The Peptide $N^\alpha$-(L-Alanyl-$d$-isoglutaminyl)-$N^\alpha$-(d-isoasparaginyl)-L-lysyl-$d$-alanine and the Disaccharide $N$-Acetylglucosaminyl-$\beta$-1,4-$N$-acetylMuramic Acid in Cell Wall Peptidoglycan of Streptococcus faecalis Strain ATCC 9790

Jean-Marie Ghuysen, Evangelos Bricas, Melina Leyh-Bouille, Marvin Lache, and Gerald D. Shochman

ABSTRACT: A major portion of the cell wall peptidoglycan in Streptococcus faecalis is composed of the disaccharide tetrapeptide $\beta$-1,4-$N$-acetylglucosaminyl-$N$-acetylMuraminyl-$N^\alpha$-(L-alanyl-$d$-isoglutaminyl)L-lysyl-$d$-alanine. The tetrapeptides are cross-linked through single $d$-isoasparaginyl residues extending from the C-terminal $d$-alanine of one tetrapeptide unit to the N-terminal L-lysine of another. This is the first time that the occurrence of an isoasparaginyl residue in a natural product has been described. The Streptomyces SA endopeptidase cleaves $d$-alanyl-$d$-isoasparaginyl links and is thus the first enzyme known to hydrolyze $d$-peptide bonds. Treatment of the disaccharide $N^\alpha$-(L-alanyl-$d$-isoglutaminyl)$-N^\alpha$-(d-isoasparaginyl)L-lysyl-$d$-alanine with 10 equiv of NaOH at $37^\circ$C for 1 hr results in deamination of the isoasparaginyl residue together with migration of the aspartyl-lysine peptide bond giving rise to a mixture of $N^\alpha$-(\textbeta-aspartyl)- and $N^\alpha$-(\textalpha-aspartyl)L-lysyl peptides. Under the same alkaline treatment, the N-acetylMuraminyl residue undergoes a lactyl elimination which results in the production of acyl peptides and a Morgan–Elson prochroomogen compound, without hydrolysis of the glycosidic linkage. This conversion, interpreted to be the result of a $\beta$ elimination, also occurs in the other disaccharide peptide monomers previously isolated from Staphylococcus aureus, Micrococcus roseus, and Streptococcus pyogenes.

The structures of the peptide subunits and of the peptide cross-links in the cell wall peptidoglycan of several Gram-positive bacterial species have been recently determined after stepwise degradation with specific Streptomyces enzymes (Ghuysen et al., 1965; Petit et al., 1966; Muñoz et al., 1966a). These studies have demonstrated that the basal peptide subunit of the wall peptidoglycan of three species (Staphylococcus aureus Copenhagen, Micrococcus roseus R27, and Streptococcus pyogenes, group A, type 14) has the structure $N^\alpha$-(L-alanyl-$d$-isoglutaminyl)L-lysyl-$d$-alanine (Muñoz et al., 1966a). On the other hand, five types of peptide cross-links between the $\epsilon$-amino group of lysine of one peptide subunit and the terminal alanine carboxyl group of another were found (Petit et al., 1966; Tipper et al., 1967). These bridges are pentaglycerine in S. aureus Copenhagen, tris-$d$-alanine in M. roseus thr, tris-$d$-alanine-$L$-threonine in M. roseus R27, di-$d$-alanine in S. pyogenes type 14, $d$-alanine in Arthrobacter crystallinophila, and direct bonding with no additional amino acids between the C-terminal alanine residue of one peptide subunit and the $\epsilon$-amino group of lysine of another in Micrococcus luteolenticus. Until now, all of the peptide bridges examined contained either glycine or neutral $\epsilon$-amino acids. $d$-Aspartic acid is present in cell walls of Streptococcus faecalis and of numerous Lactobacillus spp in amounts nearly equivalent to that of L-lysine and D-glutamatic acid (Cannins and Harris, 1956; Toennies et al., 1959; Ikawa and Snell, 1960; Ikawa, 1964; Plapp and Kandler, 1966; Shochman et al., 1967). When walls of Lactobacillus brevis were hydrolyzed in 11 N HCl at $80^\circ$ for 43 hr a derivative of aminosuccinimide, $\epsilon$-(aminosuccinyl)lysine, was isolated and on treatment with dilute sodium hydroxide was converted into a mixture of $\epsilon$-lysine and $\epsilon$-lysine.

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† Permanent address: Institut de Biochimie, Faculté des Sciences, Orsay, France.
of predominantly N\(^2\)-(3-aspartyl)lysine and a minor amount of N\(^2\)-(a-aspartyl)lysine (Swallow and Abraham, 1958). This cyclic peptide N\(^2\)-(aminosuccinyl)lysine was later shown to be a common constituent in hydrolyzates of other aspartic acid-containing bacterial walls (Ikawa and Snell, 1960; Ikawa, 1964). In the light of previous studies dealing with the occurrence and structure of peptide bridges in cell wall peptidoglycans, the isolation of N\(^2\)-(aminosuccinyl)lysine strongly suggested, as pointed out by Swallow and Abraham (1958), that the aspartic acid residues in the relevant cell walls may serve as cross-links. However, the formation of the ring through acid hydrolysis and the following interconversion of aspartylamines made it impossible to determine whether the original sequence consisted of an \(\alpha\)- or \(\beta\)-aspartyl peptide.

The present studies deal with the stereochemistry of the \(\alpha\)-aspartic residue in the cell wall peptidoglycan of \(S. faecalis\) ATCC 9790. It will be demonstrated that in a significant portion of this peptidoglycan, \(\beta\)-isoglutaminyl bridges occur between two peptide monomers linking a C-terminal \(\alpha\)-alanine of one monomer to an \(\alpha\)-NH\(_2\)- \(\beta\)-lysine of another.

