Structure of the Cell Wall of *Staphylococcus aureus*. IX. Mechanism of Hydrolysis by the L_{11} Enzyme*

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ABSTRACT: The mechanism of solubilization of the cell walls of *Staphylococcus aureus* by the L_{11} enzyme from *Flavobacterium* has been investigated. This enzyme is an endopeptidase which catalyzes the hydrolysis of both D-alanyl-glycine and glycyl-glycine linkages (in the ratio 3 to 7) in the interpeptide bridge which interconnects peptidoglycan strands in the cell wall of *S. aureus* strain Copenhagen. In intact cell walls of this strain *N*-acetylmuramyl-L-alanine linkages are also cleaved, but this linkage is not hydrolyzed in some other substrates for this enzyme. After the interpeptide bridges have been opened a relatively slow secondary hydrolysis results in the liberation of small glycine peptides. This enzyme thus belongs to a group of enzymes which are known to catalyze the hydrolysis of the pentaglycine bridge in *S. aureus*.

In recent years it has become increasingly apparent that lysozyme, the first known bacteriolytic enzyme, is only one member of a large family of bacteriolytic enzymes. Lysozyme is an acetylmuramidase (a carbohydrase) but recently a number of bacteriolytic peptidases which act on the unusual peptide linkages in bacterial cell walls have been described (see Strominger and Ghuysen, 1967, for a review). The L_{11} enzyme (Kotani *et al.*, 1959a,b; Kato *et al.*, 1960, 1962, 1963) is one member of this family of bacteriolytic peptidases. It is excreted into the culture medium by a *Flavobacterium*, isolated by enrichment culture employing cell walls in the selecting medium and catalyzes the lysis of whole cells or cell walls of *Staphylococcus aureus* or *Micrococcus lysodeikticus*, but not of a number of other bacteria. Purified preparations of this enzyme were shown to release ninhydrin-positive fragments from the cell wall of *S. aureus* but little or no reducing sugar (Kato *et al.*, 1962). The purpose of the present paper is to define the linkages which are hydrolyzed by this enzyme.

Materials and Methods

L_{11} Enzyme. A preparation purified by hydroxylapatite chromatography containing 35 units/ml and a specific activity of about 2500 units/mg of protein (Kato *et al.*, 1962) was employed.

Bacterial Cell Walls. Cell walls of *S. aureus* strain Copenhagen or strain Newman 1 were both employed. To remove ester alanine the cell walls were treated briefly at pH 9 (Tipper *et al.*, 1967). At this pH the ester alanine is removed within a few minutes and little or no hydrolysis of the *O*-acetyl esters in the cell wall occurs.

General Methods. All of the methods employed in the study of bacteriolytic enzymes in this laboratory have been described in detail (Ghuysen et al., 1966).

Results

Liberation of N- and C-Terminal Glycine and Alanine during Lysis of the Alanine-Free Cell Walls of S. aureus Strain Copenhagen. Measurement of release of total amino groups and turbidity during lysis, indicated that the solubilization of the wall by the L_{11} enzyme was due to the cleavage of a single peptide bond (Figure 1). The total increase in new amino groups was somewhat more than one residue per glutamic acid residue in the wall. The reduction of turbidity of the cell wall suspension paralleled the appearance of free amino groups, but it should be noted that solubilization (*i.e.*, decrease of turbidity) was 50% complete when only about 0.2 peptide bond had been cleaved. At the time of complete solubilization, only 0.75 peptide bond had been cleaved. Thus, at all times the per cent solubilization was somewhat greater than the per cent of peptide bonds cleaved.

There was no detectable increase in the reducing power of the preparation during solubilization (Figure 1). Thus, this enzyme preparation contains no detectable carbohydrase acting on the polysaccharide backbone of the cell wall.

Simultaneous measurements of the nature of the N- and C-terminal groups released were carried out. The N-terminal residue released was entirely glycine (Figure 2), indicating that L_{11} enzyme is an endopeptidase which catalyzes the hydrolysis of the pentaglycine bridge, which interconnects peptidoglycan strands in the cell wall. Approximately 1.1 new N-terminal glycine residues appeared.

During the first 4 hr, both C-terminal alanine and

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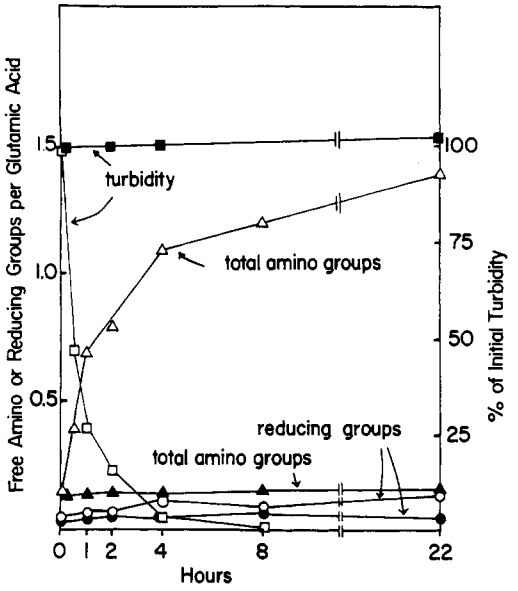


FIGURE 1: Lysis of alkali-treated cell walls of *S. aureus* strain Copenhagen by the L_{11} enzyme. Cell walls (100 μl, 10 mg/ml), 20 μl of the L_{11} enzyme, 20 μl of 0.05 M phosphate buffer (pH 6.8) containing 1.1% NaN_3, and 60 μl of water were mixed and incubated at 37°. Aliquots were removed at various intervals for measurements of turbidity, release of total amino groups, and of reducing groups. (□-△-○) Digest and (■-▲-●) control.

