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The Peptide N^{α}-(L-Alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine and the Disaccharide *N*-Acetylglucosaminyl-β-1,4-*N*-acetylmuramic Acid in Cell Wall Peptidoglycan of *Streptococcus faecalis* Strain ATCC 9790*

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ABSTRACT: A major portion of the cell wall peptidoglycan in *Streptococcus faecalis* is composed of the disaccharide tetrapeptide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-N^{α}-(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^{ϵ}-terminal L-lysine of another. It 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 linkages and is thus the first enzyme known to hydrolyze D–D peptide bonds. Treatment of the disaccharide N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine with 10 equiv of NaOH at 37° for 1 hr results in deamidation of the isoasparaginyl residue together with migration of the aspartyl–lysine peptide bond giving rise to a mixture of N^{ϵ}-(β-aspartyl)- and N^{ϵ}-(α-aspartyl)lysyl peptides. Under the same alkaline treatment, the *N*-acetylmuramyl residue undergoes a lactyl elimination which results in the production of acyl peptides and a Morgan–Elson prochromogenic compound, without hydrolysis of the glycosidic linkage. This conversion, interpreted to be the result of a β 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^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine (Muñoz *et al.*, 1966a). On the other hand, five types of peptide cross-links between the ϵ-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 pentaglycine in *S. aureus* Copenhagen, tri-L-alanine in *M. roseus* thr⁻, tri-L-alanyl-L-threonine in *M. roseus* R27, di-L-alanine in *S. pyogenes* type 14, L-alanine in *Arthrobacter crystallopoietes*, and direct bonding with no additional amino acids between the C-terminal alanine residue of one peptide subunit and the ϵ-amino group of lysine of another in *Micrococcus lysodeikticus*. Until now, all of the peptide bridges examined contained either glycine or neutral L-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-glutamic acid (Cummins and Harris, 1956; Toennies *et al.*, 1959; Ikawa and Snell, 1960; Ikawa, 1964; Plapp and Kandler, 1966; Shockman *et al.*, 1967). When walls of *Lactobacillus brevis* were hydrolyzed in 11 N HCl at 80° for 43 hr a derivative of aminosuccinimide, ϵ-(aminosuccinyl)lysine, was isolated and on treatment with dilute sodium hydroxide was converted into a mixture

* From the Service de Bactériologie, Université de Liège, Belgium, and the Department of Microbiology, Temple University School of Medicine, Philadelphia, Pennsylvania. *Received April 6, 1967.* Supported in part by a grant (UR-E4-10-2) made by the U. S. Department of Agriculture under P. L. 480 (J.-M. G.), a U. S. Public Health Service Research career program award (1-K3-A1 4792) from the National Institute of Allergy and Infectious Diseases, by a research grant (GB 4466) from the National Science Foundation (G. D. S.), and in part by a NATO fellowship (E. B.).

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of predominantly N^{ϵ}-(β-aspartyl)lysine and a minor amount of N^{ϵ}-(α-aspartyl)lysine (Swallow and Abraham, 1958). This cyclic peptide N^{ϵ}-(aminosuccinyl)-lysine was later shown to be a common constituent in hydrolysates of other D-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^{ϵ}-(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 aspartyllysines made it impossible to determine whether the original sequence consisted of an α- or β-aspartyl peptide.

