

Structure of the Cell Wall of *Staphylococcus aureus* Strain Copenhagen. VI. The Soluble Glycopeptide and Its Sequential Degradation by Peptidases*

J. M. Ghuysen, D. J. Tipper, Claire H. Birge, and J. L. Strominger

ABSTRACT: The composition of a soluble glycopeptide obtained after the lysis of cell walls of *Staphylococcus aureus* by an acetylmuramidase has been reported. This glycopeptide has been degraded sequentially by two peptidase preparations, containing six enzyme activities, viz. two glycine bridge splitting enzymes, glycine and L-alanine aminopeptidases, and glycine and D-alanine

carboxypeptidases. Studies of the kinetics of the hydrolysis and analysis of the products of hydrolysis have established that the glycine cross bridges are linked at one end to the ϵ -amino groups of lysine and that most of the peptides linked to acetylmuramic acid are tetrapeptides. Some other features of cell wall structure which are suggested by the data are discussed.

The glycopeptide is a component of all bacterial cell walls. Its constituents are invariably acetylglucosamine, acetylmuramic acid, L- and D-alanine, D-glutamic acid, and a dibasic amino acid (most commonly L-lysine or α, α' -meso-diaminopimelic acid). Additional amino acids may be present in cross bridges which link glycopeptide chains to each other (for a recent review, see Salton, 1964). In the case of *Staphylococcus aureus*, the cross bridges have been assumed to contain polyglycine (Mandelstam and Strominger, 1961). Studies of the structure of this complex three-dimensional network depend on the isolation of small soluble fragments after specific hydrolytic cleavages. The specificity of hydrolytic enzymes provides the tool for such studies.

In previous papers the isolation of a soluble glycopeptide from the cell wall of *S. aureus* strain Copenhagen after lysis of the walls with either of two acetylmuramidases has been described (Ghuysen and Strominger, 1963a,b; Ghuysen *et al.*, 1965b). In addition two peptidase preparations, capable of degrading this substrate, have been obtained from a culture filtrate of a strain of *Streptomyces albus* G selected for its high staphylytic activity (Ghuysen *et al.*, 1965a). In the present paper some characteristics of this glycopeptide will be reported as well as its sequential degradation by the two peptidase preparations.

Materials and Methods

Enzymes. *Chalaropsis* B enzyme was a gift from Dr. J. Hash of Lederle Laboratories (Hash, 1963; Tipper *et al.*, 1964). β -N-Acetylglucosaminidase was prepared from pig epididymis (Findlay and Levvy, 1960). Acetylmuramyl-L-alanine amidase and peptidases 1 and 2 from *Streptomyces* were prepared as described in preceding papers (Ghuysen *et al.*, 1962; Ghuysen *et al.*, 1965).

Cell Walls and the Soluble Cell Wall Glycopeptide (GP-1). Cell walls of *S. aureus* strain Copenhagen were prepared and digested with *Chalaropsis* B enzyme as described previously (Tipper *et al.*, 1964). The digest was fractionated on a column of Ecteola-cellulose (Ghuysen *et al.*, 1965b). The teichoic acid-glycopeptide complex was adsorbed, and the soluble glycopeptide (called GP-1) was eluted with water.

The glycopeptide fraction was applied at 2° in a volume of about 1 ml to a column of Sephadex G 50 with a V_0 of 30 ml and a V_i of 80 ml previously equilibrated with water at 2°. The elution rate was 0.5 ml/min, and 15-min fractions were collected. The fractions containing glycopeptide (see Figure 1) were lyophilized.

Analytical Procedures. 4-O- β -N-Acetylglucosaminyl-N-acetylmuramic acid was measured by the Morgan-Elson reaction with 30 min of heating, or by reducing power, as previously described (Ghuysen and Strominger, 1963a; Tipper *et al.*, 1965). Free N-acetylhexosamine was determined by the Morgan-Elson reaction with 7 min of heating (Reissig *et al.*, 1955). Total amino groups and total phosphate and O-acetyl groups were measured as previously described (Ghuysen and Strominger, 1963a,b). D- and L-alanine were determined by utilizing the specificities of D-amino acid oxidase and L-glutamate-pyruvate(L-alanine- α -ketoglutarate)-transaminase, respectively (Strominger and Threnn, 1959). Molecular weight determinations by ultracentrifugation were performed as previously described

*From the Service de Bactériologie, Université de Liège, Liège, Belgium, and the Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin. Received May 10, 1965; revised June 28, 1965. This research has been supported in part by a grant (UR-E4-10-2) made by the U. S. Department of Agriculture under public law 480. A preliminary account has been published (Ghuysen *et al.*, 1964). This work was initiated at Washington University School of Medicine and has been supported by research grants from the U. S. Public Health Service (A1-01902) and National Science Foundation (G-18742).