Materials and Methods

**Analytical Methods.** Identification and measurement of free amino acids and \(N\)-terminal groups by the fluorodinitrobenzene (FDNB)\(^1\) technique, of \(C\)-terminal groups by the hydrazinolysis technique, of \(L\)- and \(D\)-alanine by an enzymatic procedure, of acetamido sugars using the Morgan-Elson reaction after 7 or 30 min at 100° in 1% borate, and of total amino sugars have been described (Ghuyse et al., 1966). Amino acid analyses were carried out after hydrolysis in 6 N HCl for 20 hr or at 120° in sealed tubes. The same conditions of hydrolysis were used for measuring the N-terminal groups after dinitrophenylation. These relatively severe conditions were necessitated by the resistance to acid hydrolysis of the cyclic dipeptide N\(^2\)-(aminosuccinyl)lysine and of its dinitrophenyl derivative. Analyses of amino acids were also performed with either a Technicon AutoAnalyzer (Chromatobeads resin, type \(\beta\), \(1\mu\)) or with the Phoenix amino acid analyzer (Phoenix sphericus resin, type XX 8-60-1 for acidic and neutral amino acids and type XX 8-10-0 for basic amino acids), using an accelerated system. An infrared spectrum was taken on a KBr pellet (1.2 mg of compound for 303 mg of KBr) using a Perkin-Elmer Model 337 grating infrared spectrophotometer. Organic phosphorus was measured according to Lowry et al. (1954). Amide ammonia in soluble peptidoglycan fractions was estimated after hydrolysis with 4 N HCl at 100° for 4 hr. Under these conditions, production of ammonia from isoglucamine and isoglutamine is complete while the disaccharide \(\beta\)-1,4-N-acetylglucosaminyl-N-acetylmuramic acid yields no trace of ammonia. Ammonia was measured after partial neutralization of hydrolyzed samples (40 \(\mu\)l of 4 N NaOH for a 50-\(\mu\)l sample in 4 N HCl) using the technique of Ternberg and Hershey (1960) modified according to Tipper et al. (1967). Edman degradation was carried out essentially as described by Tipper et al. (1967). Lysophosphatidyl peptide (25-100 nmol of N-terminal groups) was dissolved in 70 \(\mu\)l of N-ethylmorpholine buffer. Phenyl isothiocyanate (1 \(\mu\)mol) was added and the solution heated for 45 min at 37°. After addition of 100 \(\mu\)l of water, excess reagent was removed by ether extraction (150 \(\mu\)l, twice). The aqueous phase was lyophilized and the phenylthiocarbamyl peptide was then treated with 50 \(\mu\)l of trifluoroacetic acid at 25° for 45 min in a sealed tube. Trifluoroacetic acid was removed by lyophilization and the residue was dissolved in 100 \(\mu\)l of 0.2 N acetic acid. The phenylthiocarbamoyl derivative of the NH-terminal amino acid was extracted with benzene (100 \(\mu\)l, twice) and terminal amino group analyses were performed by the FDNB technique on the aqueous phase (which contains the remainder of the peptide with its \(\alpha\)-amino group free). Cyclization of a phenylthiocarbamyl derivative of an amino group bearing an adjacent \(\alpha\)-carboxamide, with coconant elimination of ammonia, was carried out by treatment with 50 \(\mu\)l of 4 N HCl at 37° for 3 hr (instead of using 50 \(\mu\)l of trifluoroacetic acid). The solution was then partially neutralized by adding 40 \(\mu\)l of 4 N NaOH and ammonia was measured. With this HCl treatment, the yields of free ammonia were 15-20% from isoglutamine and isoglutamine and 60-70% from their phenylthiocarbamyl derivative. Dehydronation reduction (Ressler and Kashelikar, 1966) using ethylenechlorophosphite followed by sodium in liquid ammonia was carried out as previously described (Muñoz et al., 1966a). In the present experiments, the dehydrated and reduced samples were freeze dried and directly hydrolyzed at 120° for 15 hr with 6 N HCl (1 ml for 2 equim of original peptide). After freeze drying, the residue was dissolved in water and neutralized with 2 N NaOH. Free amino acids were then characterized and measured as dinitrophenyl derivatives.

**Thin Layer Chromatography.** Chromatography of dinitrophenyl compounds was performed on thin layer plates of silica gel (silica gel according to Staib, Merck), using the following solvents: (I) 1-butanol-0.15 N ammonia (1:1, upper phase); (II) chloroform-methanol-acetic acid (85:14:1) at 0°; (III) benzyl alcohol-chloroform-methanol-water-15 N ammonia (30:30:30:6:2); (IV) chloroform-methanol-acetic acid (90:9:1); (V) benzene-pyridine-acetic acid (80:20:2) (Brenner et al., 1961); (VI) chloroform-benzyl alcohol-acetic acid (70:30:3) (Brenner et al., 1961); and (VII) toluene-2-chloroethanol-pyridine-15 N ammonia (100:70:30:14) (Waltz et al., 1963). Routine analyses of dinitrophenyl amino acids were performed by sequential chromatography in the same direction using solvents 1 and II. \(N\)-DNP-lysine was measured after chromatography in solvent III. Chromatography of DANB (1,2-diaminonitrobenzene) derivatives of \(\alpha\)-keto acids (Taylor and Smith, 1955) was performed on thin layer silica gel in solvent VIII, 1-butanol-15 N ammonia (1:1, upper phase). Chromatography of nonvolatile carboxylic acids

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\(^1\) Abbreviations used: FDNB, fluorodinitrobenzene; DANB, 1,2-diaminonitrobenzene; SDS, sodium dodecyl sulfate.
was performed on thin layer plates of MN-cellulose powder 300 HR in solvent IX, ether–formic acid–water (13:3:1). Detection was carried out using 0.1% indophenol in ethanol.

Paper Electrophoresis and Paper Chromatography. Electrophoresis was carried out on Whatman No. 3MM paper (40 × 40 cm) at pH 5.0 (acetic acid–pyridine–water, 2:4:1000) or in 0.2 N acetic acid, at 20 V/cm using the Electrophoresor Plueger (Antwerp, Belgium) apparatus. Paper chromatography was carried out on Whatman No. 1 paper using the following solvents: (X) isobutyric acid–0.5 N ammonia (5:3), (XI) pyridine–water (8:2), (XII) 1-butanol–acetic acid–water (3:1:1), and (XIII) 1-butanol–pyridine–water (6:4:3). Compounds were detected with ninhydrin spray (0.05% in isopropyl alcohol–water (9:1)), with silver nitrate spray, with p-dimethylaminobenzaldehyde spray (Sallton, 1959), or by fluorescence after the paper had been dipped in a solution of 0.5 N NaOH in ethanol–I-propanol (6:4) and heated for 10 min at 120° (Sharon, 1964).

Enzymes. Purified Streptomyces VA endopeptidase and purified Streptomyces aminopeptidase (Ghysen et al., 1965; Petit et al., 1966; Muñoz et al., 1966a). Streptomyces N-acetylmutamuramyl-L-alanine amidase (Ghysen et al., 1962), Streptomyces VA endo-N-acetylmuramidase (Muñoz et al., 1966b), pig epididymis exo-

β-N-acetylglucosaminidase (Sanderson et al., 1962), and d-glucosamine 6-phosphate N-acetylase (Brown, 1962) were prepared as previously described. For most of the experiments, a crude preparation of Streptomyces peptidases (a mixture of aminopeptidase and of several endopeptidases) was employed (this preparation corresponds to fraction I of Figure 1 in Petit et al., 1966).