The present studies deal with the stereochemistry of the D-aspartic residue in the cell wall peptidoglycan of *S. faecalis* ATCC 9790. It will be demonstrated that in a significant portion of this peptidoglycan, D-isoasparaginyl bridges occur between two peptide monomers linking a C-terminal D-alanine of one monomer to an ϵ-NH_2 L-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 (Ghuysen *et al.*, 1966). Amino acid analyses were carried out after hydrolysis in 6 N HCl for 20 hr 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^{ϵ}-(aminosuccinyl)lysine and of its dinitrophenyl derivative. Analyses of amino acids were also performed with either a Technicon AutoAnalyzer (Chromo beads resin, type β, 17μ) or with the Phoenix amino acid analyzer (Phoenix spherix resins, 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 isoglutamine and isoasparagine is complete while the disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid yields no trace of ammonia. *Ammonia* was measured after partial neutralization of hydrolyzed samples (40 μl of 4 N NaOH for a 50-μl 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). Lyophilized peptide (25–100 mμmoles of N-terminal groups) was dissolved in 70 μl of *N*-ethylmorpholine buffer. Phenyl isothiocyanate (1 μl) was added and the solution heated for 45 min at 37°. After addition of 100 μl of water, excess reagent was removed by ether extraction (150 μl, twice). The aqueous phase was lyophilized and the phenylthiocarbamyl peptide was then treated with 50 μl 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 μl of 0.2 N acetic acid. The phenylthiohydantoin derivative of the NH_2-terminal amino acid was extracted with benzene (100 μl, 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 α-amino group free). Cyclization of a phenylthiocarbamyl derivative of an amino group bearing an adjacent α-carboxamide, with concomitant elimination of ammonia, was carried out by treatment with 50 μl of 4 N HCl at 37° for 3 hr (instead of using 50 μl of trifluoroacetic acid). The solution was then partially neutralized by adding 40 μl of 4 N NaOH and ammonia was measured. With this HCl treatment, the yields of free ammonia were 15–20% from isoasparagine and isoglutamine and 60–70% from their phenylthiocarbamyl derivative. *Dehydration 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 μequiv 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 Stahl, Merck), using the following solvents: (I) 1-butanol–0.15 N ammonia (1:1, upper phase); (II) chloroform–methanol–acetic acid (85:14:1) at 0°; (III) benzyl alcohol–chloroform–methanol–water–15 N ammonia (30:30:30:6:2); (IV) 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) (Walz *et al.*, 1963). Routine analyses of dinitrophenyl amino acids were performed by sequential chromatography in the same direction using solvents I and II. N^{ϵ}-DNP-lysine was measured after chromatography in solvent III. Chromatography of DANB (1,2-diaminonitrobenzene) derivatives of α-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

[1] Abbreviations used: FDNB, fluorodinitrobenzene; DANB, 1,2-diaminonitrobenzene; SDS, sodium decyl sulfate.