C-terminal glycine were released, the former more rapidly than the latter (Figure 3). At 4 hr, about 0.75 peptide bond had been split, and solubilization of the cell wall was complete (Figures 1–3). Thereafter, the only new C-terminal groups which were liberated during hydrolysis of the remaining 0.3 peptide bond were C-terminal glycine. At the completion of the hydrolysis, 0.73 C-terminal glycine and 0.37 C-terminal alanine residue had been liberated.

Configuration of the C-Terminal Alanine Released by L_{11} Enzyme. For this purpose, a lysate was prepared with L_{11} enzyme on a somewhat larger scale and subjected to hydrazinolysis. The liberated alanine was purified by chromatography on a small column of Dowex 50. Determination of the configuration of this isolated alanine by microenzymatic methods indicated that the entire sample was D-alanine (Table I).

Comparison of the Lysis of Intact Cell Walls and Alanine-Free Cell Walls of S. aureus Strain Copenhagen and Strain Newman by the L_{11} Enzyme. Several experiments had indicated that the lysis of intact cell walls might not proceed in exactly the same manner as the lysis of ester alanine-free cell walls. A careful comparison was therefore made of the lysis of both types of cell walls, from two strains of *S. aureus*. Strain Copenhagen is the strain which has been extensively studied in this laboratory, while strain Newman was employed in Japan in the original studies of the L_{11} enzyme.

The lysis of ester alanine-free cell walls of both strains, and of the intact cell walls of strain Newman, proceeded in an identical manner with liberation of N-terminal glycine only (Figures 4 and 5). However, with cell walls of strain Copenhagen, N-terminal alanine was also liberated. The liberation of N-terminal alanine in this

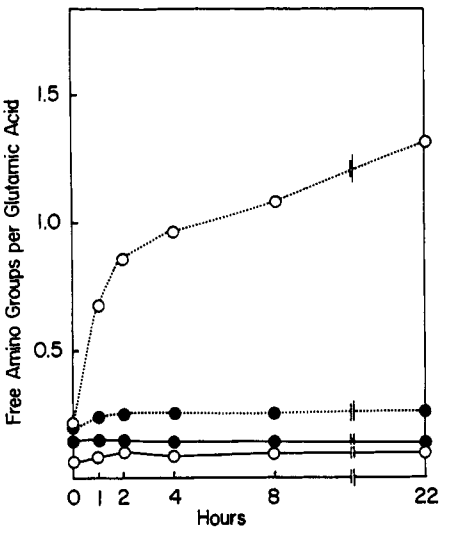


FIGURE 2: Release of N-terminal amino acids during lysis of alkali-treated cell walls of *S. aureus* strain Copenhagen by the L_{11} enzyme. Incubation mixtures were similar to those described in Figure 1. Aliquots were removed at intervals for estimation of N-terminal amino acids and of C-terminal amino acids (see following figure). (○) Digest and (●) control (○- - -○, ●- - -●) Glycine and (○——○, ●——●) alanine.

case ceased when about 0.5 residue had been liberated, and at the time when solubilization was complete. Liberation of N-terminal glycine continued after this time, and appeared to be somewhat greater in the intact cell walls than in the ester alanine-free cell walls.

Liberation of C-terminal amino acids in the four cases was identical with that previously observed with the alanine-free cell walls of strain Copenhagen (Figure 3). Thus, the liberation of the additional N-terminal alanine in the intact cell wall of strain Copenhagen was not accompanied by the liberation of a C-terminal amino acid, and hence was likely to be due to cleavage of the acetylmuramyl-L-alanine linkage.

An experiment was also carried out with a cruder preparation of the L_{11} enzyme (first ammonium sulfate fraction, Kato *et al.*, 1962) to see if this might contain a

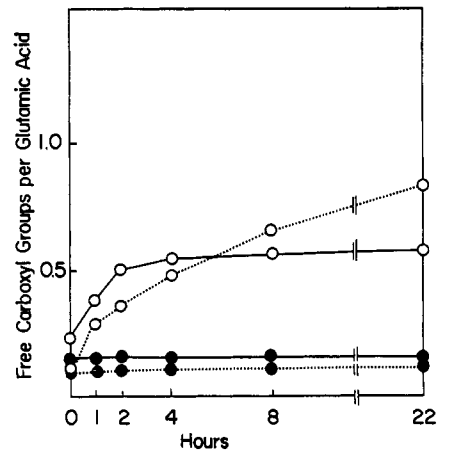


FIGURE 3: Release of C-terminal amino acids from alkali-treated cell walls of *S. aureus* strain Copenhagen. See legend to Figure 2. (○) Digest and (●) control. (○- - -○, ●- - -●) Glycine and (○——○, ●——●) alanine.

