

## Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. V. Isolation of Peptidases Active on the Peptide Moiety of the Cell Walls of Some Gram-Positive Bacteria\*

J. M. Ghuysen, L. Dierickx, M. Leyh-Bouille, J. L. Strominger, E. Bricas, and C. Nicot

**ABSTRACT:** Two preparations, termed peptidase 1 and peptidase 2, which hydrolyze peptide bonds in the cell walls of *Staphylococcus aureus* and *Micrococcus lysodeikticus* have been obtained from a culture filtrate of a strain of *Streptomyces albus*, selected for its high lytic activity on medium containing cell walls of *S. aureus*. These peptidases have been separated from each other

and from several other enzymes also present in the culture filtrate. Some of the properties of these preparations have been described. It is noteworthy that peptidase 1 which is virtually devoid of glycosidase activity is bacteriolytic, and kinetic data indicate that solubilization of cell walls by this preparation is the consequence of hydrolysis of peptide bonds.

At least three enzymes are involved in the lysis of bacterial cell walls by the extracellular filtrate of *Streptomyces albus* G: acetylmuramidase F<sub>1</sub> (Dierickx and Ghuysen, 1962), acetylmuramidase 32 (Ghuysen *et al.*, 1962; Ghuysen and Strominger, 1963a), and acetylmuramyl-L-alanine amidase (Ghuysen, 1961; Ghuysen *et al.*, 1962; Ghuysen and Strominger, 1963a). Other enzymes are also present in the culture filtrate of this organism as indicated by the observation of Salton and Ghuysen (1957) that both free alanine and free glycine were liberated from some bacterial cell walls by the crude filtrate. The data suggested that there were at least two peptidase activities present which were separated into fractions F<sub>1</sub> and F<sub>2</sub>.

The present paper describes the production, isolation, and characterization of these two peptidases. The use of these enzymes as tools with which to study the structure of the peptide moiety of the cell wall of *Staphylococcus aureus* is reported in the following paper (Ghuysen *et al.*, 1965).

### Materials and Methods

**Preparation of Cell Walls.** Cell walls of *Micrococcus lysodeikticus*, *Staphylococcus aureus* strain Copen-

hagen, and *Bacillus megaterium* strain KM were prepared by differential centrifugation after breaking the cells with glass beads by standard procedures. The preparations obtained were purified by treatment of the walls with trypsin and with ribonuclease (see Ghuysen and Strominger, 1963a; Tipper *et al.*, 1965).

**Preparation of the Soluble Glycopeptide from *S. aureus* strain Copenhagen.** *S. aureus* cell walls were first treated with an acetylmuramidase, either the 32 enzyme from *Streptomyces albus* G or the B enzyme from *Chalaropsis* (Hash, 1963; Tipper *et al.*, 1964b). Both enzymes have the same specificity and provide a soluble preparation (Ghuysen and Strominger, 1963a,b) containing a mixture of free glycopeptide (*i.e.*, a complex polypeptide linked through its N-terminal alanine groups to disaccharide units) and of teichoic acid-glycopeptide complex. The glycopeptide was separated from the teichoic acid-glycopeptide complex by chromatography on Ecteola-cellulose (Ghuysen *et al.*, 1965) and further purified by filtration on Sephadex G-50. This glycopeptide was immobile on paper chromatography with 1-butanol-acetic acid-water (3:1:1) as solvent. Further characterization will be reported in the following paper.

**Preparation of the *Streptomyces* Enzymes.** SEARCH FOR SUITABLE STRAINS. At least 16 of 100 different *Streptomyces* strains in the collection of the Bacteriology Department at Liège which had been isolated from various natural sources produced culture filtrates able to lyse suspensions of living staphylococci (Welsch, 1954; Bergamini *et al.*, 1954). Culture filtrates from several strains with high staphylolytic activity were concentrated by adsorption on Amberlite IRC 50 and elution (see below) and then incubated with the purified glycopeptide from *S. aureus*. Paper chromatography of these digests showed that most of the culture filtrates contained the acetylmuramyl-L-alanine amidase<sup>1</sup> and in addition a mixture of peptidases which liberated free alanine and free glycine and split the glycopeptide into

\* From the Service de Bactériologie, Université de Liège, Liège, Belgium; Department of Pharmacology, University of Wisconsin Medical School, Madison, Wis.; and the Institut de Biochimie, Faculté des Sciences, Orsay (S et O), France. Received May 10, 1965; revised June 28, 1965. This research has been financed in part by a grant (UR-E4-10-2) made by the U. S. Department of Agriculture under public law 480. Part of this work was initiated at Washington University School of Medicine and was supported by research grants from the U. S. Public Health Service (A1-01902) and National Science Foundation (G-18742).