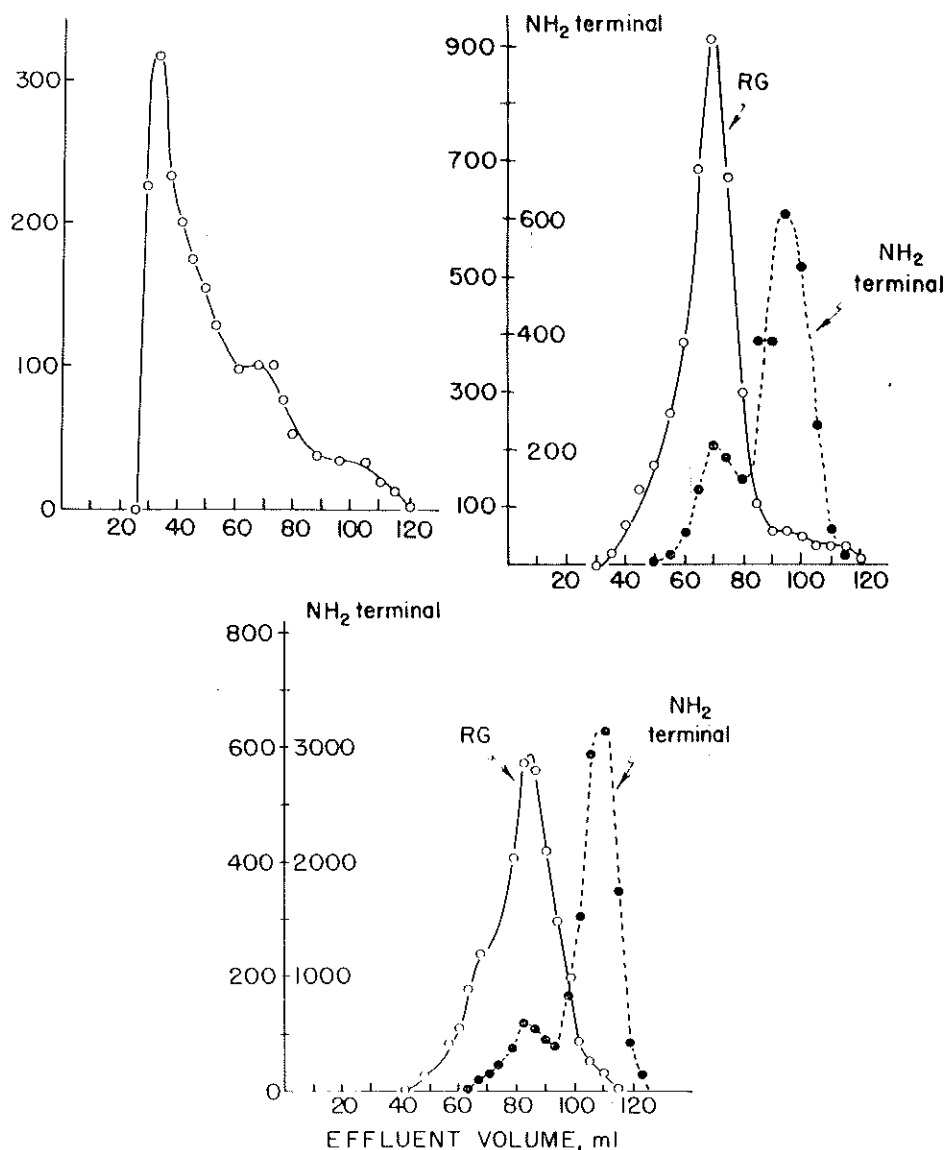


FIGURE 1: Filtration of the soluble glycopeptides GP-1 (upper left), GP-2 (upper right), and GP-3 (lower section) on a column of Sephadex G-50. Reducing groups (RG) were measured with ferricyanide and free amino groups with fluorodinitrobenzene. See text for details. The acetylmuramidase employed in the preparation of GP-1 causes complete hydrolysis of the polysaccharide to disaccharides. These disaccharides remain attached to each repeating unit of the polypeptide. Reducing groups are thus a measure of the total glycopeptide material present. Left-hand ordinate represents RG, $m\mu\text{mole/ml}$.

(Ghuysen *et al.*, 1965b). Paper chromatography for the identification of amino acids, peptides, or acetylmuramyl-peptides was carried out in butanol-acetic acid-water (3:1:1) or by two-dimensional chromatography employing isobutyric acid-1 N NH_4OH (5:3) in the first dimension and butanol-acetic acid-water in the second dimension. All compounds were detected with ninhydrin.

Determination of Total Amino Acids. Two methods were employed. Samples containing 100 to 400 $m\mu\text{moles}$ were analyzed after acid hydrolysis in the Beckman Spinco amino acid analyzer. Alternatively, samples

containing 10-30 $m\mu\text{moles}$ were hydrolyzed in sealed tubes in 50 μl of 6 N HCl at 100° for 16 hr. A standard mixture of alanine, glycine, glutamic acid, and lysine received the same treatment throughout. After lyophilization, the hydrolysates were redissolved in 1% potassium borate (100 μl). Fluorodinitrobenzene reagent (10 μl of a reagent containing 65 μl of fluorodinitrobenzene in 7.9 ml of 100% ethanol) was added and the mixture was incubated at 60° for 30 min. After acidification with 11.7 N HCl (50 μl), the dinitrophenyl derivatives were extracted with ether three times (100 μl) and the ether extracts were evaporated to dryness with

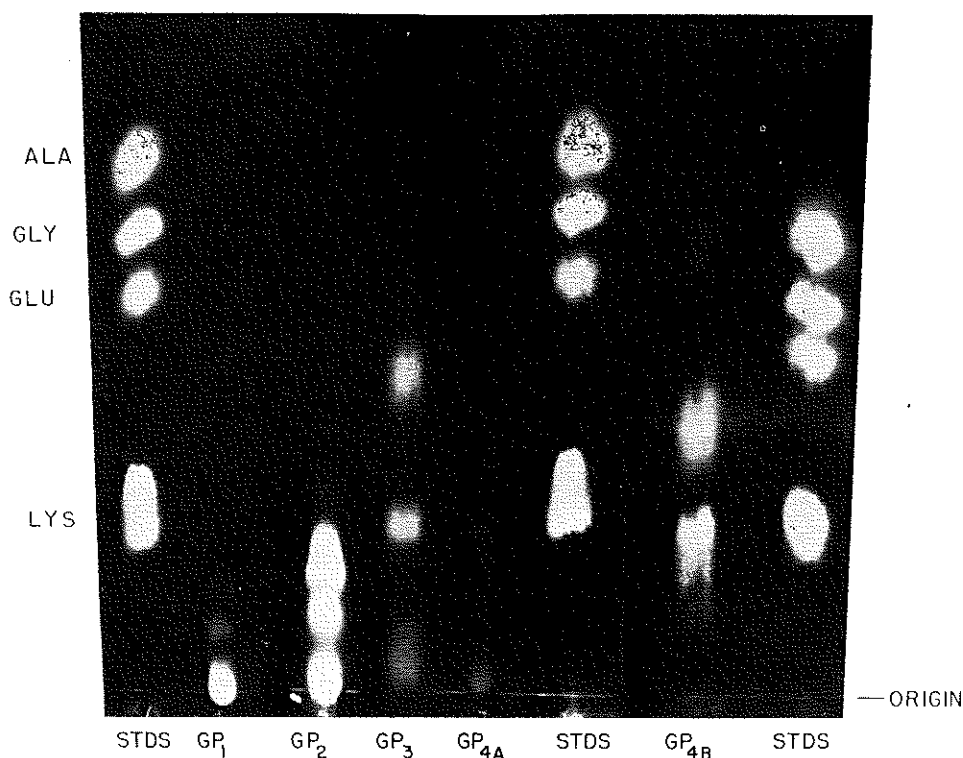


FIGURE 2: Paper chromatography of GP-1 (the soluble glycopeptide) and of its degradation products. The solvent employed was 1-butanol-acetic acid-water (3:1:1) on Whatman No. 1 paper. The paper was sprayed with ninhydrin and then photographed in fluorescent light with reflex document-copying paper.

mechanical shaking in a 37° bath. The residues were redissolved in 0.05 M ammonia (20 μ l) and aliquots were chromatographed on thin-layer plates of silica gel G (Brenner *et al.*, 1961). Sequential development in the same dimension with a basic solvent and an acidic solvent allowed the complete separation of all the dinitrophenyl (DNP) derivatives present, the sequence in order of increasing R_F being DNP-glutamic acid, DNP-glycine, DNP-alanine, bis-DNP-lysine, dinitrophenol, and dinitroaniline. The plates were first developed with solvent A (1-butanol-0.15 N ammonia, 1:1, upper phase) at room temperature for 2 hr. Then, after thorough drying in a stream of cold air, they were developed with solvent B (chloroform-methanol-acetic acid, 85:14:1) for $\frac{3}{4}$ hr at 2°. After drying, the gel in an area containing a yellow spot was loosened by scraping with a spatula and then sucked off into 1-ml tubes using a suction device. The DNP derivatives were eluted from the gel by vigorous mixing (twice for 10 sec on a Vortex mixer) with 0.05 N ammonia (200 μ l). After centrifugation, the optical density of the solutions was measured at 360 $m\mu$. Molar extinction coefficients, calculated from the standards, were approximately 15,000 for the mono-DNP derivatives, so that 10 $m\mu$ moles gave rise to an optical density of about 0.75. The whole procedure was completed in one day and was performed either in the dark or with tungsten lamp illumination to minimize photodecomposition. If required, a contact print of the chromatogram was made using reflex document

copying paper (portograph G-91, Transcopy, Inc., Newton, N. J.) and about 10 sec of illumination from a short wave ultraviolet lamp (Mineralight).