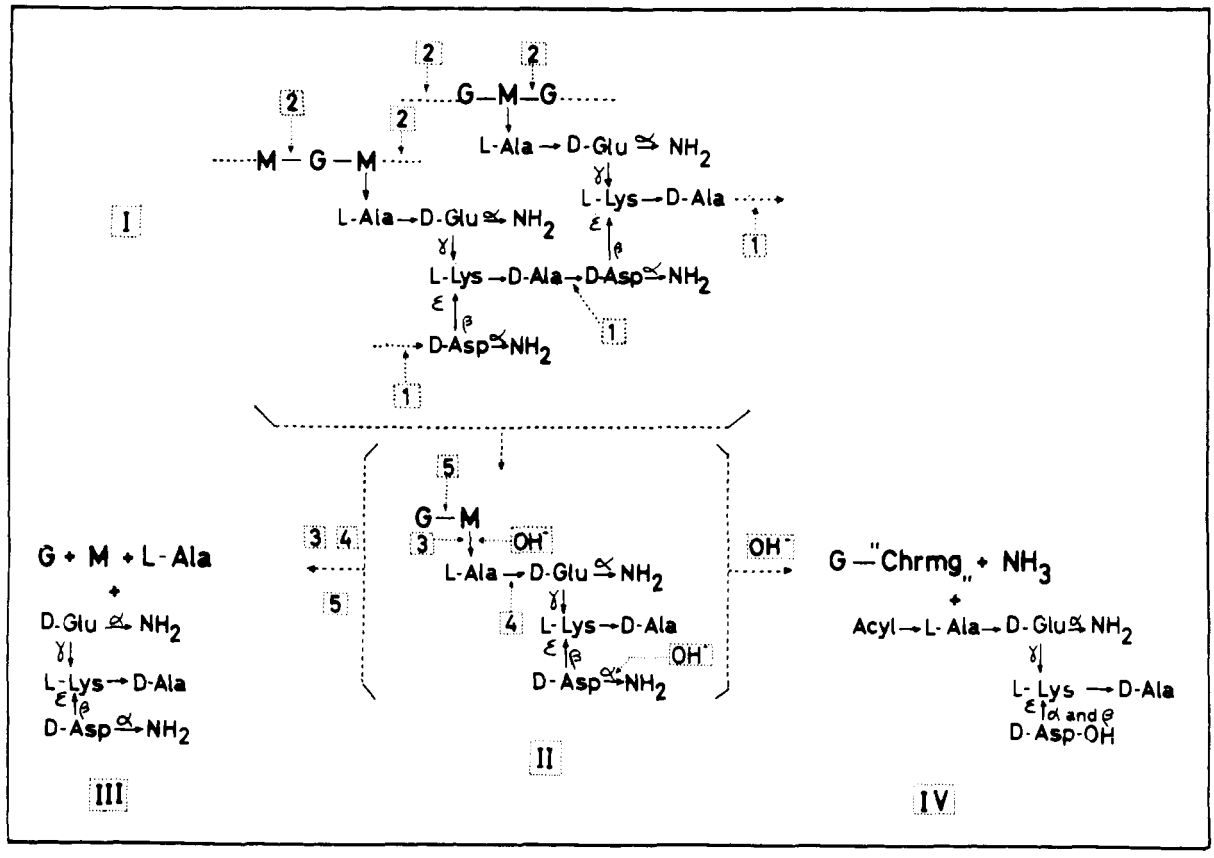

FIGURE 1: Sequential degradation of the cell wall peptidoglycan of *S. faecalis* 9790. (I) Portion of the intact peptidoglycan. (1) Site of action of SA endopeptidase. (2) Site of action of autolysin or F_1 endo-*N*-acetylmuramidase. (II) Isolated disaccharide peptide monomer. (3) Site of action of *N*-acetylmuramyl-L-alanine amidase. (4) Site of action of aminopeptidase. (5) Site of action of exo-β-*N*-acetylglucosaminidase. (OH^-) Alkaline degradation of the disaccharide peptide. Abbreviations: G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid; Chrmg, chromogenic compound derived from *N*-acetylmuramic acid.

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 Electrorheophor Pleuger (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*-dimethylaminobenzaldehydes pray (Salton, 1959), or by fluorescence after the paper had been dipped in a solution of 0.5 N NaOH in ethanol–1-propanol (6:4) and heated for 10 min at 120° (Sharon, 1964).

Enzymes. Purified *Streptomyces* SA endopeptidase and purified *Streptomyces* aminopeptidase (Ghuysen *et al.*, 1965; Petit *et al.*, 1966; Muñoz *et al.*, 1966a), *Streptomyces* *N*-acetylmuramyl-L-alanine amidase (Ghuysen *et al.*, 1962), *Streptomyces* F_1 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^{α}-(α-Aspartyl)lysylisoleucylvalylglycine and N^{α}-(β-aspartyl)lysylisoleucylvalylglycine were gifts from Dr. J. Savrda and Dr. A. Kotai (Institut de Biochimie, Orsay, France); N^{ϵ}-(aminosuccinyl)lysine from Dr. M. Ikawa, University of New Hampshire. L-Isoasparagine was a gift from Dr. C. Ressler, 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.

