DNP-isoglutaminyllysine and that the di-DNP tripeptides were indistinguishable from the synthetic di-DNP-isoglutaminyllysylalanine. The di-DNP-tetrapeptide from penicillin-treated *S. aureus* was also examined under the same conditions and found to be indistinguishable from synthetic di-DNP-isoglutaminyllysylalanylalanine (Figure 9).

Substrate Requirements for the Streptomyces Aminopeptidase Activity. As described above, the aminopeptidase is involved in two different steps of the sequential degradation of the bacterial cell walls (hydrolysis at linkages 2 and 5, Figure 1). In order to confirm our view of the mechanism of action of this enzyme, we studied its activity upon synthetic peptides whose structures are related to that of the natural substrate. Aliquots of 100 mμmoles of each of the compounds listed in Table V were treated for 1 and 6 hr, at 37°, with 10 μg of aminopeptidase in 100 μl of 0.2 M K_2HPO_4 buffer, pH 8. Variation of the buffer concentration from 0.02 to 0.2 M does not affect the velocity of the enzymatic reaction. The

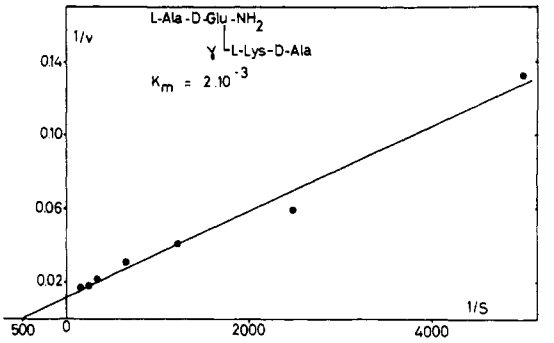

FIGURE 10: Affinity of the aminopeptidase for the synthetic tetrapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine. Tetrapeptide is hydrolyzed into L-alanine and the corresponding amidated tripeptide. The Michaelis constant K_m was calculated from the plot $1/v$ *vs.* $1/S$ according to the method of Lineweaver and Burk (1934). Aliquots (100 μl) of 2×10^{-4}–9×10^{-3} M solutions of tetrapeptide in 0.2 M phosphate buffer, pH 8, were treated for 10 min at 37° with 5 μg of aminopeptidase. Free alanine and free tripeptide were separated and measured in the form of DNP-Ala and di-DNP tripeptide amide. The Michaelis constant ($K_m = 1 \times 10^{-3}$ M) of the aminopeptidase for L-isoglutamine was similarly measured. Aliquots (100 μl) of 2×10^{-4}–7×10^{-3} M isoglutamine were treated under the same conditions as above with 2 μg of aminopeptidase. Glutamic acid was measured as DNP-Glu.

degraded products were characterized and measured after dinitrophenylation and chromatography in solvents I and II. Several conclusions can be drawn from

the data of Table V. (1) The enzyme readily liberates the N-terminal amino acid of peptides having two L-α-amino acids at the end. (2) One of these amino acids may be glycine since L-Ala-Gly, Gly-L-Ala, or Gly-Gly are hydrolyzed. In addition, L-isoglutamine is deamidated. (3) The aminopeptidase is inactive when the carboxyl group involved in the peptide bond is not in the α position; thus L-glutamine or the peptides γ-L-glutamyl-L-lysine or γ-L-glutamyl-L-lysyl-D-alanine are resistant. (4) If the amino group engaged in the linkage is not in the α position, the activity of the enzyme is diminished as observed with the dipeptide N^{ϵ}-glycyl-L-lysine. (5) The enzyme is capable of hydrolyzing peptide bonds linking an N-terminal L-amino acid to a D-amino acid although, in this case, some other substrate properties control the activity. For examples, the L-Ala-D-Glu linkage is hydrolyzed at high rates in the dipeptide L-alanyl-D-isoglutamine and in the tetrapeptide L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine. However, the L-Ala-D-Ala linkage is hydrolyzed at very low rate in the tripeptide L-alanyl-D-alanyl-D-alanine; moreover the dipeptide L-lysyl-D-alanine is not susceptible to the enzyme. (6) Peptide bonds from an N-terminal D-amino acid to an L-amino acid (and very likely from an N-terminal D-amino acid to a D-amino acid) are completely resistant.

The affinities of the aminopeptidase for two substrates, *i.e.*, L-isoglutamine and the tetrapeptide L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine, were measured in terms of Michaelis constants according to the method of Lineweaver and Burk (1934). Values of 1×10^{-3} and 2×10^{-3} M were obtained, respectively (Figure 10). The reactions catalyzed are deamidation in the former and cleaving the L-Ala-D-Glu linkage in the latter case.