Hexosamines present in the hydrolysate give rise to very low yields of dinitrophenyl derivatives, probably due to decomposition of hexosamines in the borate solution. Moreover, these derivatives are not extracted by ether from the acidified solution.

Determination of N-Terminal and Free Amino Acids. A sample of the peptide-amino acid mixture containing 5 to 30 $m\mu$ moles of each N-terminal group was neutralized, then mixed with 10% potassium borate and water to give 100 μ l of solution containing 1% borate. The dinitrophenylation was performed as described for total amino acids, and the mixture was acidified with 11.7 N HCl (50 μ l). Two aliquots of the standard mixture of amino acids received the same treatment. One of these dinitrophenylated standard mixtures and all of the dinitrophenylated peptide solutions were extracted three times with ether (100 μ l), and these extracts containing the DNP derivatives of the free amino acids were dried, chromatographed, and quantitated as described for total amino acids. The residual ether in the HCl solutions was evaporated by gentle warming. These solutions were then sealed and hydrolyzed for 6 hr at 95° together with the second dinitrophenylated standard amino acid mixture and a sample of standard ϵ -DNP-lysine (Sigma Chemical Co., St. Louis). These hydrolysates were then extracted with ether, and the ether

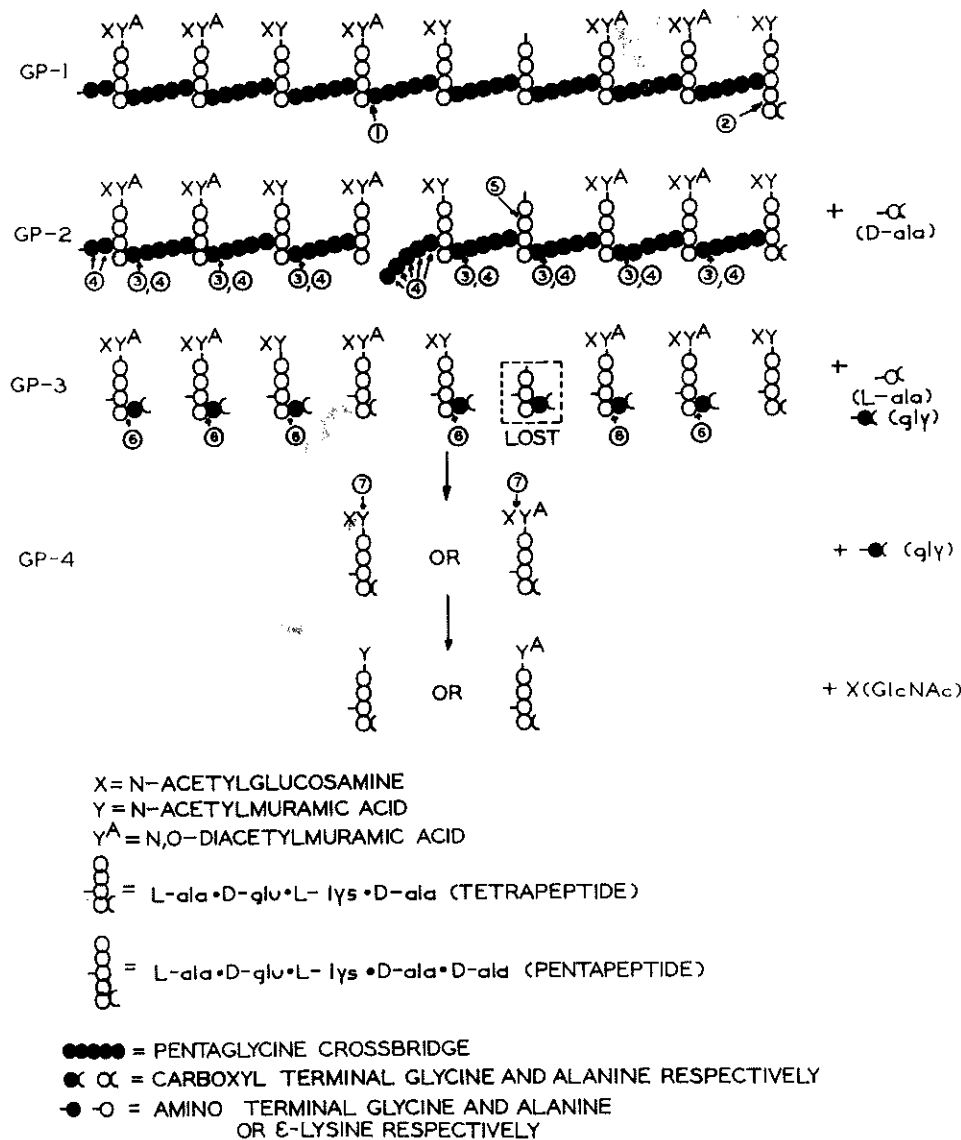


FIGURE 3: Schematic representation of proposed enzymatic degradation sequence. The enzymes are (1) endopeptidase 1 (glycine bridge splitting enzyme), (2) D-alanine carboxypeptidase, (3) endopeptidase 2 (glycine bridge splitting enzyme), (4) glycine aminopeptidase, (5) L-alanine aminopeptidase, (6) glycine carboxypeptidase, and (7) β -N-acetylglucosaminidase. The material which is not extensively degraded (e.g., GP-3₁ and GP-4A) is not represented here, nor is any differentiation made between bridges split by endopeptidases 1 and 2 since no evidence is presently available on this point. This hypothetical scheme represents a working model. The actual structure and its degradation may be more complex.

extracts were dried, chromatographed, and quantitated as described for total amino acids. These extracts contain the DNP derivatives of N-terminal glutamyl, alanyl, and glycyglycyl residues in the peptides. ϵ -DNP-lysine, derived from ϵ -N-terminal lysyl residues, remains in the HCl solutions. These solutions were also dried *in vacuo*, redissolved in 0.05 N ammonia, and chromatographed on silica gel G in solvent C (benzyl alcohol-chloroform-methanol-water-15 N ammonia, 30:30:30:6:2) in which solvent ϵ -DNP-lysine has an R_F of about 0.5. The small amounts of DNP-hexosamines present in these HCl solutions form very fast-

moving spots which do not interfere with the determination. Molar extinction coefficients are somewhat lower after this hydrolysis, averaging about 12,000, except that DNP-glycine which is more acid-labile sometimes gives an extinction coefficient of about 10,000. This procedure was validated using synthetic peptides, e.g., glycyglycine and glycyglycylalanine.