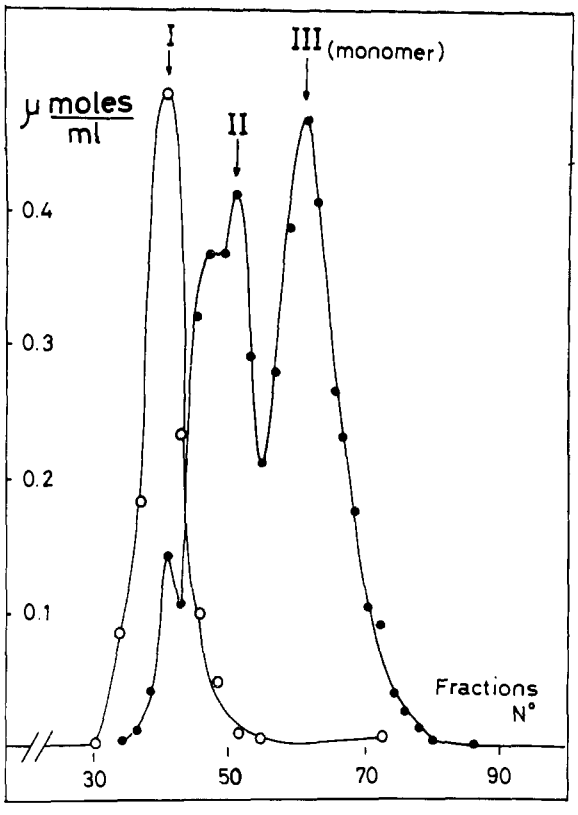

FIGURE 2: Isolation of disaccharide peptide monomer. Solubilized walls (300 mg) after steps 1 and 2 (see Figure 1) were filtered in water on an 800-ml column of Sephadex G-25 bead form. Fractions of 8 ml were collected. Total organic phosphorus, *N*-acetylglucosaminyl-β-1,4,-*N*-acetylmuramic acid (Morgan–Elson reaction after 30-min heating at 100° in 1% borate, using an ε of 7500), and amino groups (not shown in the figure) were estimated. (○) Organic P ($\times 10^{-1}$) and (●) disaccharide.

Log walls were prepared from exponential-phase cultures of *S. faecalis* 9790 grown in a synthetic medium (Shockman *et al.*, 1967). The walls were not treated by trypsin or by alkali during preparation. Analysis of the walls (Toennies *et al.*, 1959) revealed a content of about 450 mμequiv/mg of disaccharide (GlcNacMurNac) pentapeptide (L-Ala, D-Ala, L-Lys, D-Glu, and D-Asp).

Autolysis of log walls is known to involve an indigenous endo-*N*-acetylmuramidase which cleaves the glycan portion of the wall peptidoglycan into disaccharide units (Shockman *et al.*, 1967). Disaccharide peptide oligomer (fraction III in Shockman *et al.*, 1967) was prepared from autolysates of log walls. This water-soluble peptidoglycan fragment contains about 830 mμequiv/mg of disaccharide pentapeptide. SDS walls were prepared by treating log walls with sodium decyl sulfate. This treatment results in complete inactivation of the autolysin.

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* (Muñoz *et al.*, 1966a) with the two following differences. (1) After solubilization of the SDS walls by degradation with purified SA endopeptidase (hydrolysis of linkage 1, 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 F_1 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 F_1 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 F_1 endo-*N*-acetylmuramidase-solubilized SDS walls contain acetamido sugars equivalent to about 360 mμmoles of disaccharide/mg (yield, 80% on the basis of 450 mμequiv of peptidoglycan units/mg). Both autolyzed and F_1-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 mμmoles/mg to 180 mμmoles/mg. However, using the snythetic peptide N^{α}-(β-Asp)-Lys-Ile-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 mμmoles/mg in the native walls and to about 260 mμmoles/mg in the endopeptidase-treated walls. Endopeptidase treatment did not modify the number of C-terminal lysines (about 100 mμmoles/mg) but resulted in an increase of C-terminal alanines from 100 to about 250 mμmoles/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^{ϵ}-terminal lysine residues, from about 40 to about 80 mμmoles/mg of walls, has been repeatedly observed. It may be that native N^{ϵ}-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 A_2, $A_2\alpha$, and $A_2\beta$.

Results are Expressed in mmoles/1000 Total Glutamic Acid	DPO	DPM	A_2	$A_2\alpha$	$A_2\beta$
A. Disaccharide moiety					
Total HexNac[a] after exo-GlcNacase[a]		1930			
Total HexNac[a] after HCl hydrolysis	1930	2250	0	0	0
GlcNac[a] by glucosamine *N*-acetylase after HCl hydrolysis	1090	1170	0	0	0
B. Total amino acids					
Glutamic acid	1000	1000	1000	1000	1000
Lysine	1020	990	930	950	850
Aspartic acid	905	905	920	930	970
Alanine (total)	1740	1780	1840	2000	1740
L-Alanine		1280			
D-Alanine		640			
C. Amide ammonia	1710	1810	880	900	910
D. Amino-terminal groups[b]					
Alanine	0	0	0	0	0
Lysine	150	140	90	0	0
Aspartic acid	280	620	720	610	780
E. Carboxyl-terminal groups[b]					
Lysine	140	250	200		
Alanine	180	470	550		
F. Enzymatic degradation					
N-Terminal Ala after amidase		860			
Free L-Ala after amidase and aminopeptidase		920	No action	No action	No action
N-Terminal Glu after amidase and aminopeptidase		740			
G. Amino-terminal groups after one cycle of Edman degradation					
ϵ-Lysine		60	265	740	0
Aspartic acid		30	60	70	95
H. Total amino acids after Ressler–Kashelikar degradation					
Glutamic acid	305				
Lysine	115				
Aspartic acid	105				
Alanine	660				
β-Alanine[c]	20				
γ-Aminobutyric acid[c]	55				
Ornithine and α,γ-diaminobutyric acid	0				