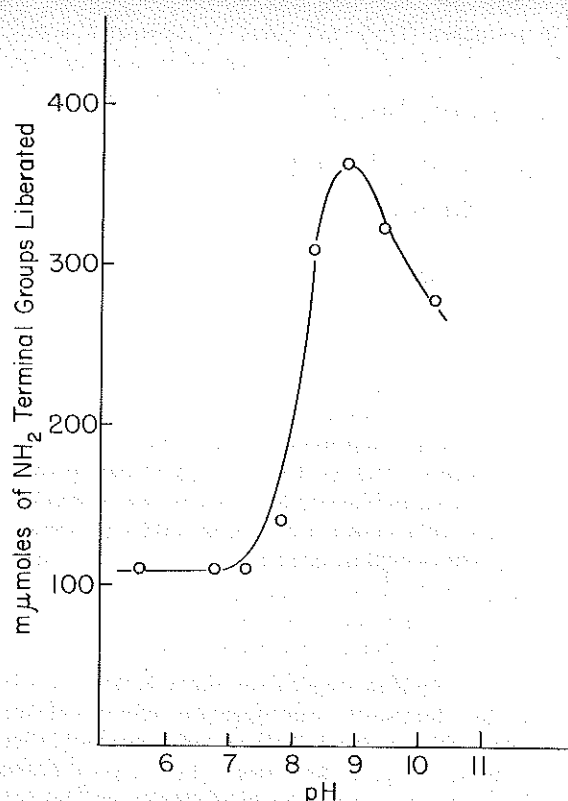


FIGURE 1: Effect of pH on the liberation of free amino groups from the glycopeptide by fraction a-9.5. Incubation was carried out in 0.01 M buffers of the following composition: pH 4.4, acetate; pH 5.6–8.05, phosphate; pH 8.2–8.8, Veronal; pH 9.2–10.2, borate. Results are expressed as mμmoles of free amino groups released from 1 mg of glycopeptide by 50 μg of enzyme protein in 8 hr at 37°. The untreated glycopeptide contained about 110 mμmoles of free amino groups/mg.

fragments which were clearly separable from the glycopeptide by paper chromatography. In order to determine which strains were most suitable for production of these peptidases, agar plates containing 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.1% MgSO<sub>4</sub>·7H<sub>2</sub>O, and either cell walls of *S. aureus* strain Copenhagen (1%) or purified glycopeptide (0.25%) were inoculated with the various strains either as isolated or after treatment with ultraviolet light. Some strains showed a 0.8–1 cm zone of lysis on each side of the mycelium after 4 days of growth at 30° on the medium containing walls. The same strains were also able to grow on the medium containing purified glycopeptide.<sup>2</sup>

<sup>1</sup> The acetylmuramyl-L-alanine amidase liberates from the glycopeptide a mixture of two disaccharides (Ghuysen and Strominger, 1963b; Tipper *et al.*, 1965). The free polypeptide still has no mobility in butanol-acetic acid-water solvent as does the intact glycopeptide. The reaction of the peptide with ninhydrin is, however, greatly increased in intensity due to the increase of terminal amino groups of alanine.

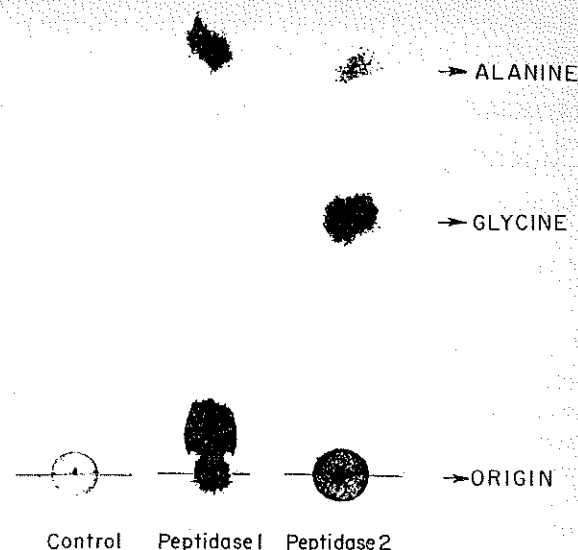


FIGURE 2: Paper chromatography of glycopeptide from *S. aureus* before and after treatment with peptidase 1 (fraction a-9.5) and with peptidase 2 (fraction C7). Incubation with fraction a-9.5 was carried out at pH 8.8 in 0.01 M Veronal buffer and with peptidase 2 at pH 8.05 in 0.01 M phosphate buffer. Aliquots of 50 μg of glycopeptide (incubated under conditions described in Figures 1 and 9) were chromatographed on Whatman No. 1 paper in 1-butanol-acetic acid-water (3:1:1). After chromatography the papers were sprayed with 0.5% ninhydrin in 90% aqueous 2-propanol.

ISOLATION OF THE CRUDE BACTERIOLYTIC ENZYME MIXTURE. The best strain found was grown for 20 hr at 28° with shaking in 20 ml of similar medium containing cell walls but without agar. At this time the logarithmic phase of growth had been reached and the cells were transferred to medium of the following composition: peptone, 1%; K<sub>2</sub>HPO<sub>4</sub>, 0.1%; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1%; KCl, 0.05%; and NaNO<sub>3</sub>, 0.2%. After two successive subcultures of increasing size, 100 l. of culture in logarithmic phase was used to inoculate two tanks, each containing 500 l. of the peptone medium. This final culture was grown at 28° for 72 hr with mechanical

<sup>2</sup> As already observed by various authors (McCarty, 1952; Ghuysen, 1957), the ability of *Streptomyces* strains to produce enzymes is highly variable, especially when the strains are repeatedly grown on artificial media. When one volume of the *Streptomyces* culture filtrate obtained in the above conditions was added to nine volumes of a cell suspension of living staphylococci (1 mg dry weight/ml), the turbidity of the suspension was reduced to 50% of its original value after 2 hr of incubation at 37°. Such high staphylolytic activity was not regularly obtained when the strain was grown under exactly the same conditions. It has also been observed that a selected strain may lose some of its ability to produce staphylolytic enzymes during storage at 0° on artificial solid media.