Results

Study of the Soluble Glycopeptide (GP-1). FILTRATION ON SEPHADEX G-50. Virtually all of GP-1 (measured by

its reducing groups) was excluded from Sephadex G-50 and came through in the void volume of the column at 30 ml (Figure 1, upper left). There were, however, two shoulders at 70 and 100 ml. As will be seen, the position of the shoulder at 70 ml corresponds to that of an enzymatic degradation product of GP-1 while that at 100 ml corresponds to low molecular weight materials.

PAPER CHROMATOGRAPHY. When 200 μ g of GP-1 was subjected to paper chromatography in 1-butanol-acetic acid-water (3:1:1) the bulk of the material remained at the origin (Figure 2). Traces of compounds at $R_{\text{alanine}} = 0.1$ and $R_{\text{alanine}} = 0.2$ were detected. These trace materials are also in the position of enzymatic degradation products of GP-1 (see below).

MOLECULAR WEIGHT MEASUREMENTS. This material had a sedimentation constant, $s_{20}^0 = 1.12$ S, a diffusion constant, $d_{20} = 9 \times 10^{-7}$ cm²/sec, and a partial specific volume, $\bar{v} = 0.67$. These data yield a weight-average molecular weight of 9200.

CHEMICAL ANALYSES. After acid hydrolysis, the material contained, per 1000 glutamic acid residues, 4620 glycine residues, 2100 alanine residues, and 1000 lysine residues in addition to glucosamine, muramic acid, and ammonia. Examination of the configuration of the alanine indicated that 1100 residues were D-alanine and 1000 were L-alanine. Using the Morgan-Elson reaction with 30 min of heating in borate buffer there were 950 disaccharide units/1000 glutamic acid residues, relative to a standard of *N*-acetylmuramyl- β -1,4-*N*-acetylglucosamine (Tipper *et al.*, 1965) and 597 *O*-acetyl groups measured with alkaline hydroxylamine. Amino end group determinations showed the extreme paucity of such groups, values of glutamic acid 0, glycine 40, alanine 70, and ϵ -amino lysine 25 residues being obtained. If the sum of amino-terminal glycine and ϵ -amino lysine is used to estimate number-average molecular weight, a value of 10,000-15,000 is obtained, but the low values for amino terminal groups are not precise. A schematic representation of the proposed structure of the soluble glycopeptide is presented in Figure 3. In this representation the free amino glycine (and ϵ -amino lysine groups) are in open bridges at the ends of chains. The glycopeptide contained no detectable organic phosphate (less than 10 residues/1000 glutamic acid residues).

Degradation of the Soluble Glycopeptide (GP-1) by Peptidase 1. In preliminary experiments incubation of 58 μ g of GP-1 with 1-6 μ g of peptidase 1 (1.0 mg of protein/ml) indicated that maximum release of free amino groups in 20 hr at 37° was obtained with 2 μ g of the enzyme. A large-scale incubation was carried out as follows: GP-1 (30.6 mg) was incubated with 0.82 mg of peptidase 1 in 1.6 ml of 0.01 M Veronal buffer, pH 8.8, for 30 hr at 37°. Aliquots containing 185 μ g of GP-1 were removed at various times and analyses for free amino acids and amino-terminal groups were carried out. Free alanine (125 residues/1000 glutamic acid residues) was released without any alteration of the amino-terminal alanine groups in the substrate (Figure 4). In addition 150 amino-terminal glycine groups appeared (Figure 5). Fresh enzyme was added to an aliquot removed at 20 hr but no additional increase in

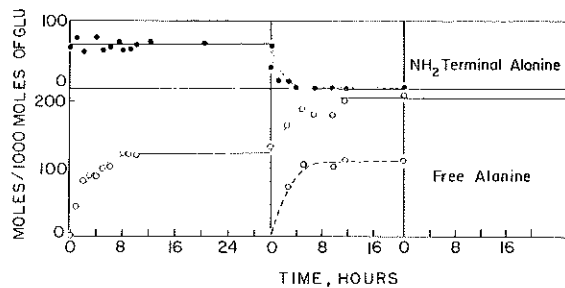


FIGURE 4: Appearance of free alanine and disappearance of amino-terminal alanine during sequential degradation of the soluble glycopeptide (GP-1) by peptidase 1 (left section), by peptidase 2 (middle section), and again by peptidase 1 (right section). The dotted lines refer to the action of peptidase 2 on GP-1. For details, see text.

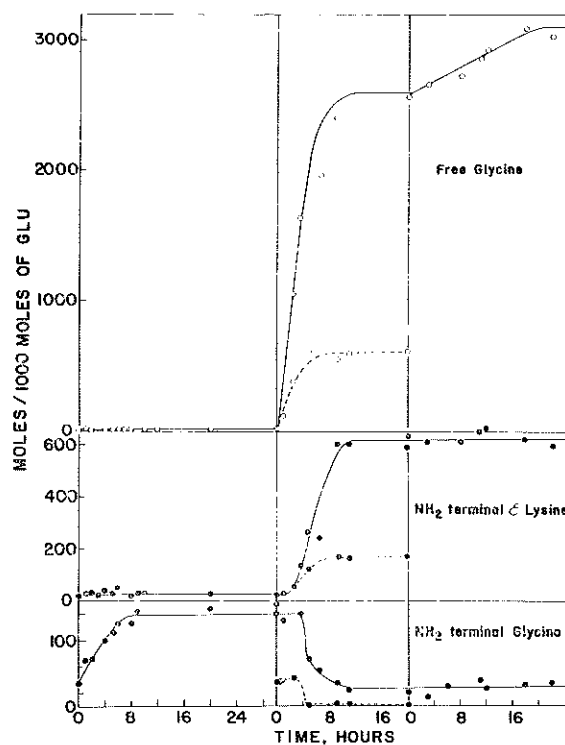


FIGURE 5: Changes in free glycine, in amino-terminal glycine, and in a free ϵ -amino group of lysine during sequential degradation of the soluble glycopeptide (GP-1) by peptidase 1 yielding GP-2 (left section), by peptidase 2 yielding GP-3 (middle section), and again by peptidase 1 yielding GP-4 (right section). The dotted lines refer to the action of peptidase 2 on GP-1. For details, see text.

amino-terminal groups occurred. In several experiments with different preparations of GP-1 the maximum amount of amino-terminal glycine released by this preparation has always been in the range of 150-250 residues.