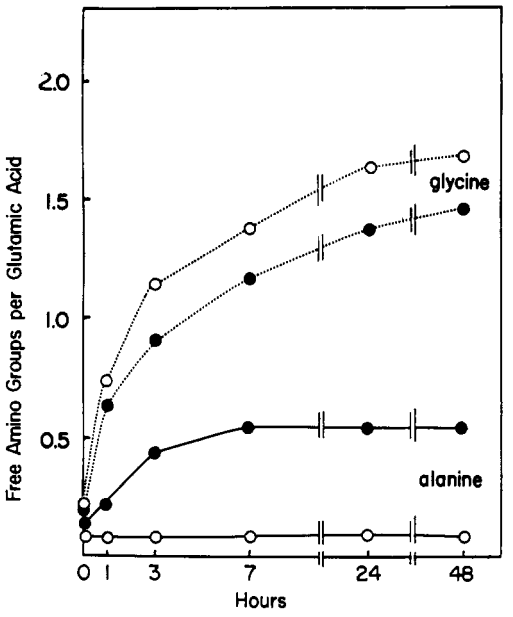


FIGURE 4: Comparison of release of N-terminal amino acids from intact cell walls and from alkali-treated cell walls of *S. aureus* strain Copenhagen. (○) Alkali-treated cell walls and (●) intact cell walls.

larger amount of the enzyme liberating N-terminal alanine. No additional activities were observed in this cruder fraction, and in particular, the relative rate and extent of liberation of N-terminal alanine was similar to that observed with the more purified fraction.

Configuration of the Liberated N-Terminal Alanine Residue. The configuration of this residue was determined by a difference method (Table II). Cell wall lysed by the L_{11} enzyme was divided into two aliquots. One aliquot (control sample) was subjected to acid hydrolysis and the amounts of D- and L-alanine in it were measured. The second aliquot was dinitrophenylated. It was then acidified and extracted with ether. The DNP-alanine in this ether extract is the D-alanine ester of the teichoic acid; this labile ester is liberated as the free DNP-amino acid under the alkaline conditions of dinitrophenylation. The material remaining was then hydrolyzed in acid and again extracted with ether; the DNP-alanine extracted is the N-terminal alanine of the peptide. Finally, the D- and L-alanine remaining as free amino acids in this hydrolysate were measured. The D-alanine which disappeared on dinitrophenylation was equivalent to the D-alanine ester of the teichoic acid. In addition, L-alanine disappeared in an amount approximately equal to the DNP-alanine of the peptide. Thus, the N-terminal alanine liberated by the L_{11} enzyme is an L-alanine residue. No L-alanine is available to dinitrophenylation when these cell walls were solubilized by an acetylmuramidase, indicating that the L_{11} is not simply unmasking free amino groups of L-alanine.

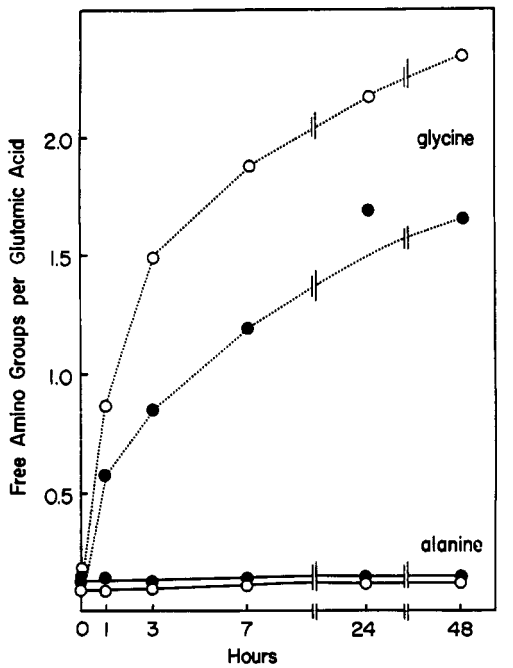


FIGURE 5: Comparison of release of N-terminal amino acids from intact cell walls and from alkali-treated cell walls of *S. aureus* strain Newman. The incubation was carried out as described in Figure 1. (○) Alkali-treated cell walls and (●) intact cell walls.

TABLE I: Configuration of C-Terminal Alanine Released after Lysis with the L_{11} Enzyme.[a]

Method	Amt in Sample (μmoles/ml)
Total Ala	0.332
D-Ala	0.342
L-Ala	0.000

[a] The cell walls of *S. aureus* strain Copenhagen (3.2 mg) from which the ester D-alanine had been removed were incubated with 80 μl of the L_{11} enzyme in 0.8 ml of phosphate buffer (pH 6.8) at 37° for 21 hr. The sample was taken to dryness and 200 μl of dry hydrazine was added. After heating for 8 hr at 100° in a sealed tube the sample was again dried over H_2SO_4. Water (3.5 ml) was added and the sample was extracted twice with 0.7 ml of benzaldehyde and then with ethyl acetate–ether. The aqueous phase was taken to dryness. Analysis of a small sample by thin-layer chromatography indicated that glycine and alanine were present in approximately equal amount. The sample was then purified on a column of Dowex 50 and total alanine and D- and L-alanine were then measured (see Ghuysen *et al.*, 1966).

Unsuccessful Efforts to Explain the Different Susceptibility of the Cell Walls of the Two Strains to the L_{11} Enzyme. Various efforts have been made to inhibit the release of N-terminal alanine from walls of strain Copenhagen by addition of ester alanine-free teichoic acid preparations, or to stimulate the liberation of N-terminal alanine from ester alanine-free walls of strain Copenhagen, by addition of teichoic acid preparations containing ester alanine. No modification of the usual responses was obtained in this way.