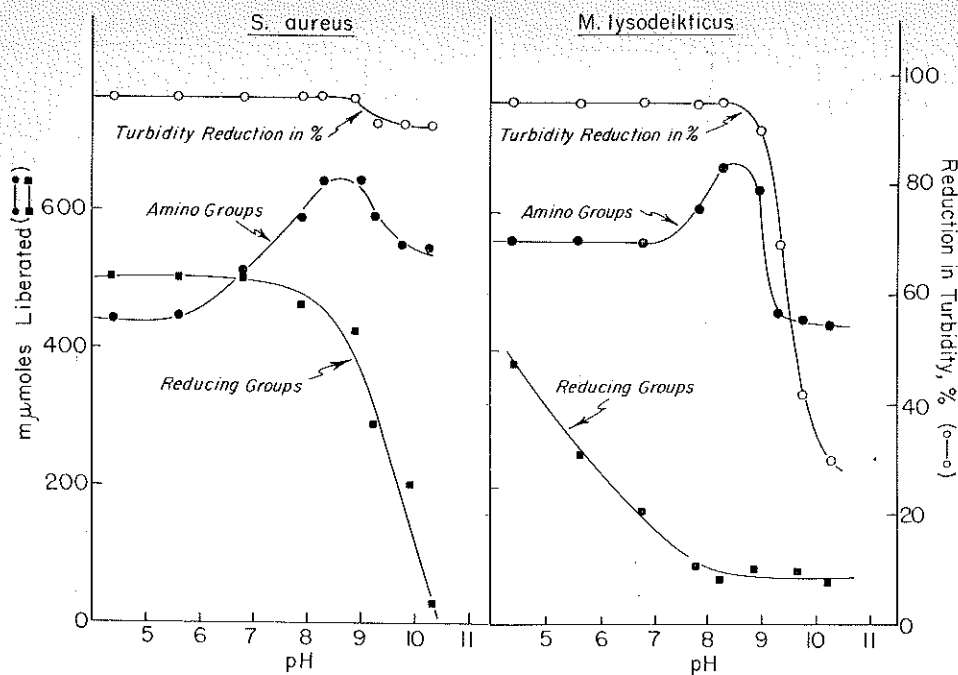


FIGURE 3: Effect of pH on digestion of cell walls of *S. aureus* and of *M. lysodeikticus* by fraction a-9.5. Incubations were carried out as described in the text. Data are expressed as % decrease in turbidity or as  $\mu$ moles of free amino groups or reducing groups liberated per mg of cell walls.

stirring (50 rpm) and an air flow rate of 100 l./min at an air pressure of 0.5 kg/cm<sup>2</sup> and then filtered and cooled at 0°.

Amberlite IRC 50(XE 64, H<sup>+</sup> form) (5 kg) was added to the 1000 l. of filtrate and the pH was adjusted to 5.2 by addition of acetic acid. The resin was collected and dried under vacuum. This material (1000 g) was re-suspended in a minimum volume of cold water and a buffer containing 8 N NH<sub>3</sub>-4 N acetic acid was added until a pH of 9 was reached. The resin was filtered off. The filtrate contained the staphylolytic enzymes. Two volumes of saturated ammonium sulfate solution were added to the filtrate at 0°. After centrifugation, the pellet obtained was resuspended in 300 ml of 0.1 M K<sub>2</sub>HPO<sub>4</sub> and stirred at 0°. The insoluble material (2 g dry weight) was discarded and the clear solution was dialyzed against water. Some material precipitated during this step but was not removed. Lyophilization yielded 5 g of powder. Finally, about 85% of this material was solubilized by prolonged dialysis at 0° against 0.05 M Tris buffer, pH 7. This solution was called the crude enzyme complex.

**FRACTIONATION OF THE CRUDE ENZYME COMPLEX.** The crude complex (800 mg, dissolved in 50 ml of Tris buffer) was filtered successively through three columns of Amberlite IRC 50 (35 × 2 cm) previously equilibrated with the same buffer at 0°. Each column was washed with the buffer (about 350 ml) until no further protein (measured by absorbancy at 278 m $\mu$ ) was present in the effluents. The effluents were dialyzed against water and then lyophilized. The dry material (referred to as

fraction C7 or peptidase 2) contained acidic proteins. The basic proteins adsorbed onto the columns were eluted with about 200 ml of 1 M Tris buffer, pH 9.5. Disappearance of absorbancy at 278 m $\mu$  was used again as an indication of completion of elution by this buffer. After dialysis, the eluates were centrifuged, if necessary, and the clear supernatant solutions were lyophilized. Column 1 yielded 115 mg of material (called peptidase 1, fraction a-9.5) and column 2 yielded 95 mg of material (called peptidase 1, fraction b-9.5). No solid was obtained from column 3 which indicated that complete adsorption of the basic proteins had occurred after passage through the first two columns.

**Analytical Methods.** Protein was determined by the modified Folin phenol procedure (Lowry *et al.*, 1954), free amino groups by reaction with 2,4-dinitrofluorobenzene (*cf.* Ghuysen and Strominger, 1963a), and reducing groups by ferricyanide reduction (Park and Johnson, 1949).