After completion of the incubation the sample was placed on the same column of Sephadex G-50 as had been employed for filtration of GP-1. The main peak of material, measured by reducing groups, now came through at a volume of 70 ml, with a shoulder remaining at 100 ml (Figure 1, upper right). Reaction with fluorodinitrobenzene revealed a large amount of amino-terminal groups eluted at 100 ml. Paper chromatography indicated that this latter material was entirely free alanine (3.0 μ moles), and analysis by enzymatic methods indicated that it was substantially all D-alanine (3.1 μ moles) with a maximum of 5–10% of L-alanine.

Analysis of GP-2. The main peak of reducing material (tubes 30–82, Figure 1, upper right) yielded 24 mg on lyophilization and was called GP-2. Paper chromatography (Figure 2) showed that two major ninhydrin-reacting components were present with R_{alanine} values of 0.15 and 0.25 and that a considerable amount of material remained at the origin. Although GP-2 is obviously heterogeneous, no effort was made to separate the various components before analysis. Amino acid and end group analyses (Table I) indicated that the

presumed effects of these enzymes on GP-1 are represented in Figure 3.

Degradation of GP-2 by Peptidase 2. In preliminary assays 56 μ g of GP-2 was incubated with 0.6–6 μ g of peptidase 2 (2.6 mg of protein/ml) in 15 μ l of 0.01 M potassium phosphate buffer, pH 8.05, for 20 hr at 37°. Measurement of amino-terminal groups indicated that maximum release under these conditions occurred with 2.4 μ g of enzyme protein.

GP-2 (15 mg) was incubated with 0.65 mg of peptidase 2 in 3.85 ml of 0.01 M potassium phosphate buffer, pH 8.05. Aliquots containing 60 μ g of GP-2 were removed at various times for analyses. During this incubation free alanine was again released (90 residues/1000 glutamic acid residues), this time with a parallel disappearance of amino-terminal alanine residues (70 residues) (Figure 4). The disappearance of amino-terminal alanine was accompanied by the appearance of some amino-terminal glutamic acid. In one experiment the amount which appeared was equivalent to the amount of amino-terminal alanine which disappeared. In later experiments (including the one described in detail here) difficulties were encountered in quantitative measurement of amino-terminal glutamic acid; this question is being investigated further.

A remarkable appearance of free glycine commenced immediately on addition of peptidase 2 and reached a value of 2600 residues (Figure 5). After a lag of 2 hours, ϵ -amino lysine groups began to appear and reached a value of 600 residues after 8 hr. After a slightly longer lag of about 4 hr (at which time about three-fourths of the free glycine had been liberated) amino-terminal glycine disappeared at a rate similar to that of the appearance of ϵ -amino lysine. At the conclusion of the reaction a small amount of amino-terminal glycine remained (20–30 residues).

The incubation mixture was subjected to filtration on the column of Sephadex G-50 (Figure 1, lower section). The main peak of reducing power now had an elution volume of 85 ml with a shoulder at 70 ml, and a very small shoulder at 100 ml. A large peak of free amino groups appeared at 110 ml. The latter was identified as glycine and alanine by paper chromatography but enzymatic analysis indicated that virtually all of the alanine was in the L configuration (total alanine, 0.56 μ mole/ml; L-alanine, 0.41 μ mole/ml; D-alanine, 0.05 μ mole/ml).

Analysis of GP-3. The main peak of reducing material (tubes 50–92, Figure 1, bottom) yielded 12 mg after lyophilization and was called GP-3. Amino acid analyses (Table I) indicated that the glycine content of GP-3 had been drastically reduced. It contained about 1 glycine residue/glutamic acid residue, compared to 4–5 residues in GP-1 or GP-2. Moreover, amino-terminal glycine and L-alanine had virtually disappeared and there were now 0.6–0.7 free ϵ -amino groups of lysine/glutamic acid residue.

Paper chromatography (Figure 2) revealed two main components, with higher mobilities (R_{alanine} 0.32 and 0.60) than the fragments found in GP-2. Ninhydrin-reactive material was still present near the origin (R_{alanine}

TABLE I: Analyses of the Soluble Glycopeptide and Its Degradation Products.^a

	Analyses				
	GP-1	GP-2	GP-3	GP-4B1	GP-4B2
A. Total amino acids					
Glutamic acid	1000	1000	1000	1000	1000
Lysine	1000	1000	1000	900	890
Glycine	4620	4630	1100	250	160
Alanine	2100	1960	1800	2050	2000
D-Alanine	1120	1050	1000	1090	1150
L-Alanine	1010	990	890	1180	1250
B. Amino terminal groups					
Glutamic acid	0	0	0	0	0
Glycine	40	140	30	0	0
Alanine	70	70	0	0	0
ϵ -Lysine	25	25	650	1260	1240
C. Disaccharide					
Total	950			1010	1230
O-Acetyl	600	610	590	0	980

^a Data are expressed as moles/1000 moles of glutamic acid for convenience.

only change in this material which had occurred was liberation of 10% of the D-alanine residues (so that the ratio of total alanine to glutamic acid was now 1960:1000) and appearance of 100 new amino-terminal glycine residues/1000 glutamic acid residues.

The degradation sequence and analysis of GP-2 indicate that peptidase 1 contains a glycine bridge splitting enzyme and a D-alanine carboxypeptidase. The

0 to 0.1). A small amount (3 mg) of GP-3 was separated into its three components by paper chromatography. These compounds were then recovered by elution [GP-3₁ (R_{alanine} 0-0.1), GP-3₂ (R_{alanine} 0.32), and GP-3₃ (R_{alanine} 0.60)].

Amino acid analyses of these separated materials indicated that the glycine content of GP-3₁ was considerably higher than that of GP-3₂ or GP-3₃ or of the starting material, GP-3. Moreover, GP-3₂ and GP-3₃ each contained 1.0 free ϵ -amino groups of lysine while GP-3₁ contained only 0.5. GP-3₁ appears to represent a small amount of a glycopeptide fragment which was only partially degraded by the enzymes employed. GP-3₁ contained about 0.5 *O*-acetyl groups, as did the starting material, GP-1. GP-3₂ and GP-3₃ differed from each other in that GP-3₂ contained no *O*-acetyl groups while GP-3₃ contained 0.9 *O*-acetyl group/glutamic acid residue.

GP-3₂ and GP-3₃ (400 μ g) were each treated with 8 μ g of acetylmuramyl-L-alanine amidase in 30 μ l of 0.025 M sodium acetate buffer, pH 5.4. GP-3₂ yielded disaccharide 1 (*N*-acetylglucosaminyl-*N*-acetylmuramic acid) while GP-3₃ yielded disaccharide 2 (*N*-acetylglucosaminyl-*N,O*-diacetylmuramic acid) (Ghuysen and Strominger, 1963b; Tipper, *et al.*, 1965). These disaccharides were detected with diphenylamine-trichloroacetic acid reagent (Hough *et al.*, 1950) after paper chromatography in 1-butanol-acetic acid-water (3:1:1). The peptides released on treatment with amidase (detected with ninhydrin) had identical paper chromatographic mobilities.