Synthetic Compounds. N\(^\omega\)-(α-Aspartyl)lysylisoleucyl

valylglycine and N\(^\omega\)-(β-aspartyl)lysylisoleucylvalylglycine were gifts from Dr. J. Savard and Dr. A. Kotai (Institut de Biochimie, Orsay, France); N\(^\omega\)(aminosuccinic)lysine from Dr. M. Ikawa, University of New Hampshire. L-Isosparagin was a gift from Dr. C. Resler, Institute for Muscle Disease, New York, and from Dr. W. K. Paik, Temple University. Synthetic N-acetylmuramic acid was a gift from Dr. R. Jeanloz, Harvard University.

Cell Walls and Soluble Disaccharide Peptide Oligomer.
Experimental Section

I. Isolation of the Disaccharide Pentapeptide Monomer. Preliminary Assays. The sequential enzymatic degradation of *S. faecalis* cell walls essentially followed the procedures previously applied to the cell walls of *S. aureus*, *M. roseus*, and *S. pyogenes* (Mukerji et al., 1965a) with the two following differences. (1) After solubilization of the SDS walls by degradation with purified SA endopeptidase (hydrolysis of linkage I, Figure 1), the newly appeared N-terminal aspartic acid could not be liberated as free aspartic acid by further treatment with *Streptomyces* aminopeptidase. Thus a crude *Streptomyces* peptidase preparation could be used in place of the purified endopeptidase with identical results. (2) The lytic F1 endo-N-acetylmuramidase hydrolyses most of the N-acetylmuramyl linkages in the SA endopeptidase-solubilized SDS walls (hydrolysis of linkage 2, Figure 1), yielding disaccharide units which are still peptide substituted. However, the *S. faecalis* autolysin present in log walls (which has the same specificity as the F1 endo-N-acetylmuramidase) can work in conjunction with the SA endopeptidase (in 0.01 M Veronal-HCl buffer, pH 8.4). Thus, incubation of log walls with endopeptidase induced not only the appearance of new terminal amino groups of aspartic acid but also the cleavage of glycosidic linkages. Therefore, the one incubation resulted in the liberation of disaccharide peptide monomers.

At completion of the degradation, both autolyzed log walls and F1 endo-N-acetylmuramidase-solubilized SDS walls contain acetamido sugars equivalent to about 360 μmoles of disaccharide/mg (yield, 80% on the basis of 450 μeq/mg of peptidoglycan units/mg) of both autolyzed and F1-degraded cell walls have equivalent amounts of N- and C-terminal groups, the content of which, therefore, can be visualized as being native to these wall preparations. Under action of the SA endopeptidase, the number of N-terminal aspartic acid residues is significantly increased from 80 μmoles/mg to 180 μmoles/mg. However, using the synthetic peptide N*-Lys-Lys-Val-Gly* as a model for the N-terminal estimations, the recovery of DNP-aspartic acid was only 60-70% of the theoretical value. When corrected on this basis, N-terminal aspartic acid amounts to about 100 μmoles/mg in the native walls and to about 250 μmoles/mg in the endopeptidase-treated walls. Endopeptidase treatment did not modify the number of C-terminal lysines (about 100 μmoles/mg) but resulted in an increase of C-terminal alanines from 100 to about 250 μmoles/mg. It appears, therefore, that peptidase action results at least mainly in the cleavage of the peptide bond between the amino group of aspartic acid and the carboxyl group of alanine. It should be noted, however, that, under SA endopeptidase treatment, an apparent small increase in the number of N-terminal lysine residues, from about 40 to about 80 μmoles/mg of walls, has been repeatedly observed. It may be that native N-terminal lysine residues are more accurately measured when the FDNB procedure is applied to a peptide moiety of reduced size.

Preparative Run. Log walls (300 mg) were treated at
TABLE I: Analysis of *S. faecalis* Disaccharide Peptide Monomer (DPM), Disaccharide Peptide Oligomer (DPO), and the Acyl Peptides Aα, Aβ, and Aαβ.

<table>
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<th>Results are Expressed in mmoles/1000 Total Glutamic Acid</th>
<th>DPO</th>
<th>DPM</th>
<th>Aα</th>
<th>Aβ</th>
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<td>A. Disaccharide moiety</td>
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<tr>
<td>Total HexNac* after exo-GlcNacase*</td>
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<tr>
<td>Total HexNac* after HCl hydrolysis</td>
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<td>1090, 1170</td>
<td>0</td>
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<td>B. Total amino acids</td>
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<td>970</td>
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<td>E. Carboxyl-terminal groups*</td>
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<td>F. Enzymatic degradation</td>
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<td>G. Amino-terminal groups after one cycle of Edman degradation</td>
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<td>H. Total amino acids after Ressler-Kashelikar degradation</td>
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* HexNac, N-acetyhexosamine; GlcNac, N-acetylglucosamine; exo-β-GlcNacase, exo-β-N-acetylglucosaminidase.
* Data for N- and C-terminal groups are not corrected. * The yield of transformation can be expressed in per cent of β-alanine in the mixture β-alanine and aspartic acid as they are recovered (16%) or in per cent of γ-aminobutyric acid in the mixture γ-aminobutyric acid and glutamic acid as they are recovered (17%).

37° with 18 mg of protein of the crude *Streptomyces* peptidase preparation in 14 ml of 0.01 M Veronal buffer (pH 8.4) for 11 hr. The small residual turbidity remaining was removed by centrifugation and the supernatant concentrated by lyophilization and filtered in water on a column of Sephadex G-25 bead form (V₀ + Vᵰ = 800 ml). As shown in Figure 2, a Morgan–Elson-positive, FDNB-positive peak, tentatively identified as disaccharide peptide monomer (fractions 54–69), was obtained relatively well separated from the other substances present. The first peak of gel-excluded material contained organic phosphorus (from teichoic acid) and only a relatively small amount of Morgan–Elson-positive material (fractions 30–44). The second peak fraction (fractions 44–54) is poorly resolved and probably contains incompletely degraded peptidoglycan. Fractions 54–69 were combined, lyophilized, and after a second filtration on the same Sephadex column yielded, irrespective of the component used for the estimation, 48 μmoles of monomeric disaccharide pentapeptide. This represents 35% of the theoretical yield. Similar yields were obtained from other degradations carried out under similar conditions.
FIGURE 3: Dehydration reduction of *S. faecalis* diisaccaride peptide oligomer according to Ressler and Kashelkar (1966). Two-dimensional chromatography on thin layer silica gel of the dimethylphenyl derivatives of a control mixture of amino acids and of the amino acids from the dehydrated and reduced oligomer. Abbreviations: 1, DNPH; 2, γ-aminobutyric acid; 3, β-alanine; 4, lysine; 5, alanine; 6, glutamic acid; 7, aspartic acid; 8, ornithine; 9, α,γ-diaminobutyric acid. For solvents, see Material and Methods.

out under identical conditions. These figures are low in comparison to the yields of monomeric disaccharide peptide (50–60%) previously obtained from cell walls of *S. aureus* and *M. roseus* (Muñoz et al., 1966a).