## Results

**Action of Fraction a-9.5 on the Soluble Glycopeptide from *S. aureus*.** The glycopeptide (210  $\mu$ g) was incubated with 11  $\mu$ g of protein of fraction a-9.5 in 30  $\mu$ l of various buffers from pH 4 to 10.2, all at 0.01 M. After 8 hr of incubation at 37° free amino groups were estimated with dinitrofluorobenzene. Fraction a-9.5 contained a peptidase or peptidases which liberated such groups from the glycopeptide with an optimum pH of about 8.8 in 0.01 M Veronal buffer (Figure 1). Paper

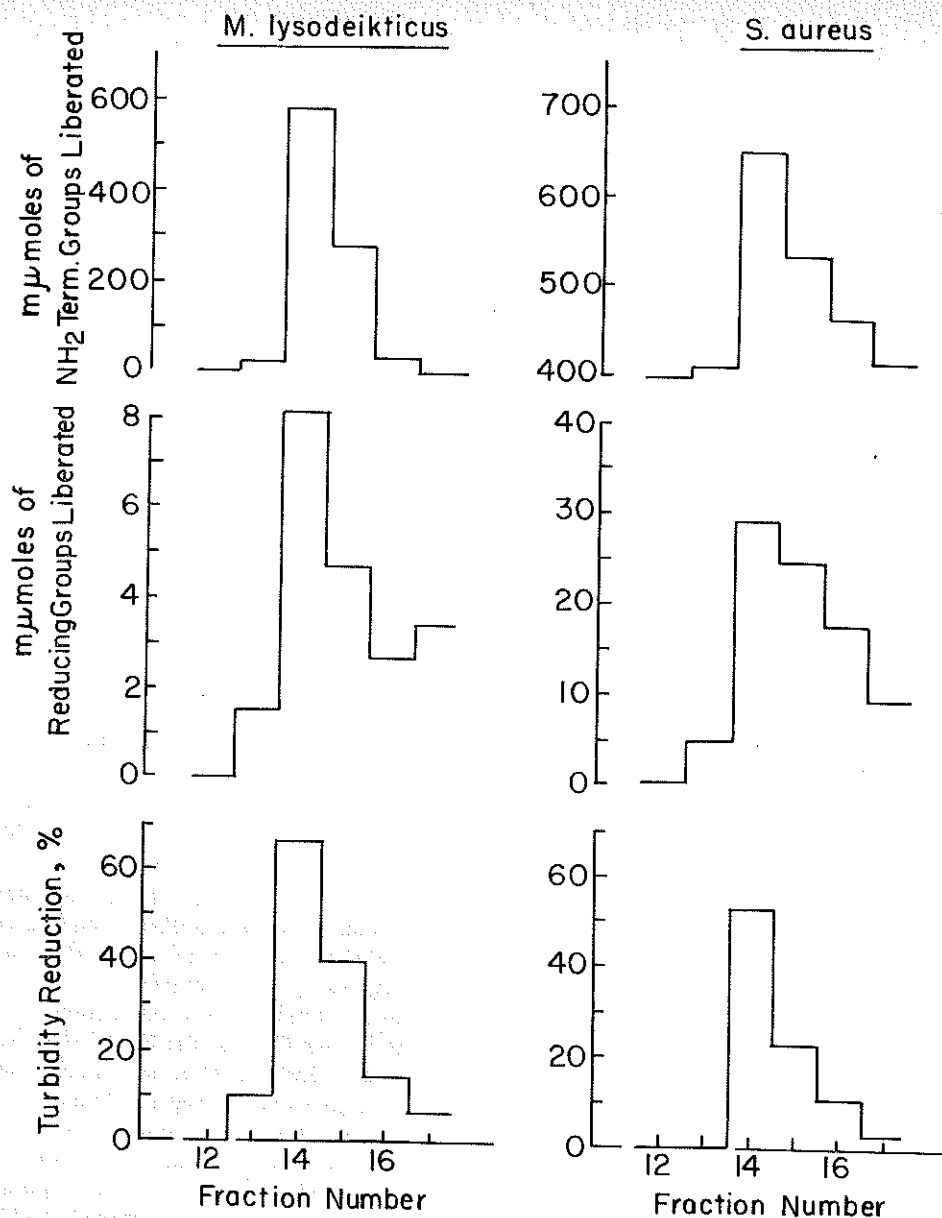


FIGURE 4: Distribution of various activities with cell wall as substrates after sucrose gradient electrophoresis at pH 8.8 in 0.05 M Veronal phosphate buffer of fraction b-9.5. Fraction b-9.5 had been introduced at the level of fractions 14-15. Data are expressed as in Figure 3. Each fraction (7 ml) was dialyzed against water. Cell walls from *S. aureus* or *M. lysodeikticus* (750  $\mu$ g) were incubated with 26  $\mu$ l of enzyme in 80  $\mu$ l of 0.01 M Veronal buffer, pH 8.8, for 21 hr at 37°.

chromatography of the digest showed the presence of free alanine. In addition the remaining polymer had been degraded as evidenced by the distinct increase in mobility in the solvent employed and a greatly enhanced sensitivity to ninhydrin (Figure 2).

*Action of Fraction a-9.5 on Bacterial Cell Walls.* Fraction a-9.5 was powerfully bacteriolytic against walls of *S. aureus* and *M. lysodeikticus*. When the incubations were carried out at various pH values, however, it was possible to detect two types of activity in this prepara-

tion, peptidase(s) and glycosidase. The glycosidase was presumably the F<sub>1</sub> acetylmuramidase, a basic protein known to be adsorbed onto Amberlite IRC 50 at pH 7 at low ionic strength (Ghuysen, 1957).