TABLE II: Configuration of N-Terminal Alanine Released after Lysis with the L_{11} Enzyme.[a]

	Free DNP-Ala	D-Ala	DNP-Ala after Acid Hydrolysis	L-Ala
Control sample		180		107
Dinitrophenylated sample	84[b]	88	71[a]	44
Δ		92[b]		63[c]

[a] Intact cell walls (8.89 mg) were incubated with 20 μl of L_{11} enzyme in total volume of 240 μl of 0.004 M phosphate buffer (pH 6.8) for 8 hr at 37°. After incubation the reaction mixture was subjected to dinitrophenylation and the DNP-amino acids were extracted with ether. Then the aqueous phase was heated at 100° for 8 hr in 6 N HCl. DNP-amino acids liberated by the acid hydrolysis were again extracted with ether. HCl was removed from the aqueous phase *in vacuo* and the amounts of D- and L-alanine in this residual material were determined. In the control sample cell walls were subjected to acid hydrolysis and the amounts of D- and L-alanine in it were measured directly. For details of Methods, see Ghuysen *et al.* (1966). [b] The free DNP-Ala after dinitrophenylation is the ester D-Ala of the teichoic acid. The disappearance of D-Ala on dinitrophenylation is equivalent to it. Thus, there is no additional D-Ala in peptide linkage which is susceptible to dinitrophenylation. [c] About two-thirds of the L-Ala disappears on dinitrophenylation and is equivalent in amount to the DNP-Ala formed from material in peptide linkage.

Measurement of the *O*-acetyl content of the walls indicated that walls of strain Copenhagen contained 0.6 *O*-acetyl residue/acetylmuramic acid residue, before removal of ester alanine, and 0.4 residue after removal of ester alanine. Similarly walls of strain Newman contained 0.6 residue before and 0.6 residue after, removal of ester alanine. Thus, no striking differences in content of *O*-acetyl ester were observed. However, walls of strain Copenhagen contained more ester D-alanine residues (0.8 residue/glutamic acid residue) than did walls of strain Newman (0.4 residue), which may be a reflection of a difference in the extent of esterification or in the amount of teichoic acid present. This difference in the walls of the two strains does not readily explain the difference in susceptibility of the acetylmuramyl-L-alanine linkage to enzymatic attack.

Hydrolysis of Soluble Glycopeptides by the L_{11} Enzyme. When the cell wall of strain Copenhagen is solubilized by the B enzyme from *Chalaropsis* (an acetylmuramidase), a soluble glycopeptide (disaccharide units linked to a cross-linked polypeptide) is formed. This polymer (GP_1) has been extensively studied (Ghuysen *et al.*, 1963, 1965b). Treatment of it with the L_{11} enzyme resulted in liberation of N-terminal glycine and C-terminal alanine and glycine in the same proportion as from the intact cell wall (Figures 6 and 7). The rate of liberation of C-terminal alanine was, however, less than the rate of liberation of C-terminal glycine and appeared to parallel it throughout the course of hydrolysis. No N-terminal alanine was released during hydrolysis of this substrate.

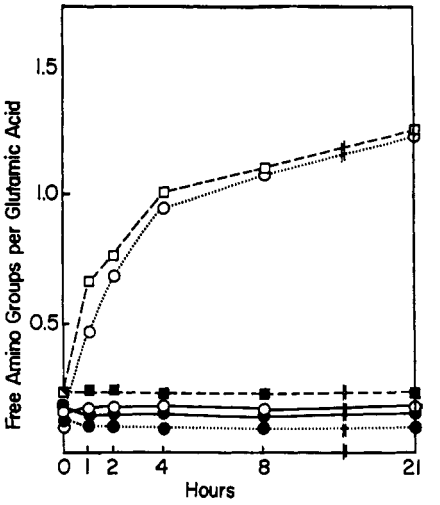


FIGURE 6: Release of N-terminal amino acids from the soluble glycopeptide obtained from *S. aureus* strain Copenhagen. The soluble glycopeptide GP-1 (8.8 mg/ml, 400 μl), prepared with the B enzyme from *Chalaropsis*, was mixed with 80 μl of the L_{11} enzyme, 80 μl of 0.05 M phosphate buffer (pH 6.8), and 240 μl of water. Incubation was carried out at 37°. (□, ○) Digest and (■, ●) control. (□- - -□, ■- - -■) Total amino groups, (○· · ·○, ●· · ·●) glycine, and (○——○, ●——●) alanine.

Further degradation of GP_1 by endopeptidase 1 from *Streptomyces albus* G resulted in cleavage of about 20% of the glycine bridges with liberation of N-terminal glycine and an equivalent amount of C-terminal alanine (Ghuysen *et al.*, 1965a,b). The hydrolysis then ceased. No C-terminal glycine was liberated by this enzyme. The product of this hydrolysis was called GP_2. Treatment of

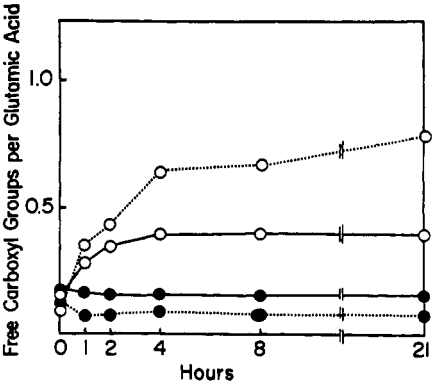


FIGURE 7: Release of C-terminal amino acids from the soluble glycopeptide from *S. aureus* strain Copenhagen on treatment with the L_{11} enzyme. See legend to Figure 6.