II. Structure of the Disaccharide Peptide Monomer and of the Disaccharide Peptide Oligomers. Disaccharide moiety. Analyses of HCl hydrolysates by two-dimensional chromatography in solvents XI and XII or with the autoanalyzer showed the presence of muramic acid and glucosamine in approximately equivalent amounts. The disaccharide moiety was more accurately estimated from (Table I A); (1) total hexosamine content of acid hydrolysates (3 N HCl, 3 hr, 100°C) using the Morgan–Elson reaction (7 min at 100°C) after chemical recrystallization; (2) glucosamine content of these HCl hydrolysates using the same procedure but after specific enzymatic recrystallization with the β-glucosaminidase; and (3) N-acetylglucosamine liberated by the exo-β-N-acetylglucosaminidase (hydrolysis of linkage 3, Figure 1), once again determined by the Morgan–Elson reaction as above. Finally, the disaccharide was liberated from the peptide moiety through the action of the N-acetylglucosaminyl-γ-alanine amidase (hydrolysis of linkage 3, Figure 1). It was characterized as β-1,4-N-acetylglucosaminyl-N-acetylmuramic acid by paper chromatography in solvents X, XII, and XIII using authentic disaccharide as standard, and by determining its molar extinction coefficient (ε 9500) in the Morgan–Elson reaction after 30 min at 100°C in 1% borate. This value distinguishes this disaccharide from a β-1,6-linked disaccharide (Sharon et al., 1966) and from the isomeric β-1,4-N-acetylglucosaminyl-N-acetylmuramic disaccharide (Tipper and Strominger, 1966).

Amino acids, C- and N-terminal groups. Within the limit of experimental error, the disaccharide peptide monomer fraction contains essentially one N-terminal group (actually 0.14 N-terminal lysine and 0.62 aspartic acid) and one C-terminal group (actually 0.25 lysine and 0.47 alanine) per glutamic acid, thus establishing its monomeric structure (Table II B, D, and F). The presence of small amounts of N-terminal lysine and C-terminal lysine is indicative of small amounts of incomplete monomeric units. The monomeric structure was confirmed by paper chromatography; in solvent X the fraction migrates with *R*~f~ 0.58 which is identical with that of the *S. aureus* and *M. roseus* disaccharide peptide monomers which were run as standards. The disaccharide peptide oligomer fraction has the same over-all chemical composition as the disaccharide peptide monomer fraction but contains much fewer N- and C-terminal groups. Its polymeric structure is further confirmed by its exclusion from Sephadex G-25 gel and by its paper chromatography in solvent X (smears with *R*~f~ of 0.31–0.43).

Enzymatic degradation. The disaccharide peptide monomer was sequentially degraded (Table II F) first by the N-acetylglucosaminyl-γ-alanine amidase (hydrolysis of linkage 3, Figure 1) which exposed essentially one N-terminal γ-alanine per glutamic acid (actually 0.86) and next by the *Streptomyces* aminopeptidase (hydrolysis of linkage 4, Figure 1) which liberated one γ-alanine residue (actually 0.92) and exposed one N-terminal glutamic acid (actually 0.74). These degradations were carried out exactly as previously applied to other disaccharide peptide monomers (Muñoz et al., 1966a).

Amide determination. Hydrolysis of the disaccharide peptide monomer or of the disaccharide peptide oligomer in 3 N HCl for 4 hr at 100°C yielded virtually 2 moles of ammonia/mole of glutamic acid (Table III C). This observation coupled with the fact that both fractions are neutral on paper electrophoresis at pH 5 strongly suggests that the carboxyl groups of both glutamic acid and aspartic acid, which are not engaged in peptide linkages, are amide substitutted. The neutral properties of the two compounds could also be explained if only glutamic acid was amidated and if aspartic acid was present as a N-aminosuccinyllysyl ring derivative. This, of course, would not explain the presence of two amide ammonia moieties per glutamic acid and has been further ruled out by the infrared spectrum of the disaccharide peptide oligomer. No traces of bands at 1705 and 1785 cm⁻¹, which are known to be characteristic of the carboxyl group of the succinimide ring (Swallow and Abraham, 1958), could be detected.
Dehydration Reduction. The nature of an amide-substituted glutamic or aspartic acid residue can be determined by dehydration reduction of a peptide in which the relevant residues are in an endo position (Ressler and Kashelkar, 1966). γ or β substitution will produce ornithine (from a glutaminyl residue) or α,γ-diaminobutyric acid (from an asparaginyl residue). Conversely, isoglutamine and isoasparaginyl will give rise to γ-amino-butyric acid and β-alanine, respectively. Degradation of the disaccharide peptide oligomer, in which the glutamic acid residues and most of the aspartic acid residues are in endo positions, was attempted despite the fact that this fraction was not soluble or only poorly soluble in the solvent triethyl phosphate used for the dehydration. This is probably due to the polymeric structure of the compound and is probably the cause of the poor recovery of amino acids normally untouched by the procedure (Glu, 30%; Ala, 38%; and Lys, 12%) and of the poor conversion of the amidated glutamic and aspartic acids (15–17%). The results are given in Table IV. No traces of α,γ-diaminobutyric acid or ornithine were detected. β-Alanine and γ-aminobutyric acid were unequivocally characterized in the forms of dinitroneyl (DNP) derivatives and were further identified by thin layer chromatography in various solvent systems combining solvent I or VII in a first direction and solvent IV, V, or VI in the second direction. This is illustrated in Figure 3.