*DIGESTION OF CELL WALLS OF S. aureus.* Cell walls (0.5 mg) were incubated at various pH values with 12  $\mu$ g of protein of fraction a-9.5 in 70  $\mu$ l of 0.01 M buffers. After 6 hr at 37°, dissolution of the walls was virtually complete at all pH values. After centrifugation the reducing groups and free amino groups of the lysates were



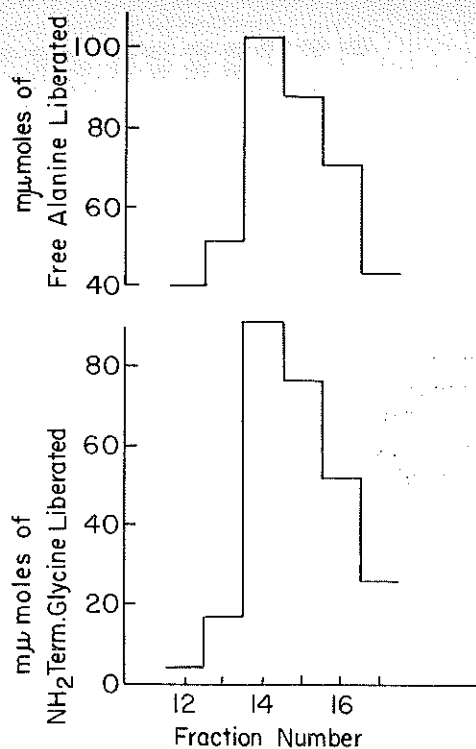


FIGURE 5: Distribution of various activities with soluble glycopeptide as substrate after sucrose gradient electrophoresis of fraction b-9.5. The fractions were the same as those used in Figure 4 and data are expressed as  $m\mu$ moles of free alanine or of N-terminal glycine liberated/mg of substrate. Soluble glycopeptide ( $300 \mu\text{g}$ ) was incubated with  $7 \mu\text{l}$  of dialyzed enzyme in  $20 \mu\text{l}$  of  $0.01 \text{ M}$  Veronal buffer, pH 8.8, for 21 hr at  $37^\circ$ . Analyses were carried out employing thin-layer chromatography of dinitrophenyl amino acids as described in the following paper (Ghuysen *et al.*, 1965).

estimated (Figure 3). At acidic pH values many reducing groups had been liberated.<sup>3</sup> The free amino groups of the lysates at pH 6 or below ( $440 m\mu$ moles/mg of cell wall) represent the free amino groups of ester D-alanine residues in the teichoic acid.<sup>4</sup> When the incubations were carried out at pH values higher than 7, increasing amounts of free amino groups were found in the lysates with a maximum increase of about  $200 m\mu$ moles/mg occurring at pH 8.5. In contrast the appearance of reducing groups was greatly decreased at pH values higher than 8. The low liberation of reducing groups at pH 10.2 ( $20 m\mu$ moles/mg) accompanied by complete lysis strongly suggests that the peptidase(s) alone was lytic.

<sup>3</sup> Appearance of material reacting in the Morgan-Elson reaction after 30 min of heating in 1% sodium borate buffer paralleled the appearance of reducing groups.

<sup>4</sup> The same amount of free amino groups was released from walls of *S. aureus* strain Copenhagen after treatment at pH 9 at  $37^\circ$  for 20 hr. This material was identified as free alanine.

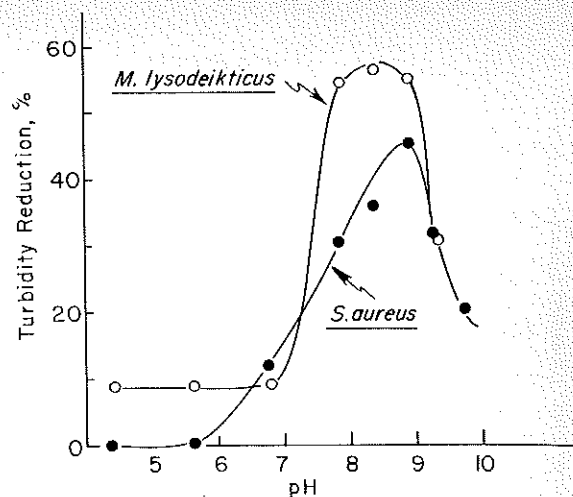


FIGURE 6: Effect of pH on lysis of cell walls of *S. aureus* and *M. lysodeikticus* by purified peptidase 1. Cell walls ( $500 \mu\text{g}$ ) were incubated with  $3.2 \mu\text{g}$  of enzyme protein in  $70 \mu\text{l}$  of various buffers (see Figure 1) for 16 hr at  $37^\circ$ . Lysis was then measured by decrease in turbidity.

DIGESTION OF CELL WALLS OF *M. lysodeikticus*. The same conclusion was derived from an experiment performed with walls of *M. lysodeikticus* (Figure 3). Some differences were observed however. The optimum pH for the glycosidase was more acidic (pH 4.4 or less), and activity diminished as the pH was increased above 4.4. Large amounts of free amino groups were detected in the digests, even at acidic pH values. Free amino groups, present mainly as  $\epsilon$ -amino groups of lysine, are much more frequent in the glycopeptide of *M. lysodeikticus* (Salton, 1961) than in the glycopeptide of *S. aureus*, although *M. lysodeikticus* walls do not contain ester alanine with free amino groups. The free amino groups found in lysates obtained by digestion between pH 4 and 7 presumably represent the normally occurring free amino groups of the glycopeptide moiety of *M. lysodeikticus*. An additional liberation of free amino groups also was found with walls from *M. lysodeikticus* at a pH optimum of 8.5–9. Unlike walls of *S. aureus*, however, both lysis and appearance of new free amino groups were abruptly diminished at pH values higher than 9 (Figure 3).