This degradation sequence and analysis of GP-3 indicate that peptidase 2 contains a second glycine bridge splitting enzyme (see Discussion) and both glycine and L-alanine aminopeptidases. The presumed effects of these enzymes on GP-2 are represented in Figure 3.

Degradation of GP-3 by Peptidase 1. Peptidase 1 did not liberate any free glycine from GP-1 or GP-2. However, incubation of GP-3 (which contains no amino-terminal glycine) with peptidase 1 resulted in liberation of free glycine (Figure 5). In this experiment 7.4 mg of GP-3 was incubated with 0.36 mg of peptidase 1 in 1.65 ml of 0.01 M Veronal buffer, pH 8.8. After 22 hr the incubation was stopped; about 500 residues of glycine had been liberated per 1000 glutamic acid residues. The remaining material (equivalent to about 6 mg of GP-3) was filtered on a column of Sephadex G-25 (Figure 6). Analyses of reducing groups and free amino groups indicated the presence of three materials, *viz.*, GP-4A with a peak at 130 ml containing reducing groups (but no detectable free amino groups), GP-4B with a peak at 170 ml containing both reducing groups and free amino groups, and a peak at 220 ml containing only free amino groups. The latter was identified as free glycine.

Analyses of GP-4A and GP-4B. These two materials were recovered from the column eluates by lyophilization. GP-4A was similar to the undegraded material, GP-3₁, observed previously. It still contained a large amount of glycine. The only detectable free amino groups were 0.5 free ϵ -amino groups of lysine. On paper

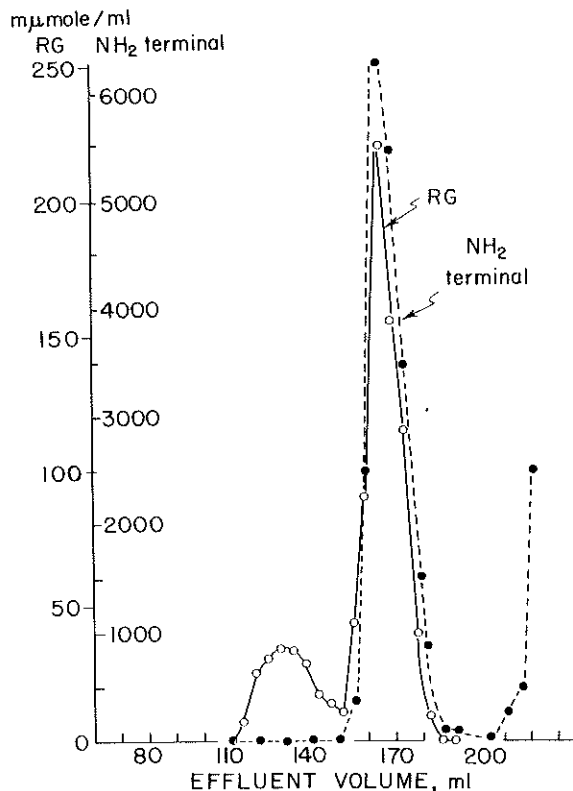


FIGURE 6: Filtration of GP-4 on a column of Sephadex G-25. This column had a V_0 of 90 ml of a V_1 of 125 ml. Fractions of 5 ml were collected at 10-min intervals. Reducing groups (RG) and free amino groups were measured. See text for details.

chromatography (Figure 2) GP-4A behaved similarly to GP-3₁, remaining near the origin.

On the other hand GP-4B contained only a small amount of glycine. The only detectable free amino groups were 1000 free ϵ -amino groups of lysine/1000 glutamic acid residues. GP-4B was separated by paper chromatography into two components, GP-4B₁ and GP-4B₂, with mobilities nearly identical with those of GP-3₂ and GP-3₃, respectively (Figure 2). The remainder of GP-4B (2-3 mg) was separated into its two components by paper chromatography and, after spraying of guide strips with ninhydrin, GP-4B₁ and GP-4B₂ were recovered from the chromatograms. Analyses of these materials (Table I) indicated that they contained glutamic acid, lysine, and alanine in the ratio of 1:1:2 and only small amounts of glycine. Each contained a free ϵ -amino group in lysine and no other free amino groups, and each contained 1 disaccharide unit. Enzymatic analysis for L- and D-alanine indicated, moreover, that each contained one L- and one D-alanine residue. The two compounds differed in that 4B₁ contained no *O*-acetyl groups while 4B₂ contained one *O*-acetyl group.

The degradation sequence and analyses indicate that peptidase 1 contains a glycine carboxypeptidase in addition to the activities previously mentioned. The pre-

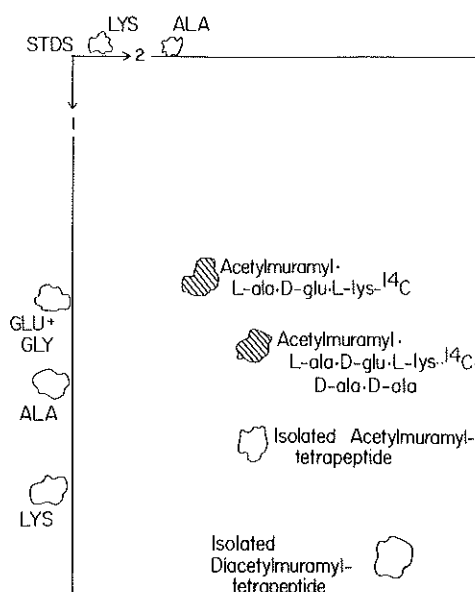


FIGURE 7: Two-dimensional paper chromatography of *N*-acetylmuramyl-tetrapeptide and *N,O*-diacetylmuramyl-tetrapeptide, the final products of the degradation sequence, with authentic reference compounds, acetylmuramyl-L-Ala-D-Glu-L-Lys- ^{14}C -D-Ala-D-Ala and acetylmuramyl-L-Ala-D-Glu-L-Lys- ^{14}C . Whatman No. 1 paper was used with the solvents (1) isobutyric acid-0.5 N NH_4OH (5:3) and (2) 1-butanol-acetic acid-water (3:1:1). The degradation products were located with ninhydrin and the authentic materials by radioautography. Separate chromatograms were run for each of the degradation products and this illustration is a composite of the results.

sumed effect of this enzyme on GP-3 is represented in Figure 3.