Edman Degradation. The disaccharide peptide monomer in which isoasparagine is in the N-terminal position was also submitted to an Edman degradation. After one cycle of the degradation, N-terminal aspartic acid almost completely disappeared and was not replaced by any other N-terminal group (Table III). Aspartic acid is thus linked through its β-carboxyl group to the remainder of the peptide, which is in agreement with its occurrence in the form of an isoasparaginyl residue. However, after coupling the disaccharide peptide monomer to phenyl isothiocyanate, cyclization in 3 N HCl for 3 hr at 37°C did not induce liberation of free ammonia (while a partial liberation of ammonia is observed from an isoasparagine control; see Methods). It is felt, however, that this negative result is not inconsistent with an N-(isoasparaginyl)lysyl peptide structure which, as shown in the following paragraph, presents peculiar chemical reactivities.

Deamination and interconversion of the N′-isoasparaginyl-β-lysyl moiety into N′-β- and α-aspartyl-β-lysyl derivatives. Preliminary assays. Initially it was observed that ammonia was produced when equivalent volumes of a disaccharide peptide monomer solution and of a saturated K₂CO₃ solution were mixed in a diffusion vial (as used for the ammonia determination) and rotated at 25°C for 1–2 hr. It was then found that treatment at 37°C for 1 hr of the disaccharide peptide monomer with 10 equiv of NaOH (example, 50 μmoles in 10 μl of 0.05 N NaOH) resulted in the liberation of 1 mole of ammonia/mole of disaccharide. After such treatment, the disaccharide peptide monomer still contained 1 mole of amide ammonia. The disaccharide N′-(β-alanyl)-isoasglutaminyl-β-lysyl-d-alanine compounds isolated from S. aureus, M. roseus, and S. pyogenes cell walls were not deamidated under the same conditions. Therefore the remaining mole of ammonia was very probably in the form of an isoglutaminyl residue. It was also observed that, under the above
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Figure 5: Paper electrophoresis in 0.2 n acetic acid of NaOH-treated disaccharide peptide monomers, fraction Aα, and synthetic N°N-(α- and -β-aspartyl)lysyl peptides. Aliquots were spotted at 10 cm from the anode and electrophoresis was carried out for 2 hr at 20 v/cm. Nishhydrin (peptide moiety) and fluorescence (disaccharide moiety, not shown) were used for detection. Neutrality is at the level Aβ. Abbreviations: SA, disaccharide peptide monomer of S. aureus; SF, disaccharide peptide monomer of S. faecalis; Aα, see Figure 4; α, N°N-(α-Asp)-Lys-Val-Gly; β, N°N-(α-Asp)-Lys-Val-Gly. The four untreated disaccharide peptide monomers from S. aureus, M. rosenus, S. pyogenes, and S. faecalis and the three acyl peptides from the NaOH-treated S. aureus, M. rosenus, and S. pyogenes disaccharide peptide monomers are basic and migrate identically. The S. faecalis acyl peptide dissociates into two components (Aβ and Aα).

NaOH treatment, the S. faecalis disaccharide peptide monomer, as well as the three other disaccharide peptide monomers just mentioned, were cleaved at the junction N-acetylmuramyl-γ-l-alanine giving rise to an acyl peptide plus a neutral lactyl-less disaccharide (see part III). The acyl peptides did not contain any N-terminal alanine and treatment with the N-acetylmuramyl-γ-l-alanine amidase failed to produce such an amino group.

Isolation of the S. faecalis Acyl Peptides. On paper electrophoresis at pH 5, the S. faecalis acyl peptide is acidic (compound Aβ in Figure 4) and separates well from both the lactyl-less disaccharide (compound N, detected by fluorescence, Figure 4) and the untreated disaccharide peptide monomer which are neutral. Compound N is contaminated by only traces of original disaccharide peptide monomer (as evidenced by the weakly ninhydrin-positive spot). Also, as shown in Figure 4, a trace of a third compound (compound Aγ), slightly less acidic than the main compound Aα, was detected. Compounds N, Aα, and Aγ from S. faecalis were obtained by preparative paper electrophoresis and further purified by gel filtration on Sephadex G-25 in water (V₄ + V₁ = 160 ml) (elution volumes, 105 ml for Aγ and Aα; 135 ml for N). The acyl peptide Aα had no acetylaminosugars and contained 78% of the amino acids present in the original S. faecalis disaccharide peptide monomer preparation. The neutral lactyl-less disaccharide (compound N) contained 92% of the N-acetylmuraminosugars of the original disaccharide peptide monomer (estimated after enzymatic release of the muraminosugars in an HCl hydrolysate). The Aγ compound was probably a trace of the original disaccharide peptide monomer which had only undergone deamidation of its lysosparaginyl residue. This compound will not be discussed further. On further paper electrophoresis in 0.2 n acetic acid (Figure 5), the acyl peptide Aα from S. faecalis dissociated into two components: compound Aβ which is now neutral and compound Aα which is still acidic. Preparative electrophoresis of fraction Aα in 0.2 n acetic acid was used to isolate these two compounds which were further purified by gel filtration on Sephadex G-25. The relative yields were 80% of Aβ and 20% of Aα.

Structure of the S. faecalis Acyl Peptides. Compounds Aα, Aβ, and Aγ have the same amino acid composition and virtually the same number of amino- and carboxyl-terminal groups as the untreated disaccharide peptide monomer (Table I). One exception is the smaller amount of unsubstituted N'-lysine peptide in compound Aα and its absence in compounds Aα and Aβ. The two striking differences are the presence of only one amide amonia and the complete absence of N-acetylmuraminosugars residues in the three acyl peptide compounds. After Edman degradation of compound Aα, N-terminal aspartic acid disappeared and was quantitatively replaced by N'-terminal lysine, conclusively showing that it is an N°N-(α-aspartyl)lysyl peptide. Conversely, compound Aβ is the N°N-(β-aspartyl)lysyl peptide since, under the same conditions, the disappearance of N-terminal aspartic acid did not result in the exposure of any other terminal amino group. Quite clearly, fraction Aα is a mixture of two acyl peptides, Aγ and Aβ. The yield of the Edman degradation also confirms the ratio Aβ:Aγ of 4:1 found by paper electrophoresis in 0.2 n acetic acid. From the foregoing, the structure N°N-(β-aspartyl)-N°N-(γ-l-alanyl-d-α-lysaminomethyl)-γ-l-lysyl-d-alanine can be assigned to the main compound in fraction Aβ and the structure N°N-(α-aspartyl)-N°N-(γ-l-alanyl-d-β-lysaminomethyl)-γ-l-lysyl-d-alanine to the main compound in fraction Aα.