*Purification of Peptidase from Fraction b-9.5.* Analysis of the activities of fraction b-9.5 showed that this preparation also contained alanine-liberating and glycopeptide-degrading peptidase(s) as did fraction a-9.5. Fraction b-9.5, however, contained only a small amount of acetylmuramidase. This preparation was then subjected to sucrose gradient electrophoresis in  $0.05 \text{ M}$  Veronal phosphate buffer, pH 8.8. After 46 hr at  $10 \text{ v/cm}$ , peptidase and bacteriolytic activities were found in fractions 12 to 17, closely associated with a peak of protein which had not migrated in the electrical field. The same distribution of activities was found whether the substrate was walls of *S. aureus* or *M.*

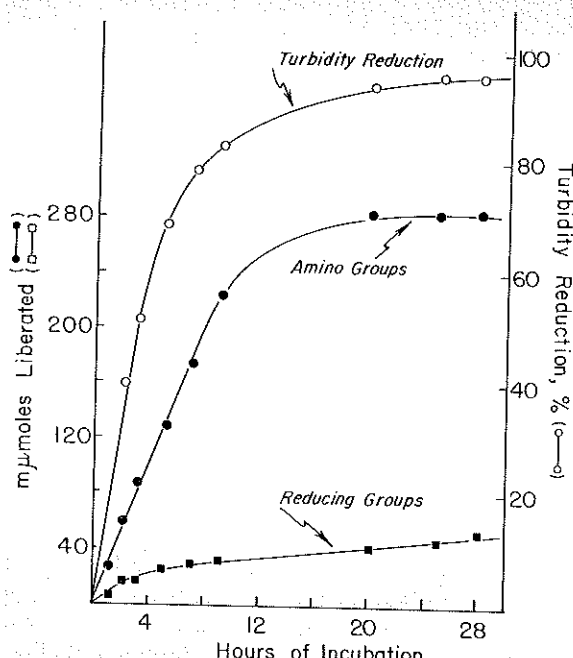


FIGURE 7: Kinetics of digestion of cell walls of *S. aureus* by peptidase 1. Incubation conditions are described in the text. Data are expressed as in Figure 3.

*lysodeikticus* (Figure 4) or soluble glycopeptide from *S. aureus* (Figure 5). Turbidity reduction, release of free amino groups, and a small release of reducing groups from both cell walls were parallel in different fractions (Figure 4). In addition to liberation of free alanine, new N-terminal glycine groups, still bound to the glycopeptide, appeared from the soluble glycopeptide of *S. aureus* (Figure 5). The splitting of glycopeptide into large, chromatographically mobile fragments by this enzyme (Figure 2) may be related to the appearance of the new N-terminal glycine amino groups.<sup>6</sup>

Fractions 14 and 15 were pooled, dialyzed against water, and lyophilized. This material (2 mg of protein, dissolved in 1 ml of water) is the best preparation of peptidase 1 presently available.

**Kinetics of Digestion of Cell Walls by Peptidase 1.** The effect of pH on the bacteriolytic activities of the purified peptidase 1 was examined using walls of *S. aureus* and of *M. lysodeikticus* as substrates. As previously observed with the soluble glycopeptide from *S. aureus* with fraction a-9.5 (Figure 1), the lysis of both bacterial walls was optimum in a narrow pH range, 8-9 (Figure 6).

In order to study the liberation of free amino groups from walls of *S. aureus* they were first freed of the ester D-alanine residues of the teichoic acid by treatment at

<sup>6</sup> In recent experiments (J. M. Ghuyssen, unpublished) the peptidase which lyses walls of *S. aureus* was separated by chromatography on CM-cellulose from the enzyme which lyses the cell walls of *M. lysodeikticus*. In these experiments, the alanine-liberating enzyme has also been separated from the two lytic fractions.

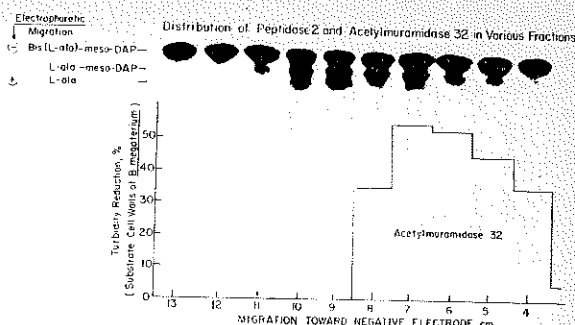


FIGURE 8: Purification of peptidase 2 by sucrose gradient electrophoresis of fraction C7. The separation of 15 mg of this fraction was carried out in 0.03 M phosphate buffer, pH 6.45, as described by Ghuyssen *et al.* (1962). Fractions of 5 ml, representing 1 cm of the column, were collected. To assay peptidase 2, 10  $\mu$ l of 0.01 N NH<sub>4</sub>OH (to bring the pH to 8) and then 10  $\mu$ l of a solution containing 10  $\mu$ g of bis-L-Ala-meso-DAP was added. After incubation for 20 hr at 37° the mixtures were subjected to paper electrophoresis in acetic acid-pyridine-water (9:2:1000), pH 4, for 2 hr at 20 v/cm in the Pleuger electrophoresis apparatus, with spots applied 5 cm from the anode. The dried electrophoreograms were sprayed with ninhydrin. All compounds migrated toward the cathode, with alanine having the lowest migration. Only a portion of the electrophoreogram is shown. To assay acetylmuramidase 32, 20  $\mu$ l of each fraction was added to 200  $\mu$ g of cell walls of *Bacillus megaterium* KM in 300  $\mu$ l of 0.01 M phosphate buffer, pH 6.45. These cell walls were employed in the assay because they are not solubilized by peptidase 2. Turbidity was measured after 1 hr at 37°.