Discussion

Bacterial cell wall peptidoglycans are polymers composed of three basal components: (1) polysaccharide chains; (2) peptide subunits branching from the polysaccharide chains; and (3) bridges cross-linking the peptide subunits. The covalent association between these three components results in a two- or three-dimensional network encompassing the entire bacterium. Initially attention was focused on the structure of the polysaccharide chains. In all cases studied, as in cell walls of *E. coli* (Weidel and Pelzer, 1964), *Micrococcus lysodeikticus* (Salton and Ghuysen, 1960; Perkins, 1960; Sharon *et al.*, 1966; Leyh-Bouille *et al.*, 1966), *S. aureus* (Ghuysen and Strominger, 1963-a,b; Tipper *et al.*, 1965; Tipper and Strominger, 1966), *A. crystallopoietes* (Tipper *et al.*, 1967b), and *M. roseus* (Muñoz *et al.*, 1966), the polysaccharides were shown to be water-soluble chitinlike chains consisting of alternating GlcNAc and MurNAc residues. Recent studies (Petit *et al.*, 1966; Weidel and Pelzer, 1964; Tipper *et al.*, 1967b) of the nature of the groups which cross-link the peptide subunits provided evidence for the existence of various types of peptide crosslinkages. In all cases examined, one amino group of the diamino acid (most often lysine or diaminopimelic acid) of one peptide subunit was found to be joined to the carboxyl group of a terminal D-alanyl of a second peptide subunit, either through direct bonding or through an intervening peptide, the chemical composition of which was shown to vary according to the nature of the bacterium: pentaglycine in *S. aureus*, tri-L-Ala-L-Thr in *M. roseus* R 27, and tri-L-Ala in another strain of *M. roseus* (Petit *et al.*, 1966).

Through controlled degradations involving five successive enzymatic hydrolyses of specific linkages in the peptidoglycans, the peptide subunits have now been isolated from three L-lysine containing cell walls: *S. aureus*, *M. roseus*, and *S. pyogenes*, *via* the intermediate isolation of disaccharide peptide subunits. These peptide subunits have been characterized as the tetrapeptide amide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine. The structure is supported by the following chemical and enzymatic evidence. (1) Amino acid composition and characterization of the C- and N-terminal groups of the disaccharide peptide. (2) Cleavage of this compound by *N*-acetylmuramyl-L-alanine amidase into disaccharide and peptide with the concomitant appearance of an N-terminal L-alanine. (3) Further degradation of the tetrapeptide by an aminopeptidase resulting in the liberation of an L-alanine residue and the concomitant appearance of an N-terminal D-isoglutaminyl residue. (4) Isolation of the residual tripeptide in the form of a di-DNP derivative. This DNP tripeptide is indistinguishable and inseparable from synthetic di-DNP-isoglutaminyl-lysylalanine. In the chromatographic system employed for the comparison, the isomers, differing by containing α- or γ-linked glutamic acid, or glutaminyl residues, were clearly distinguishable. (5) Electrophoretic mobilities of the disaccharide tetrapeptide, of the tetrapeptide, and of the tripeptide are consistent with α-amide substitution of the glutamic acid residue. (6) Chemical dehydration and reduction of the disaccharide peptide to γ-aminobutyric acid is further proof for the presence of an isoglutaminyl residue in the *endo* position.

Except for an isoglutaminyl residue in place of a γ-glutamyl residue, the tetrapeptide subunit has the sequence previously proposed for the nucleotide pentapeptide, uridinediphospho-N-acetylmuramyl-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala, that *S. aureus* utilizes as a precursor for cell wall peptidoglycan biosynthesis (Ito and Strominger, 1964). Analysis of an *endo-N*-acetylmuramidase digest of cell walls of *S. aureus* (Ghuysen *et al.*, 1965c; Tipper and Strominger, 1965) indicated that such a pentapeptide accounts for only a small percentage of the peptide subunits. Cell walls of *S. aureus* which contain a high proportion of such an amidated pentapeptide subunits, substituted by uncross-linked pentaglycine chains, were prepared according to the technique of Tipper and Strominger (1965), by growing cells of *S. aureus* in the presence of sublethal doses of penicillin. The disaccharide pentapeptide pentaglycine was isolated after *endo-N*-

acetylmuramidase degradation of the cell walls with an actual yield of 18%, in terms of disaccharide units. After enzymatic degradation as indicated above, the residual peptide Glu-Lys-Ala-Ala had chromatographic properties identical with those of the synthetic tetrapeptide isoglutaminyllysylalanylalanine, in agreement with previous observations of Tipper and Strominger (1965) according to which the amidation of the nucleotide pentapeptide precursor takes place before closure of the peptide bridges and is not inhibited by penicillin. These data are in accord with the structure previously deduced for the disaccharide-pentopeptide-pentaglycine from analysis of its constituents and end groups (Tipper and Strominger, 1966; Tipper, 1966).