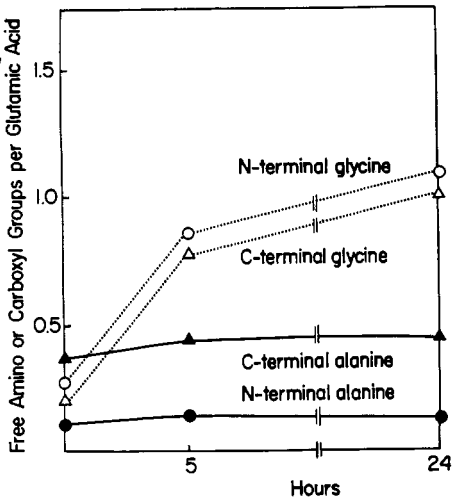


FIGURE 8: Release of N-terminal and C-terminal amino acids from a further degraded soluble glycopeptide from *S. aureus* strain Copenhagen on treatment with the L_{11} enzyme. The soluble glycopeptide GP-1 employed in Figures 6 and 7 was further degraded by endopeptidase 1 from *S. albus* G (Ghuysen *et al.*, 1965a,b). The product GP-2 (10 μl) was mixed with 12 μl of the L_{11} enzyme containing 0.05 M phosphate buffer (pH 6.8) and 44 μl of water and incubated at 37°.

GP_2 with L_{11} enzyme resulted in liberation of equivalent amounts of N-terminal glycine and C-terminal glycine (Figure 8). No further liberation of C-terminal alanine occurred beyond that which had been liberated by endopeptidase 1. Again, no N-terminal alanine was liberated during hydrolysis of this substrate of the L_{11} enzyme.

Isolation of Product from Large-Scale Lysis of Cell Walls of Strain Copenhagen. In order to obtain confirmatory evidence for the proposed mechanism of lysis, the lysis was carried out on a large scale. Initially the lysate was fractionated by passing it over a column of Sephadex G-25 which separated the polymers in the lysate from low molecular weight products and phosphate buffer (Figure 9). The high molecular weight material was further fractionated by passing it over a

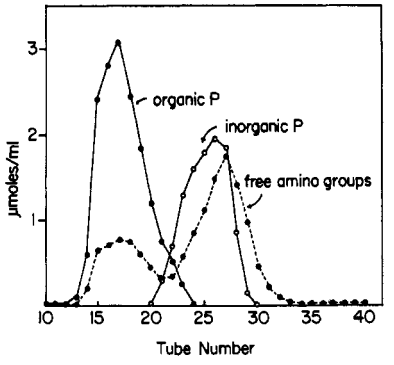


FIGURE 9: Separation of the products of a large-scale lysis of cell walls of *S. aureus* strain Copenhagen with the L_{11} enzyme on a column of Sephadex G-25. Cell walls (200 mg) were mixed with 2 ml of L_{11} enzyme and 8 ml of water and incubated for 24 hr at 37°. The lysate was applied to a column of Sephadex G-25 (2 × 90 cm) and followed by water. Fractions of 9.5 ml were collected every 30 min.

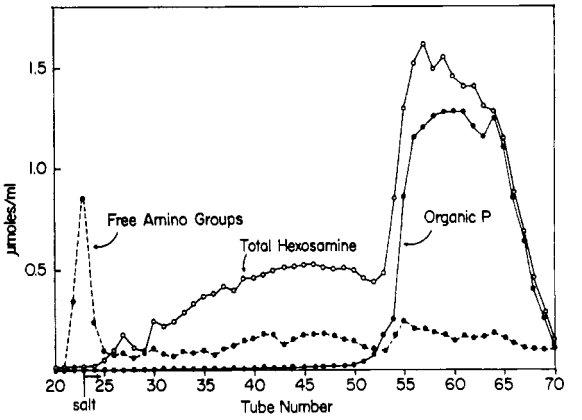


FIGURE 10: Separation of glycopeptide and teichoic acid–glycopeptide complex on a column of ECTEOLA-cellulose. Fractions 14–20 (Figure 10) were lyophilized and redissolved in a small volume of water. This material was applied to a column of ECTEOLA-cellulose and developed as described previously (see Figure 3 in Tipper *et al.*, 1967). The gradient of LiCl was started at tube 150.

column of ECTEOLA-cellulose, followed by elution with water and then by a gradient of LiCl (Figure 10). A small amount of peptide peak came straight through this column. It was presumably material identical with the peptide fraction in the Sephadex G-25 column. It contaminated the polymers since it was not completely separated from them in the Sephadex G-25 column. Water slowly eluted a glycopeptide fraction (which contains both amino sugars and peptides) and then the LiCl gradient eluted a teichoic acid–glycopeptide complex. There were, therefore, three fractions to consider: the glycopeptide, the teichoic acid–glycopeptide complex, and the peptides.

Analyses of the individual peptide fractions (Table III) indicated that it was a mixture of materials. The early fractions (Figure 9, fractions 23–25) were composed mainly of peptides containing all of the cell wall amino acids in the molar ratio of Glu 1:Ala 2:Lys 1:Gly 4. These fractions contained, per glutamic acid residue, 1 N-terminal glycine and 1 N-terminal alanine residue and a total of 1 C-terminal group, two-thirds of which was glycine and one-third of which was alanine. Thus, the material in these fractions had the analyses expected for the small peptide subunit of the wall except for the fact that it contained 4 rather than 5 glycine residues. Later fractions (Figure 9, tubes 26–28) yielded similar analyses except that they contained increased amounts of glycine (ratio Glu 1 : Gly 10) and an excess of both C-terminal and N-terminal groups. In addition to the components of the early fractions, these fractions contained a small glycine peptide, probably diglycine, as evidenced by its position in thin-layer chromatography. Still later fractions (Figure 9, tubes 28–30) contained a large amount of free alanine (measured as DNP-alanine, extractable with ether after dinitrophenylation). This latter substance arises from the labile D-alanine ester of the teichoic acid by a slow nonenzymatic hydrolysis during incubation with the L_{11} enzyme at pH 7. Thus, it was apparent from the study of these fragments that the relatively slow liberation of N-terminal glycine above a