pH 9 for 24 hr at 37°. These ester alanine-free walls (1.2 mg) were incubated with 20  $\mu$ g of protein of peptidase 1 in 100  $\mu$ l of 0.01 M Veronal buffer, pH 8.8 (Figure 7). Under these conditions, 50% of the turbidity disappeared in 3 hr at 37°. At completion of the reaction, about 300 m $\mu$ moles of free amino groups was liberated from 1 mg of cell wall. Lysis of the walls and liberation of free amino groups occurred at the same velocity. However, the release of reducing groups, due to contamination by some glycosidase, was very slow.<sup>6</sup>

**Isolation of Peptidase 2.** Sucrose gradient electrophoresis of the acidic fraction C7 gave an electrophoreogram (Figure 8) very similar to that given by the F<sub>2</sub>B preparation from *Streptomyces* (Ghuyssen *et al.*, 1962) with typical peaks of acetylmuramyl-L-alanine amidase, of caseinase A, of caseinase B (in very small

<sup>6</sup> Extrapolation of the curve for liberation of reducing groups to zero time suggests that the walls themselves contain about 20 m $\mu$ moles of reducing groups/mg. This value is in agreement with data obtained for the size of the intact polysaccharide isolated from these walls (Tipper *et al.*, 1964; Ghuyssen *et al.*, 1964).

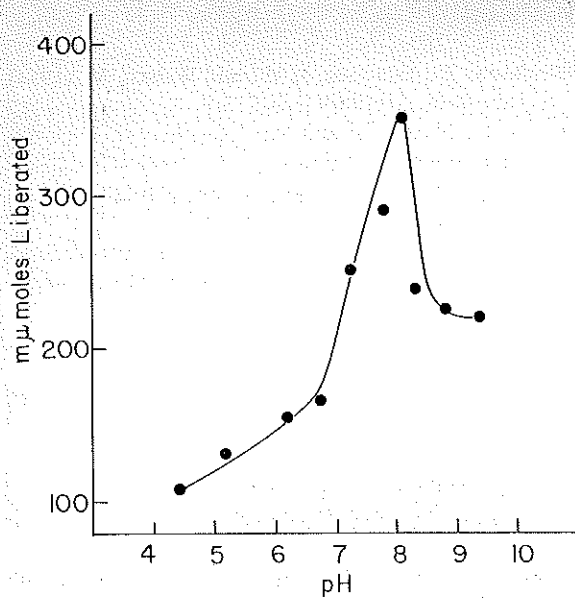


FIGURE 9: Effect of pH on the action of peptidase 2 on soluble glycopeptide. Data are expressed as mμmoles of free amino groups liberated/mg of glycopeptide. For details, see text.

amounts),<sup>7</sup> and of acetylmuramidase 32. The first three of these enzymes are more negatively charged than acetylmuramidase 32 and hence do not appear in Figure 8. In addition to these activities, fraction C7 (and to a lesser extent the F<sub>2</sub>B preparation) was able to liberate from the soluble glycopeptide of *S. aureus* both a small amount of free alanine (as does peptidase 1) and a larger amount of free glycine (Figure 2). Moreover, both of these preparations also hydrolyzed the synthetic tripeptide, bis-L-alanyl-meso- $\alpha,\alpha'$ -diaminopimelic acid (Bricas *et al.*, 1962; Nicot *et al.*, 1965) into free alanine and the monosubstituted derivative, L-alanyl-meso- $\alpha,\alpha'$ -diaminopimelic acid.<sup>8</sup> On sucrose gradient electrophoresis, the protein which hydrolyzed the synthetic tripeptide was a little more basic at pH 6.45 than the acetylmuramidase 32 (Figure 8). In order to reduce the contamination of this peptidase by acetyl-

<sup>7</sup> This small amount of caseinase B found in fraction C7, compared with that present in the older preparation F<sub>2</sub>B, obtained by a similar procedure, is another example of the variability in the production of specific extracellular enzymes by *Streptomyces* under the present conditions.

<sup>8</sup> Several synthetic substrates were employed: meso-diaminopimelic acid-bis(L-alanine) (I); meso-diaminopimelic acid-bis(D-alanine) (II); bis-L-alanyl-meso-diaminopimelic acid (III), and bis-D-alanyl-meso-diaminopimelic acid (IV). Peptides II and IV were completely resistant to hydrolysis while about 25% of the alanine of peptide I and 50% of the alanine of peptide III were released. In both cases, only one of two alanine residues was susceptible to hydrolysis. The monosubstituted diaminopimelic acid derivatives could be separated from substrates by paper chromatography using the solvent of Rhuland *et al.* (1955). The structure of the two monosubstituted diaminopimelic acid derivatives and, therefore, the precise configuration of the linkages hydrolyzed were not established.