Conclusion. The main component of the S. faecalis disaccharide peptide monomer preparation must, therefore, have the sequence β-Lα-N-acetylmuraminosugars N-acetylmuramyl-γ'-l-alanyl-d-Gluα-Gluα-Me-CONH₂-γ-l-Aspα-CONH₂-l-Lys-d-Ala. Minor amounts of other disaccharide monopeptides are present which either lack a terminal d-alanine residue or have an unsubstituted N'-lysine residue (see Table I). Disaccharide peptide oligomers appears to be an assortment of oligomers in which disaccharide peptide monomers are cross-linked through d-alanyl-d-aspartyl linkages.

III. The Base-Catalyzed Lactyl Elimination from N-Acetylmuramic Acid. Alkaline Degradation of Synthetic N-Acetylmuramic Acid. Treatment of syn-
thetic N-acetylmuramic acid with 10 equiv of NaOH for 1 hr at 37° gave rise to a neutral, reducing compound which reacted as a Morgan-Elson chromogen without heating in borate, fluoresced with Sharon’s reagent, migrated on paper in solvent XIII with an $R_p$ of 0.65 (Figure 6), and was no longer detectable by indophenol spray after cellulose thin layer chromatography in solvent IX. Under the last conditions, the NaOH-treated N-acetylmuramic acid yielded a spot with the same $R_p$ value as a sample of authentic hexitc acid ($R_p$ 0.85). This value was different from that of N-acetylmuramic acid (strenk, $R_p$ 0.6-0.9) or pyruvic acid ($R_p$ 0.15). The NaOH-treated N-acetylmuramic acid was also treated with DANB (see Methods) and the mixture was submitted to silica gel thin layer chromatography in solvent VIII. No spot corresponding to the quinoxoline derivative of pyruvic acid could be detected. These data, taken altogether, provide evidence for the elimination of lactate from N-acetylmuramic acid and, by extension, for the elimination of lactoyl peptide from the disaccharide peptide monomer as recently proposed by Perkins (1967).

The *S. faecalis* Neutral Lactyl-Less Disaccharide. The Dimeric Structure. As already pointed out, disaccharide N is neutral. It migrates as a single spot in solvent XII (relative migrations in centimeters: N = 13, N-acetylglycosamine = 15, and N-acetylmuramic acid = 25) and solvent XIII (Figure 6). It can be detected using the silver nitrate or the p-dimethylaminobenzaldehyde sprays or by fluorescence. It gives a positive Morgan-Elson reaction only after appropriate heating in 1% borate. Its elution volume on Sephadex G-25 (see above) is consistent with a disaccharide structure. Its content of N-acetylglycosamine can be specifically estimated after enzymatic racemization of an HCl hydrolysate, using the 7-min Morgan-Elson reaction. Exo-$\beta$-N-acetylglycosaminidase cleaves the compound into N-acetylglycosamine and a compound migrating in solvent XIII with $R_p$ 0.65 (Figure 6), i.e., the same as that of NaOH-treated N-acetylmuramic acid (see above). This latter compound is neutral on electrophoresis at pH 5 and develops a color that has a maximum at 585 $\mu \lambda$, characteristic of acetamido sugars, when directly treated with Ehrlich’s reagent for 20 min at 37°. It can thus be concluded that compound N is a dimer with an N-acetylglycosamine residue at the nonreducing end, glycosidically $\beta$ linked to a reducing neutral compound. When freed from N-acetylglycosamine, this reducing compound has chromogenic properties in the presence of Ehrlich’s reagent.

Molar Extinction Coefficient in the Morgan-Elson Procedure of the Lactyl-Less Disaccharide. Disaccharide N can be estimated from the specific enzymatic determination of its glycosamine content or by determining its total hexosamine content after NaBH$_4$ reduction (for conditions of reduction, see Lévy-Boulle et al., 1966). The molar extinction coefficient of disaccharide N in the Morgan-Elson procedure was determined. Disaccharide N was dissolved separately both in water and in 1% borate. Aliquots (125 $\mu l$) of the borate solution were heated in boiling water for 7 and 30 min. The borate and water solutions (125 $\mu l$) were then mixed with 500 $\mu l$ of the Morgan-Elson reagent (Ghuyse et al., 1966) and heated at 37° for 20 min. Extinction coefficients are shown in Table II. Compound N has virtually the same coefficient as the untreated disaccharide N-acetylglycosaminyl-$\beta$-1,4-N-acetylmuramic acid after heating in borate for 30 min. However, since development of coloration requires prior hydrolysis of the 1,4 linkage (Tipper et al., 1965), this linkage in compound N seems to be more sensitive to alkali than that in the untreated disaccharide. Indeed, 7 min at 100° in borate is almost sufficient to induce full color development with the lactyl-less disaccharide.

Molar Extinction Coefficient in the Morgan-Elson Procedure of the Free Chromogen Arising from the N-Acetylmuramic Acid Residue. Aliquots of the purified *S. faecalis* compound N were quantitatively hydrolyzed with exo-$\beta$-N-acetylglycosaminidase (final volume: 25 $\mu l$ of 0.01 M citrate buffer, pH 4.2). Some aliquots were placed at 37° for 20 min in the presence of 600 $\mu l$ of Ehrlich’s reagent (Ghuyse et al., 1966). Others were first mixed with a borate solution (final volume: 125 $\mu l$ containing 1% borate), exposed to 100° for 7 min, and then treated with 500 $\mu l$ of the Ehrlich’s reagent as above. Since N-acetylglycosamine has a molar extinction coefficient in the Morgan-Elson reaction of 0.0 in the absence of borate treatment and of 20,000 after 7 min at 100° in 1% borate (Ghuyse et al., 1966), the molar extinction coefficient relative to the chromogenic compound originating from the N-acetylmuramic acid residue could be calculated. The results are reported in Table II. The free chromogen has the same molar coefficient (19,000-20,000) as authentic N-acetylmuramic acid, providing that both compounds are exposed to 1% borate for 7 min at 100° before the addition of Ehrlich’s reagent. In the absence of the borate treatment, the chromogen prepared as indicated has a coefficient of 40,000.