Degradation of GP-4B₁ and GP-4B₂ by β -*N*-Acetylglucosaminidase. The small amounts of materials remaining were then treated with β -*N*-acetylglucosaminidase from pig epididymis (Figure 3). 4B₁ yielded 0.96 residue of *N*-acetylglucosamine while 4B₂ yielded 0.90 residue. Two-dimensional paper chromatography of the residual incubation mixtures followed by detection with ninhydrin indicated that the acetylmuramyl-tetrapeptide remaining from 4B₁ and the diacetylmuramyl-tetrapeptide remaining from 4B₂ gave single spots. Acetylmuramyl-L-Ala-D-Glu-L-Lys- ^{14}C and acetylmuramyl-L-Ala-D-Glu-L-Lys- ^{14}C -D-Ala-D-Ala were employed as internal markers and were located by radioautography prior to ninhydrin development. The *N*-acetylmuramyl-tetrapeptide derived from 4B₁ by treatment with the β -*N*-acetylglucosaminidase had a faster mobility in both solvents than either of the reference compounds (Figure 7) and, similarly, the *N,O*-diacetylmuramyl-tetrapeptide derived from 4B₂ had a still faster mobility (Figure 7).

Degradation of the Soluble Glycopeptide (GP-1) by Peptidase 2. The hydrolysis of GP-1 by peptidase 2

without prior treatment by peptidase 1 has also been examined. A relatively small amount of glycine was liberated from GP-1 by the glycine aminopeptidase accompanied by the disappearance of amino terminal glycine and the appearance of free ϵ -amino groups in lysine (Figure 5). These are derived, at least in part, from hydrolysis of the minor components present in GP-1 (R_{alanine} 0.1 and 0.2; see Figure 2). These minor components disappeared during the hydrolysis and a new material with R_{alanine} 0.26 appeared. Similarly peptidase 2 results in the liberation of free L-alanine from GP-1 with the disappearance of amino-terminal alanine (Figure 4).

Discussion

Peptidase 1, therefore, contains at least three enzymatic activities, an endopeptidase (termed endopeptidase 1) which hydrolyzes the polyglycine cross bridges, a D-alanine carboxypeptidase, and a glycine carboxypeptidase. Peptidase 2 also contains three activities, endopeptidase 2 (see below) which also hydrolyzes polyglycine cross bridges, L-alanine aminopeptidase, and glycine aminopeptidase. It remains to be established whether or not there is more than one aminopeptidase or carboxypeptidase involved in catalyzing these reactions.

Several important features of cell wall structure have been established by these studies. In the first place, they establish that the polyglycine cross bridges which cross-link glycopeptide chains are attached at the C terminal end to the ϵ -amino groups of lysine, as had previously been postulated (Mandelstam and Strominger, 1961). Second, they establish that the major cell wall peptide is an acetylmuramyl-tetrapeptide, despite the fact that the precursor of this structure is a UDP¹-acetylmuramyl-pentapeptide (Park, 1952; Strominger, 1959).

The opening of the polyglycine cross bridges is apparently accomplished by two enzymes. Endopeptidase 1 is capable of hydrolyzing only 15-25% of the bridges with liberation of amino-terminal glycine. The action of peptidase 2, containing endopeptidase 2 and glycine aminopeptidase, results in opening of at least 50% of the remaining glycine bridges and liberation of free glycine from the open amino end of these bridges. The kinetics of action of peptidase 2 indicate that at first free glycine appears with little or no change in the amino-terminal glycine. After a few hours, when almost three-fourths of the free glycine has been liberated, free ϵ -amino groups of lysine begin to appear. It is necessary to postulate the existence of endopeptidase 2 in peptidase 2 because the amount of free ϵ -amino groups of lysine which finally appear is so much greater than the number of glycine bridges which are opened by endopeptidase 1. The close correspondence between the appearance of these free ϵ -amino groups and the disappearance of amino terminal glycine is the first ex-

¹ Abbreviation used in this work: UDP, uridine diphosphate.

perimental evidence to support the previous hypothesis that the polyglycine bridges are attached to the ϵ -amino group of lysine at the carboxyl end of the bridge.

These data also suggest the possibility that there may be at least two types of glycine cross bridges, differentiated by endopeptidases 1 and 2. The action of endopeptidase 2 results in the hydrolysis of a bridge in such a manner that a C-terminal glycine is left in the structure. This C-terminal glycine is then liberated by the glycine carboxypeptidase present in peptidase 1. The action of endopeptidase 1, however, does not result in the appearance of any C-terminal glycine since no free glycine is produced by peptidase 1 (which contains both endopeptidase 1 and glycine carboxypeptidase). An alternative explanation of the limited hydrolysis of polyglycine bridges by endopeptidase 1 could relate to the substrate requirements of this enzyme. It is possible that it cleaves polyglycine bridges only in the cell walls or in the large polymer, GP-1, and that, when the size of the substrate GP-1 is reduced by cleavage of a few bonds (about 1 out of 4 to 6), this enzyme is no longer able to act. This explanation is supported by the fact that when endopeptidase 1 is employed to lyse bacterial cell walls, a considerably larger fraction of the polyglycine bridges are opened (see preceding paper, Figure 7, and J. M. Ghuysen, unpublished experiments). On the contrary, endopeptidase 2 is unable to lyse bacterial cell walls or GP-1, and it is possible that this enzyme acts only on polyglycine bridges in small soluble fragments.

Some of the glycopeptide appears to be unaffected by both of these preparations as is indicated by the fact that the maximum of the ϵ -amino groups of lysine which are eventually freed is only 60% of the total lysine present. Moreover, compounds GP-3, and GP-4A appear to represent relatively undegraded glycopeptide. Thus, there could conceivably be more than two kinds of polyglycine bridges, although other unusual structural features could explain the failure of these bridges to be opened by the two endopeptidases. These possibilities will be easier to examine when the peptidase preparations are further purified. In building the three-dimensional structure of the cell wall, it may be necessary to provide for "horizontal" as well as for "vertical" cross bridges (Ghuysen *et al.*, 1965b), and it is conceivable that these two types of bridges are chemically different.

The main final product of the sequential splitting of the soluble glycopeptide by the two peptidase preparations and by β -*N*-acetylglucosaminidase is an acetylmuramyl-tetrapeptide and its *O*-acetyl derivative. This fact is of considerable interest since the precursor of this structure is an acetylmuramyl-pentapeptide. It has been repeatedly observed that the glycopeptide components of a number of bacterial cell walls contain about two alanine residues (one L and one D) per glutamic acid residue (see Salton, 1964). In view of the fact that UDP-acetylmuramyl-L-Ala-D-Glu-L-Lys (but not UDP-acetylmuramyl-L-Ala-D-Glu-L-Lys-D-Ala) is a precursor in the synthesis of UDP-acetylmuramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (Ito and Strominger, 1962), one possible interpretation of cell wall composi-

tion was that the glycopeptide contained approximately equal amounts of acetylmuramyl-tripeptide and acetylmuramyl-pentapeptide so that the average composition would be two alanines/glutamic acid residue (Mandelstam and Strominger, 1961). The present experiments indicate that acetylmuramyl-tetrapeptide derived by degradation of the wall behaves as a single compound on two-dimensional paper chromatography and that it is readily distinguished by this means from authentic acetylmuramyl-tripeptide and acetylmuramyl-pentapeptide. Further studies of the isolated acetylmuramyl-tetrapeptide will be reported when larger amounts of this compound have been isolated.