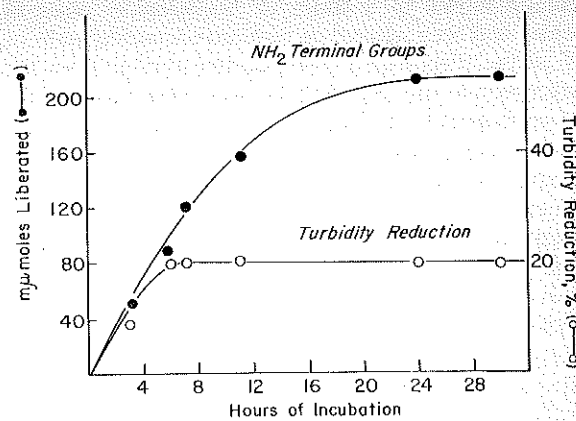


FIGURE 10: Kinetics of lysis of cell walls of *S. aureus* by peptidase 2. For details, see text.

muramidase 32, the fractions at 8–11 cm were dialyzed, lyophilized, and then subjected to a second electrophoresis under the same conditions. The final preparation, which represented about 7% of the protein in fraction C7, contained the enzyme(s) responsible for both the liberation of glycine and of alanine from the soluble glycopeptide.<sup>9</sup> It will be referred to as peptidase 2.

**Action of Peptidase 2 on the Soluble Glycopeptide from *S. aureus*.** The glycopeptide (200 μg) was incubated with 4 μg of protein of peptidase 2 in 30 μl of 0.01 M buffers of various pH values from 4 to 10. After 4 hr at 37° free amino groups of the incubation mixtures were estimated (Figure 9). The maximum activity was observed at pH 8.05 in 0.01 M phosphate buffer. Paper chromatography of a digest at this pH (Figure 2) revealed the liberation of a small amount of alanine and of a large amount of glycine distinguishing this preparation from peptidase 1. Moreover, the paper chromatographic mobility of the residual glycopeptide was not altered by peptidase 2 although the intensity of reaction with ninhydrin was increased (Figure 2).

**Action of Peptidase 2 on Cell Walls of *S. aureus*.** Cell walls of *S. aureus* (560 μg, previously freed of the ester D-alanine residues of the teichoic acid) were incubated with 20 μg of protein of peptidase 2 in 40 μl of 0.01 M phosphate buffer, pH 8.05 (Figure 10). After 20% of the walls had been solubilized the reduction of turbidity abruptly ceased. Despite this, however, free amino groups continued to be liberated to a maximum of 214 mμmoles/mg of walls.

## Discussion

Two enzyme preparations which hydrolyze the peptide moiety of the glycopeptide of *S. aureus* strain Copenhagen have been isolated from the bacteriolytic

<sup>9</sup> This preparation retained the ability to hydrolyze peptide III (bis-L-alanyl-meso-diaminopimelic acid), but the activity originally present on peptide I (diaminopimelic acid-bis-L-alanine) had been lost in the purification.

culture filtrate of a strain of *Streptomyces albus* G which had been selected on medium containing walls of *S. aureus*. Peptidase 1 liberated free alanine from the glycopeptide and degraded the remaining polymer into large fragments, as shown by the appearance of new N-terminal glycine residues and by the paper chromatographic behavior of the digested material. Peptidase 1 is an enzyme or enzyme mixture, which is basic at pH 7 (and hence is adsorbed on Amberlite IRC 50 at this pH and low ionic strength) but neutral at pH 8.8. Peptidase 1 is virtually devoid of glycosidase activity but nevertheless lyses the cell walls of *S. aureus* and of *M. lyso-deikticus*. Similarly, the autolytic acetylmuramyl-L-alanine amidase of *Bacillus subtilis* which lyses the cell walls of this organism (Young *et al.*, 1964) and the bacteriolytic enzyme from *Cytophaga* sp. which lyses cell walls of a number of organisms (Ensign and Wolfe, 1964; Tipper *et al.*, 1964a) have no glycosidase activity. It appears, therefore, that lysis of bacterial cell walls can be brought about by hydrolysis of peptide chains as well as of the polysaccharide component.

Peptidase 2 liberated a mixture of free alanine and free glycine from the glycopeptide. However, it did not appear to degrade the remaining polymer. Peptidase 2 is an enzyme or enzyme mixture which is not adsorbed on Amberlite IRC 50 at pH 7. At pH 6.45 it is slightly more basic than acetylmuramidase 32 and was difficult to separate from this enzyme. Peptidase 2 was able to reduce the turbidity of walls of *S. aureus* by only 20%. The reason why turbidity decrease ceases at this level is not clear at present.

The earlier observations of Salton and Ghuysen (1957) of the presence of peptidases in the filtrate of *Streptomyces* cultures have been confirmed, therefore, and extended in the present study. It should be emphasized that the purification of these enzymes has been carried out employing qualitative assays. Synthetic or other well-defined substrates have not been available and the nature of the hydrolysis of peptide bonds has not been known until recently (see following paper). The absolute activity cannot be defined further than has been described in the experiments above, and the absolute purification of the various preparations with respect to protein of the starting material is unknown. These preparations have nevertheless been extremely useful in the study of the structure of the glycopeptide from *S. aureus* which will be reported in the following paper (Ghuysen *et al.*, 1965). The nature of the bonds cleaved by these preparations has also been defined in the course of that work.

## References

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