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**Table II:** Molar Extinction Coefficients in the Morgan-Elson Procedure.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No Borate</th>
<th>7 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-1,4-X-Y</td>
<td>0</td>
<td>3,500</td>
<td>9,500</td>
</tr>
<tr>
<td>$\beta$-1,4-X-Chrmg (i.e., <em>S. faecalis</em> fraction N)</td>
<td>0</td>
<td>8,000</td>
<td>10,500</td>
</tr>
<tr>
<td>Chrmg arising from Y, calculated after exo-$\beta$-GlcNucase hydrolysis of compound N</td>
<td>40,000</td>
<td>20,000</td>
<td></td>
</tr>
</tbody>
</table>

* X, N-acetylglycosamine; Y, N-acetylmuramic acid; Chrmg, chromogenic compound.
**Figure 6**: Paper chromatography in solvent XIII of the chromogenic compound arising from N-acetylglucosamine and of related compounds. Abbreviations: X, N-acetylglucosamine (Rf 0.5); Y, N-acetylmuramic acid (Rf 0.38); X-Y, N-acetylglicosaminyl-β-1,4-N-acetylmuramic acid (Rf 0.35); N, compound N or N-acetylglicosaminyl-β-1,4-prochormogen (Rf 0.48); Nh, compound N after hydrolysis by exo-β-N-acetylglicosaminidase; Y1, NaOH-treated N-acetylmuramic acid (Rf 0.65). Detection was made using Saltman's (1959) reagent or by fluorescence after heating in NaOH.

**Requirements for Lactyl Elimination.** The alkali-catalyzed conversion of N-acetylmuramic acid into a chromogenic compound under the conditions described requires the presence of the reducing group, the acetamido group, and the lactyl substituent on C-3. Indeed, such a chromogen is produced from synthetic and natural N-acetylmuramic acids (see above and Figure 6) and from the β-1,4-N-acetylglicosaminyl-N-acetylmuramic acid disaccharide but not from muramic acid or from the isomeric β-1,4-N-acetylmuraminyl-N-acetylglicosaminyl disaccharide. Moreover, glucosamine and N-acetylglicosamine are not converted into a chromogen by NaOH under the conditions used.

**Discussion**

The *S. faecalis* Peptidoglycan. Bacterial cell wall peptidoglycans can be visualized as a network resulting from the covalent association of three basal components: polysaccharide chains, peptide subunits branching off from the polysaccharide chains, and the bridges which cross-link the peptide subunits. These three components in the *S. faecalis* peptidoglycan have been studied by means of sequential enzymatic and chemical degradations.

The *S. faecalis* polysaccharide moiety has been quantitatively degraded into β-1,4-N-acetylglicosaminyl-N-acetylmuramic acid disaccharide units with the help of either the indigenous wall autolysin (Shockman et al., 1967) or the Streptomyces F1 endo-N-acetylmuraminidase. It is thus composed of alternating N-acetylglicosamine and N-acetylmuramic acid, a structure which increasingly appears to be ubiquitous in the bacterial world. All of the N-acetylmuramic acids are peptide substituted. Such a high degree of substitution was previously found in *S. aureus*, *M. roseus*, and *S. pyogenes* peptidoglycans (Muñoz et al., 1966a). However, this cannot be considered to be a general characteristic of all bacterial peptidoglycans. *M. lysodeikticus* peptidoglycan is the best known example of a peptidoglycan characterized by a very low degree of such peptide substitution (Leyh-Bouille et al., 1966).

The *S. faecalis* peptide subunit has the sequence N°-(a-alanyl-α-isoglutamyl)-1-lysyl-d-alanine, i.e., exactly that sequence previously found in *S. aureus*, *M. roseus*, and *S. pyogenes* (Muñoz et al., 1966a). It should be noted, however, that among L-lysine-containing peptidoglycans, this sequence is also susceptible to minor variations. In *M. lysodeikticus*, a glycine residue, instead of an amide, is present on the α-carboxyl group of glutamic acid (Tipper and Strominger, 1965; Mirelman and Sharon, 1965; Tipper et al., 1967).

The *S. faecalis* peptide cross-links consist of single N-isoasparaginyl residues extending from the C-terminal d-alanine of one peptide subunit to the N°-lysine of another. The proposed foregoing structure rests upon isolation from cell walls of the disaccharide pentapeptide β-1,4-N-acetylglicosaminyl-N-acetylmuramyl-N°-(a-alanyl-α-isoglutamyl)-1-lysyl-d-alanine. The location of the N-isoasparaginyl residue as a substituent of the N°-lysine in this disaccharide pentapeptide monomer emerges from a series of comprehensive determinations: (1) presence of two amidine ammonias per monomer unit; (2) disappearance through Edman degradation of the terminal amino groups of asparagine without consequent exposure of any other terminal amino group (which is compatible with a peptide bond involving the β-carboxyl group of asparagine; (3) production of β-alanine through dehydration reaction according to Ressler and Kashelikar (although the yield of transformation is poor; see Experimental Section); (4) absence of an aminosuccinimide ring as shown by the infrared spectrum; (5) deamination of the isoasparaginyl residue followed by interconversion into a mixture of N°-(β-aspartyl)lysyl and N°-(α-aspartyl)lysyl derivatives; and (6) confirmation by Edman degradation of the presence of the β and α links in the two former peptides.

N-Isasparagine is the first dicarboxylic, the first amidated, and the first D isomer found as a cross-linking amino acid in the bacterial wall peptidoglycans. Studies dealing with the occurrence of D-aspartic acid in bacterial walls suggest the possibility that such a peptide bridge might be a taxonomic characteristic of certain groups of bacteria, i.e., *Lactobacilli* and some related microorganisms. To the authors' knowledge, this *S. faecalis* peptidoglycan is the first natural peptide or proteinlike material in which the occurrence of an isoasparaginyl residue has been demonstrated. *β-Aspartyl*
peptides have been identified in human urine, mainly the dipeptide β-aspartylglycine (Buchanan et al., 1962; Dorer et al., 1966), and in enzymatic hydrolysates of proteins of various origin (Haley et al., 1966).