The appearance of 0.1 residue of D-alanine as the consequence of action of the D-alanine carboxypeptidase is also of interest. Early analyses of the cell wall glycopeptide from *S. aureus* indicated that there was 1.1 D-alanine residue/glutamic acid residue (Mandelstam and Strominger, 1961), and this has now been confirmed by analyses of a soluble glycopeptide. The glycopeptide (GP-2) which results from the action of D-alanine carboxypeptidase has 1.0 D-alanine residue. Moreover, the cell wall glycopeptide itself contains only 0.1 C-terminal alanine residue (Salton, 1961). An attractive hypothesis is that 10% of the repeating units are acetylmuramyl-pentapeptide units (perhaps the growing points of the wall) and that only these are the substrates for the D-alanine carboxypeptidase. Evidence relating to this hypothesis could be obtained by further studies of the specificity of the D-alanine carboxypeptidase and by the isolation of an intact acetylmuramyl-pentapeptide from the wall.

Finally, the origin of the amino-terminal L-alanine, removed by the L-alanine aminopeptidase, is not entirely clear. The action of an autolytic acetylmuramyl-L-alanine amidase, which has been found in several other bacterial species (Pelzer, 1963; Young *et al.*, 1964) during preparation of the walls, could account for the presence of this material which amounts to about 7% of the total L-alanine in the wall.

References

- Brenner, M., Niederwieser, A., and Pataki, G. (1961), *Experientia* 17, 145.
 Findlay, J., and Levvy, S. A. (1960), *Biochem. J.* 77, 170.
 Ghuysen, J. M., Dierickx, L., Leyh-Bouille, M., Strominger, J. L., Bricas, E., and Nicot, C. (1965a), *Biochemistry* 4, 2237 (this issue; preceding paper).
 Ghuysen, J. M., Leyh-Bouille, M., and Dierickx, L. (1962), *Biochim. Biophys. Acta* 63, 286.
 Ghuysen, J. M., and Strominger, J. L. (1963a), *Biochemistry* 2, 1110.
 Ghuysen, J. M., and Strominger, J. L. (1963b), *Biochemistry* 2, 1119.
 Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1964), Abstracts, 6th International Congress of Biochemistry, New York, 508.
 Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1965b), *Biochemistry* 4, 474.

- Hash, J. (1963), *Arch. Biochem. Biophys.* 102, 397.
- Hough, L., Jones, J. K. N., and Wadman, W. H. (1950), *J. Chem. Soc.*, 1702.
- Ito, E., and Strominger, J. L. (1962), *J. Biol. Chem.* 237, 2689.
- Mandelstam, M. H., and Strominger, J. L. (1961), *Biochem. Biophys. Res. Commun.* 5, 466.
- Park, J. T. (1952), *J. Biol. Chem.* 194, 877, 885, 897.
- Pelzer, H. (1963), *Z. Naturforsch.* 18b, 950.
- Reissig, J. L., Strominger, J. L., and Leloir, L. (1955), *J. Biol. Chem.* 217, 959.
- Salton, M. R. J. (1961), *Biochim. Biophys. Acta* 52, 329.
- Salton, M. R. J. (1964), *Bacterial Cell Walls*, Amsterdam, Elsevier.
- Strominger, J. L. (1959), *Compt. Rend. Trav. Lab. Carlsberg* 31, 181.
- Strominger, J. L., and Threnn, R. H. (1959), *Biochim. Biophys. Acta* 33, 280.
- Tipper, D. J., Strominger, J. L., and Ghuysen, J. M. (1964), *Science* 146, 781.
- Tipper, D. J., Ghuysen, J. M., and Strominger, J. L. (1965), *Biochemistry* 4, 468.
- Young, F. E., Tipper, D. J., and Strominger, J. L. (1964), *J. Biol. Chem.* 239, 3600.

Determination of Amino Acid Sequences in Oligopeptides by Mass Spectrometry. IV. Synthetic *N*-Acyl Oligopeptide Methyl Esters*

E. Bricas,† J. van Heijenoort,† M. Barber,‡
W. A. Wolstenholme,‡ B. C. Das,§ and E. Lederer§

ABSTRACT: A series of *N*-acylated di-, tri-, tetra-, and octapeptide methyl esters has been prepared, the *N*-acyl groups used being acetyl, decanoyl, stearoyl, and an equimolecular mixture of heptadecanoyl and octadecanoyl. In all cases, a good molecular ion peak as well as intense peaks corresponding to the cleavage of the peptide bonds were obtained.

In the first paper of this series the structure of fortuitine, a natural eicosanoyl nonapeptide methyl ester of molecular weight 1359, was determined by mass spectrometry (Barber *et al.*, 1965a). In the second paper the structure proposed for peptidolipin NA, a natural macrocyclic *N*-acyl heptapeptide of molecular weight 963, was confirmed (Barber *et al.*, 1965b). The third paper describes the determination by mass spectrometry of the structure of a natural peptidolipid from *Mycobacterium johnei*. This compound is an *N*-acyl pentapeptide methyl ester of molecular weight 945 (Lanéelle *et al.*, 1965). In each of these three compounds, the principal fragmentation observed was that of the peptide bond, thus allowing an unambiguous determina-

The basic fragmentation pattern of the peptide derivative is the same regardless of the *N*-acyl group, but the determination of amino acid sequences is easier with the *N*-stearoyl peptide esters and even more so with the peptide esters acylated with an equimolecular mixture of heptadecanoic and octadecanoic acids.

tion of the sequence of amino acids in these peptide derivatives.

The principal difference with respect to previous mass spectrometric studies of oligopeptide derivatives (Stenhagen, 1961; Heyns and Grützmaier, 1963a,b; Weygand *et al.*, 1963) was the presence in these compounds of a long-chain *N*-terminal acyl group. We have, therefore, undertaken a study of a series of synthetic long-chain *N*-acyl di-, tri-, tetra-, and octapeptide methyl esters.

For comparing the effect of the length of the *N*-acyl group we have prepared *N*-acetyl-, -decanoyl-, -stearoyl-, and mixed -heptadecanoyl and -stearoyl tetrapeptide methyl esters.

Experimental Section

Preparation of N-Acyl Oligopeptide Methyl Esters. The *N*-acyl oligopeptide methyl esters were obtained by acylation of the corresponding peptide methyl esters. Their homogeneity was confirmed by elemental analysis, by determination of physical constants (melting point

* Received July 19, 1965. Inquiries should be sent to E. Lederer.

† From the Institut de Biochimie, Faculté des Sciences Paris-Orsay, Orsay, France.

‡ From the Scientific Apparatus Department, Associated Electrical Industries, Manchester, England.

§ From the Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.