TABLE III: Analyses of Peptides, Glycopeptide, and Teichoic Acid–Glycopeptide Complex.[a]

	MurNAc	GlcNAc	Glu	Gly	Ala	Lys	N-Terminal Ala	N-Terminal Gly	Excess N-Terminal Gly	C-Terminal Ala Plus Gly	Excess C-Terminal	Free Ala
Peptides[b]												
Fraction 23	0	0	0.85	3.2	2.8	1.1	0.6	0.5	0	0.6	0	0
Fraction 26	0	0	0.98	9.1	2.2	1.2	1.0	1.9	0.9	1.1	0.2	0
Fraction 29	0	0	0.60	5.3	1.8	0.68	0.5	0.9	0.3	1.0	0.4	0.9
Glycopeptide[c]	1.24	1.19	0.20	0.77	0.43	0.24	0.03	0.06		0.11		0
Teichoic acid–glycopeptide complex[c]	0.39	1.41[d]	0.07	0.22	0.27[d]	0.06	0.01	0.02		0.08		0.12

[a] The peptide fractions analyzed are those from Figure 9 and the glycopeptide and teichoic acid–glycopeptide complex are from Figure 10. [b] Data are expressed as micromoles per milliliter in the sample analyzed. [c] Data are expressed as micromoles per milligram of material. [d] The total phosphate was 1.2. Most of the GlcNAc and Ala are components of the teichoic acid portion of the complex rather than of the glycopeptide.

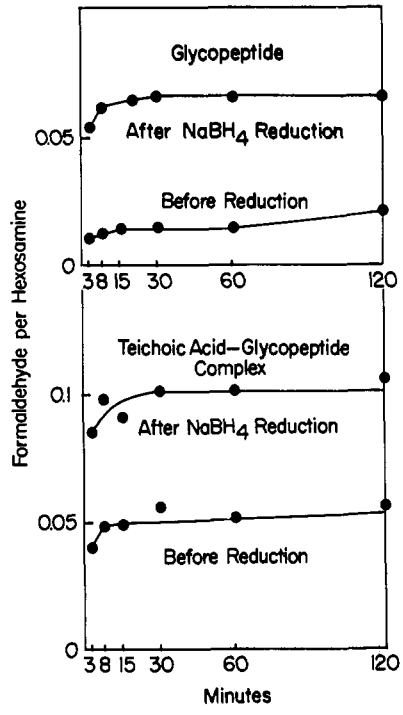


FIGURE 11: Release of formaldehyde during periodate oxidation of the glycopeptide and teichoic acid–glycopeptide complex before and after reduction with $NaBH_4$. The method was essentially that employed by Tipper *et al.* (1967).

value of 1 N-terminal/glutamic acid (Figure 4) was due to a second point of cleavage in the glycine bridge, resulting in liberation of a small glycine peptide. No hexosamine was detectable in any of the fractions.

Analyses of the soluble glycopeptide (Figure 10, tubes 27–51) indicated that it was a polysaccharide containing equal amounts of acetylglucosamine and acetylmuramic acid and about 0.2 peptide unit/disaccharide (Table III). This peptide had a composition similar to that found in the free peptide; the reduced amount of glycine relative to glutamic acid was again noteworthy. The *O*-acetyl content per disaccharide (0.3) was less than that found in the intact cell wall, and the chain length measured by formaldehyde production after reduction with $NaBH_4$ was about 20 (Figure 11).[1]

Analyses of the teichoic acid–glycopeptide complex (Table III) indicated that its glycopeptide component was similar to that described above. The chain length of the glycopeptide was 14–15 (estimated from the increase in formaldehyde production on $NaBH_4$ reduction) (Figure 11). Its teichoic acid had a greatly reduced amount of ester alanine (0.1/phosphate), as expected from the presence of free alanine in the peptide fraction. The number-average chain length of the teichoic acid was about 20. This value was estimated from the formaldehyde without $NaBH_4$ reduction, which is derived from the ribitol end group of the teichoic acid. This

[1] The formaldehyde is produced from C-6 of the sugar at the reducing end of the glycan. Any *O*-acetyl ester present at this position would block formaldehyde production. Virtually all of the *O*-acetyl groups are, however, removed by the procedure employed in the determination (Tipper *et al.*, 1967).

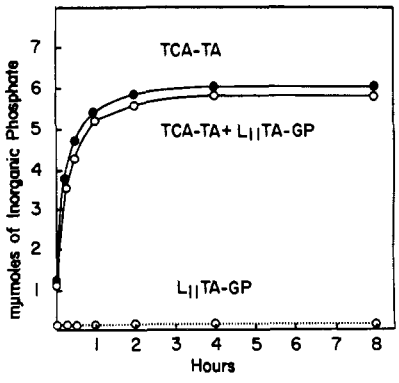


FIGURE 12: Release of phosphomonoester groups from the teichoic acid–glycopeptide complex by *Escherichia coli* phosphomonoesterase. The method was essentially the same as that employed by Tipper *et al.* (1967). The sample of teichoic acid–glycopeptide complex (L_{II} TA–GP) was assayed alone and after mixture with a sample of teichoic acid prepared after extraction of cell walls with trichloroacetic acid (TCA–TA). The mixing experiment assures both that the release of phosphomonoester groups was complete and that there was no inhibitor present in L_{II} TA–GP.