The disaccharide N\(^{-}\)-(L-alanyl-D-isoglutaminyl)-N\(^{-}\}(D-isosparaginyl)-L-lysyl-D-alanine results from the cleavage through an endo-N-acetylmuramidase of the N-acetylmuramyl linkages in the polysaccharide chains and from hydrolysis by the Streptomyces SA endopeptidase of D-alanyl-D-isosparaginyl linkages in the peptide moiety. Evidence for the opening of these D-alanyl-D-isosparaginyl linkages is provided by the C- and N-terminal group analyses carried out on cell walls which undergo solubilization in the presence of the endopeptidase, on the purified disaccharide peptide oligomer fraction obtained after treatment of the walls by an endo-N-acetylmuramidase alone, and on the disaccharide peptide monomer obtained after treatment with the endo-N-acetylmuramidase and the SA endopeptidase working in conjunction. Previous work had shown that in S. faecalis aspartic acid was virtually exclusively in the \(d\) form, which explains the complete resistance of the D-isosparaginyl residue in the disaccharide pentapeptide toward the Streptomyces aminopeptidase. On the other hand, the sequential degradation of the S. faecalis disaccharide peptide monomer with the N-acetylmuramyl-L-alanine amidase and then the Streptomyces aminopeptidase conclusively shows that the portion of the alanine content which is in the \(L\) form substitutes the N-acetylmuramic acid residues of the polysaccharide chains as it does in many other bacterial peptidoglycans. Consequently, the alanine residues engaged in the alanyl-D-isosparaginyl linkages are in the \(D\) form. SA endopeptidase is able to dissolve walls by hydrolyzing D-alanyl-glycyl linkages in S. aureus and D-alanyl-L-alanyl linkages in M. roseus and S. pyogenes (Petit et al., 1966; Muñoz et al., 1966a). As a result of the present work, SA endopeptidase or another enzyme present in the same preparation appears also to be able to split D-alanyl-D-isosparaginyl linkages and thus, to our knowledge, the first enzyme described capable of hydrolyzing D-D-peptide linkages.

The disaccharide pentapeptide isolated from S. faecalis represents a major part of the cell wall peptidoglycan. However, the actual over-all yield (35%) and the gel filtration patterns of the enzyme-treated cell walls (Figure 2) indicate that more than a negligible portion of the peptidoglycan is insensitive to the SA endopeptidase. This observation is strengthened by the fact that S. faecalis cell walls, when prepared from stationary-phase (threonine deprived) cultures, are dissolved by endo-N-acetylmuramidases (Streptomyces F\(_1\), S. faecalis autolysis, and lysozyme) but are only very slowly and very incompletely solubilized by the SA endopeptidase.
Whether this resistance is a consequence of some structural peculiarities in the peptide moiety or results from the inhibition of the enzyme by other cell wall components is not known.

Deamination and Interconversion of the N'-Isopropargyllysyl Peptide. The base-catalyzed deamination and interconversion of the N'-isopropargyllysyl peptide yield 4 moles of the N'-α-aspartyllysyl derivative mole of the N'-α-aspartyllysyl derivative. The succinimide derivative N'-α-(aminosuccinyl)lysine is known to be rapidly converted (as a result of the ring opening by cold dilute alkali) into a mixture of β- and α-aspartyllysines in which the β isomer also greatly predominates (Swell and Abraham, 1958; Iwama, 1964). It seems plausible that the transformation, with the concomitant loss of 1 mole of ammonia, of the N'-isopropargyllysyl portion of the S. fecalis disaccharide peptide monomer into the β- and α-aspartyllysyl peptides involved the transitory formation of a cyclic aminosuccinimide derivative. The alkaline-catalyzed formation of an imide from an α-carboxamide β peptide was not expected. It does not occur with the isoglutaminyl residue in the disaccharide peptide monomer itself and might be restricted to a N'-isopropargyllysyl structure. The mechanism of this reaction might be related to the well-known imide formation from an amide ester (i.e., cyclization of N-acetyleasparagine or N-acetyleasparagine esters; Sondeheimer and Holley, 1954, 1957) or from a peptide ester (i.e., cyclization of esters of N-benzoyl-(α or β)-aspartyl peptides; Battersby and Robinson, 1955). These latter cyclizations are performed with extreme ease at room temperature by treatment with 1 equiv, or even less, of NaOH. Further alkaline treatment of these imides results in the ring opening in both possible ways.

The Base-Catalyzed Lactyl Elimination from N-Acetylglucosamine. The S. fecalis disaccharide peptide monomer (as well as other disaccharide peptide monomers from S. aureus, M. roseus, and S. pyogenes), when treated for 1 hr at 37° with 10 equiv of NaOH, is cleaved at the junction N-acetylglucosamyl-α-alanine. An acetyl peptide (most likely a lactyl peptide) and a neutral still-reducing disaccharide are produced. The N-acetylglucosamine acid moiety which has undergone the lactyl elimination remains glycosidically linked to N-acetylglucosamine (which is at the nonreducing end of the disaccharide). This dimer does not react directly with Ehrlich’s reagent (Table II) unless it is heated (100°) in borate. Ero-β-N-acetylglucosaminidase liberates the lactyl-less modified N-acetylmuramic acid residue from the dimer at pH 4. In the free form, it behaves as a neutral and reducing compound characterized by a well-defined $R_F$ in solvent XIII and by its ability to directly develop a purple color with Ehrlich’s reagent in acetate acid–hydrochloric acid with a calculated molar extinction coefficient of 40,000 (Table II). However, when this chromogen is exposed to 1% borate for 7 min at 100° before the addition of Ehrlich’s reagent, the calculated molar extinction coefficient is reduced to 20,000 (Table II)—the value given by free N-acetylmuramic acid or free N-acetylglucosamine in our usual Morgan–Elson procedure. These observations can be explained by the sequence of reactions shown in Figure 7. The base-catalyzed elimination of the peptide-substituted lactyl group from the N-acetylmuramic acid residue of the disaccharide moiety of the disaccharide peptide could result from a β elimination involving the acidic proton on C-2, in the position α to the carbonyl, and the O-lactyl substituent on C-3. The introduction of unsaturation between C-2 and C-3 would give rise to the prochrohogen glycoside (Figure 7, B) (i.e., compound N which is probably identical with the slow-moving chromogen described by Perkins, 1960). Incidentally, the lability of the 1,4 glycosidic linkage in this prochrohogen glycoside (as observed with compound N) may arise from the allylic position of the 1,4 glycosidic bond. The prochrohogen liberated by exo-β-N-acetylglucosaminidase action is probably 2,3-dihydro-N-acetylglycosamine (Figure 7D)—the compound detected by Kuhn et al. (1954) and tentatively identified by BeMiller and Whistler (1962) as an intermediate in chromogen formation when free N-acetylglycosamime is heated under alkaline conditions. During the course of its enzymatic liberation, free prochrohogen could then cyclize into a Δ4-dihydrofurane derivative or chromogen I (Figure 7) (which would be characterized by a molar extinction coefficient of 40,000 after direct addition of Ehrlich’s reagent without pretreatment in borate at 100°). Further modifications of chromogen I into a furan derivative, such as chromogen III (Figure 7) described by Kuhn and Kruger (1957), could result from further treatment with borate at 100°.

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