In the case of *M. roseus* R 27, degradation of the cell walls yielded not only the tetrapeptide subunit L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine but also a tripeptide subunit L-alanyl-D-isoglutaminyl-L-lysine, the structure of which has been thoroughly established by means of the methods summarized above. The dipeptide remaining after removal of the terminal L-alanine of the tripeptide subunit was found to be chromatographically identical with the synthetic dipeptide isoglutaminyllysine in a system which separates the two dipeptides α-Glu-Lys and γ-Glu-Lys and their respective amide derivatives. Tripeptide subunits and tetrapeptide subunits probably occur in a ratio 1:3 in the *M. roseus* cell walls as they are prepared (Petit *et al.*, 1966). A solution of F_1 *endo-N*-acetylmuramidase-degraded cell walls of *M. roseus* was fractionated by filtration on the combined Sephadex G-50 and G-25 columns used for the isolation of the monomeric disaccharide pentapeptide pentaglycine (see Figure 5). Analyses of the fractions showed that the tripeptide subunits do not occur in the cell walls as uncross-linked monomeric substituents of the polysaccharide chains. They can be located only at the C-terminus end of tetramer peptide units.

The yield of cell wall tripeptide from normal *S. aureus*, or of tripeptide plus dipeptide from *M. roseus* amounted to about 40% of the quantity to be expected on the basis of the glutamic acid content of the intact cell walls. This value does not include those peptide subunits (1) which could not be isolated in the form of disaccharide peptide owing to their association with the nonpeptidoglycan components of the cell walls (15–25%); (2) which were consumed by analyses performed to follow the enzymatic degradations and to monitor the columns; (3) which were not transformed into *di*-DNP derivatives (some peptide subunits were recovered as mono-DNP derivatives). It can thus be concluded that these peptides are the basal subunits of at least a major portion of the respective peptidoglycans.

The actual yield for the isolation of the tripeptide in the case of *S. pyogenes* is much lower, about 12%. Evidently, this is due to structural peculiarities of the glycan portion of the peptidoglycan which make it partially resistant to degradation into disaccharide units through the action of the F_1 *endo-N*-acetylmuramidase (Muñoz *et al.*, 1966). All data relative to the degradations of the peptide moiety are consistent with the hypothesis that a large part of this polymer consists of tetrapeptides interlinked through D-Ala-L-Ala-L-Ala-ϵ-Lys bridges to form oligomers of small sizes.

A ratio of lysine to hydroxylysine 10:1 has been found in *S. pyogenes* cell walls as well as in the purified disaccharide peptide fraction (see Table II and Cell Walls in Materials). However, all of the other unusual amino acids seen in minor amounts in the undegraded cell walls occurred only as trace contaminants in the purified disaccharide peptide. This suggests that *S. pyogenes* is capable of utilizing hydroxylysine to build up at least a minor part of its peptidoglycan; about 10% of the peptide subunits would contain this unusual diamino acid. *Streptococcus faecalis* is the only bacterium which has been reported to contain hydroxylysine in place of lysine in its cell wall peptidoglycan, the former being utilized by the cells only in the case of lysine deprivation (Shockman *et al.*, 1965).

S. aureus, *M. roseus*, and *S. pyogenes* present striking similarities as far as the peptide subunits of their cell wall peptidoglycans are concerned. It is, however, too early to extend this conclusion to all cell wall peptidoglycans. Particularly, the presence of an amide function of the α-carboxyl group of the glutamic acid residue, which is not involved in linkage peptide, may not be a general property of bacterial cell walls. For example, the α-carboxyl group of glutamic acid in cell walls of *M. lysodeikticus* is glycine substituted (Tipper *et al.*, 1967a).

Finally, the substrate requirements for aminopeptidase activity, as elucidated with synthetic peptides of known structure, well explain the behavior of the cell wall peptide subunits toward this enzyme. (1) The degradation of the peptide subunits from their N-terminal ends stops as soon as N-terminal glutamic acid residues are exposed because glutamic acid is in the D form and because it is engaged in amide linkage to lysine through its γ-carboxyl group. (2) Deamidation of the α-amide in the peptide subunits cannot occur again because glutamic acid is in the D form. (3) The opened peptide bridges are readily degraded by the aminopeptidase until the last amino acid residue has its N-terminal group exposed. Indeed, the bridges in the three cell walls examined do not contain any D-amino acid and all linkages within the bridges are peptide bonds between α-amino acids. However, the last residue, which is linked to lysine, is removed at low rates because now the ϵ-amino group of lysine is involved in the amidic linkage to be split.

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