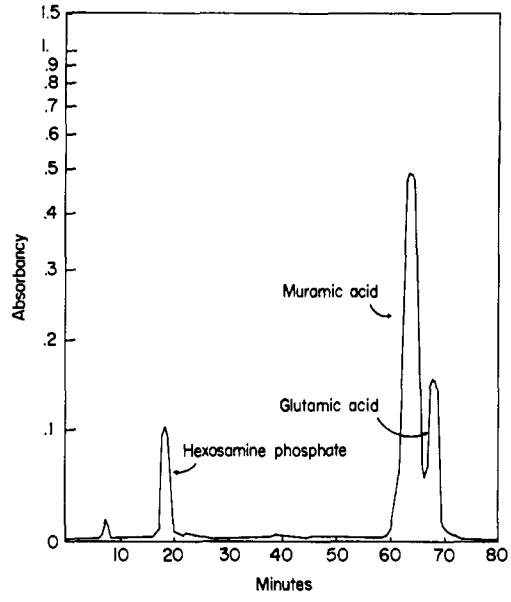


FIGURE 13: Presence of hexosamine phosphate in the teichoic acid–glycopeptide complex. A sample was hydrolyzed in 6 N HCl and components were separated on a Beckman–Spinco amino acid analyzer.

teichoic acid–glycopeptide complex contained no detectable phosphomonoester group (Figure 12). One other feature of its analysis is especially noteworthy. After acid hydrolysis of the teichoic acid–glycopeptide complex an unknown material detected with ninhydrin was eluted from the long column of the Beckman–Spinco amino acid analyzer near the position of serine phosphate (Figure 13). No similar material was present in the acid hydrolysate of the glycopeptide. The material contained approximately equal amounts of amino groups, hexosamine, and organic phosphate, and no amino acids could be detected after further acid hydrolysis. The amount of the material present was 1/30–40 phosphates. We assume that this material represents the linkage point of the teichoic acid to the glycopeptide; it is being investigated further.

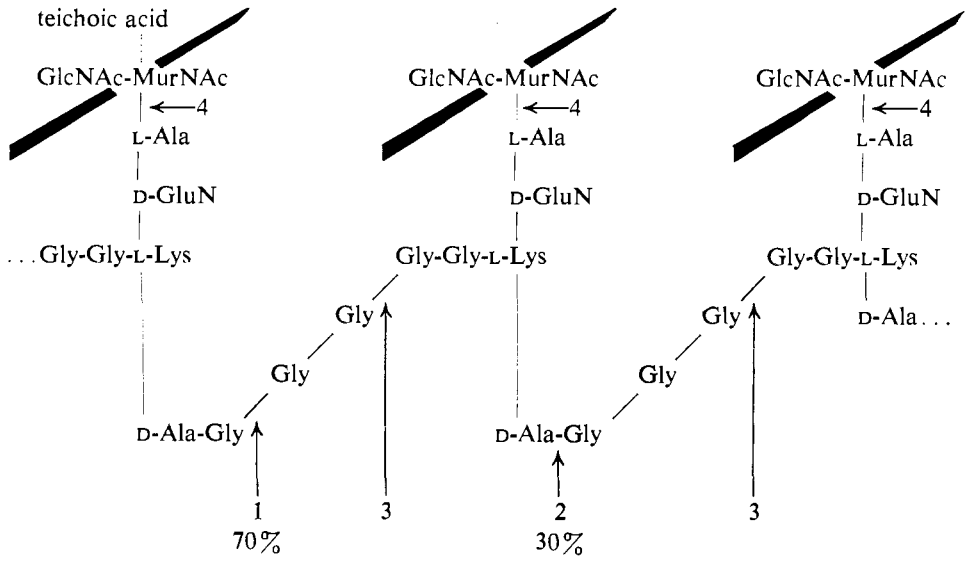
Discussion

Thus the bacteriolytic enzyme, L_{II} from *Flavobacterium*, studied here belongs to a group of endopeptidases which solubilize bacterial cell walls as a consequence of hydrolysis of the interpeptide bridges which interconnect peptidoglycan strands (see Strominger and Ghuysen, 1967). In addition to the L_{II} enzyme the two endopeptidases from *S. albus* G (Ghuysen *et al.*, 1965a,b) lysostaphin from a *Micrococcus* species (Schindler and Schuhardt, 1964; Browder *et al.*, 1965), the AL_1 enzyme from *Myxobacterium* (Ensign and Wolfe, 1965, 1966; Tipper *et al.*, 1967), a staphylococcol virolysin (Doughty *et al.*, 1966), and an enzyme from a *Pseudomonas* species (Lache *et al.*, 1966) all catalyze the hydrolysis of the pentaglycine bridge in *S. aureus*. The observation that the L_{II} enzyme and several of the other enzymes in this group catalyze the hydrolysis of this bridge with the simultaneous liberation of C-terminal D-alanine and N-terminal glycine was important in establishing that the interpeptide bridge is connected to the terminal D-alanine residue of the tetrapeptide subunit of the peptidoglycan, a fact which played a prominent part in establishing the mode of action of penicillins (Tipper and Strominger, 1965).

The precise nature of the linkages cleaved in various substrates by the L_{II} enzyme is of some interest. In cell walls of *S. aureus*, each pentaglycine bridge is hydrolyzed once, 30% of the bridges at a D-alanyl-glycine linkage and 70% at a glycyl-glycine linkage. A further hydrolysis of glycyl-glycine linkages then results in a relatively slow liberation of glycine peptides.[2] SA endopeptidase I from *S. albus* G catalyzes the hydrolysis of only about 30% of the interpeptide bridges in GP-1, a soluble product derived from the cell wall of *S. aureus;* this hydrolysis is entirely at a D-alanyl-glycine linkage (Ghuysen *et al.*, 1965a,b). GP-2, the product obtained from the hydrolysis of GP-1 by SA endopeptidase I, is a substrate for further hydrolysis by the L_{II} enzyme. However, the L_{II} enzyme catalyzes a cleavage of only glycyl-glycine linkages in this substrate. One possible interpretation of these data is that there are two different kinds of pentaglycine bridges in the cell wall of *S. aureus*, *e.g.*, positionally different, vertical and horizontal bridges, and that one of these types of bridges is cleaved either by the L_{II} enzyme or by SA endopeptidase I at a D-alanyl-glycine linkage, but that the other type of bridge is hydrolyzed by the L_{II} enzyme at a glycyl-glycine linkage. Another possible interpretation, however, is that whether a D-alanyl-glycine or a glycyl-glycine linkage is split depends upon the size of the substrate. In the large macromolecular substrate the L_{II} enzyme can catalyze the hydrolysis of both types of linkages but, in smaller substrates, resulting from prior solubilization on treatment with another enzyme, then only glycyl-

[2] The nature of the peptides released has now been investigated in detail (Kato *et al.*, 1968).

SCHEME 1: Schematic Representation of Structure of Cell Wall of *S. aureus* Strain Copenhagen and Points of Cleavage by L_{11} Enzyme.[a]



[a] Glycine bridges are split at 1 to yield N- and C-terminal glycine or at 2 to yield N-terminal glycine and C-terminal D-alanine. A secondary split at 3 yields small glycine peptides. Hydrolysis at 4 results in liberation of N-terminal alanine. The teichoic acid–glycopeptide complex and glycopeptide result from hydrolysis at 1 or 2. Carbohydrate-free peptides are released by further hydrolysis at 3 and 4. The heavy line represents the glycan of which only one disaccharide unit is shown.

glycine linkages can be hydrolyzed. Substrate size appears to play an important role in the hydrolysis catalyzed by SA endopeptidase I (Muñoz *et al.*, 1966). The question of whether or not there may be different types of bridges in the cell wall of one organism is of some interest (see, for example, Krulwich *et al.*, 1967).

In some types of substrates but not in others, the L_{11} enzyme can also catalyze the cleavage of *N*-acetylmuramyl-L-alanine linkages. The factors which determine whether or not this linkage is hydrolyzed are not known.

References

Browder, H. P., Zygmunt, W. A., Young, J. R., and Tavormina, P. A. (1965), *Biochem. Biophys. Res. Commun. 19*, 383.

Doughty, C. C., Shoemaker, N. L., and Mann, J. A. (1966), *Bacteriol. Proc.*, 85.

Ensign, J. C., and Wolfe, R. S. (1965), *J. Bacteriol. 90*, 395.

Ensign, J. C., and Wolfe, R. S. (1966), *J. Bacteriol. 91*, 524.

Ghuysen, J.-M., Dierickx, L., Leyh-Bouille, M., Strominger, J. L., Bricas, E., and Nicot, C. (1965a), *Biochemistry 4*, 2237.

Ghuysen, J.-M., and Strominger, J. L. (1963), *Biochemistry 2*, 1110, 1119.

Ghuysen, J.-M., Tipper, D. J., Birge, C. H., and Strominger, J. L. (1965b), *Biochemistry 4*, 2245.

Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1966), *Methods Enzymol. 8*, 685.

Kato, K., Hirata, T., Murayama, Y., Suginaka, H., and Kotani, S. (1968), *Biken's J.* (in press).

Kato, K., Kotani, S., Imanishi, M., and Amano, T. (1963), *Biken's J. 6*, 223.

Kato, K., Kotani, S., Matsubara, T., Kogami, J., Hashimoto, S., Chimori, M., and Kazekawa, I. (1962), *Biken's J. 5*, 155.

Kato, K., Matsubara, T., Mori, Y., and Kotani, S. (1960), *Biken's J. 3*, 201.

Kotani, S., Hirano, T., Kitaura, T., Kato, K., and Matsubara, T. (1959a), *Biken's J. 2*, 143.

Kotani, S., Kato, K., Matsubara, T., Hirano, T., and Higashigawa, M. (1959b), *Biken's J. 2*, 211.

Krulwich, T., Ensign, J., Tipper, D. J., and Strominger, J. L. (1967), *J. Bacteriol. 94*, 734, 741.

Lache, M., Kyskind, J. W., and Hearn, W. R. (1966), *Federation Proc. 25*, 752.

Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J.-F., Heymann, H., Bricas, E., and Lefrancier, P. (1966), *Biochemistry 5*, 3748.

Schindler, C. A., and Schuhardt, V. T. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 414.

Strominger, J. L., and Ghuysen, J.-M. (1967), *Science 156*, 213.

Tipper, D. J., and Strominger, J. L. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1133.

Tipper, D. J., Strominger, J. L., and Ensign, J. (1967), *Biochemistry 6*, 906.