[a] HexNac, *N*-acetylhexosamine; GlcNac, *N*-acetylglucosamine; exo-β-GlcNacase, exo-β-*N*-acetylglucosaminidase. [b] Data for N- and C-terminal groups are not corrected. [c] 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 ($V_0 + V_i$ = 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

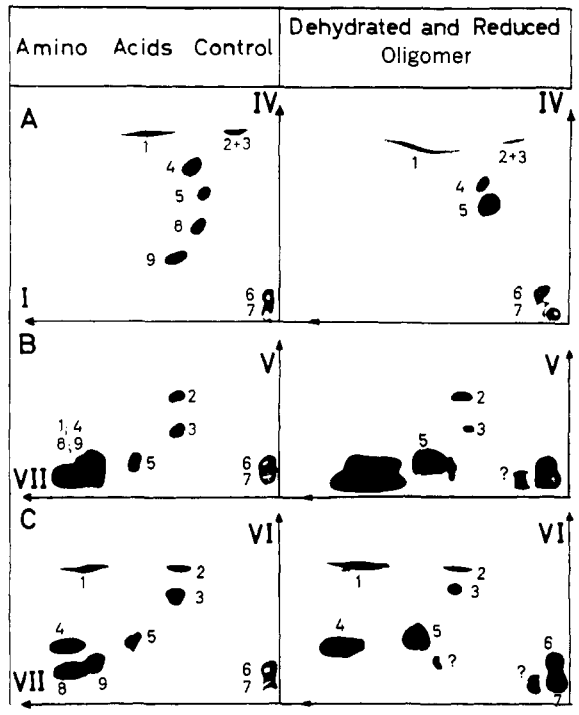

FIGURE 3: Dehydration reduction of *S. faecalis* disaccharide peptide oligomer according to Ressler and Kashelikar (1966). Two-dimensional chromatography on thin layer silica gel of the dinitrophenyl derivatives of a control mixture of amino acids and of the amino acids from the dehydrated and reduced oligomer. Abbreviations: 1, DNPOH; 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 Oligomer. 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 IA): (1) total hexosamine content of acid hydrolysates (3 N HCl, 3 hr, 100°) using the Morgan–Elson reaction (7 min at 100°) after chemical reacetylation; (2) glucosamine content of these HCl hydrolysates using the same procedure but after specific enzymatic reacetylation with the D-glucosamine *N*-acetylase; and (3) *N*-acetylglucosamine liberated by the exo-β-*N*-acetylglucosaminidase (hydrolysis of linkage 5, 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*-acetylmuramyl-L-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° 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*-acetylmuramyl-*N*-acetylglucosamine 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^{ϵ}-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 IB, D, and E). The presence of small amounts of N^{ϵ}-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 IF) first by the *N*-acetylmuramyl-L-alanine amidase (hydrolysis of linkage 3, Figure 1) which exposed essentially one N-terminal L-alanine per glutamic acid (actually 0.86) and next by the *Streptomyces* aminopeptidase (hydrolysis of linkage 4, Figure 1) which liberated one L-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 and of the disaccharide peptide oligomer in 3 N HCl for 4 hr at 100° yielded virtually 2 moles of ammonia/mole of glutamic acid (Table IC). 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 substituted. 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^{ϵ}-(aminosuccinyl)lysyl 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 carbonyl group of the succinimide ring (Swallow and Abraham, 1958), could be detected.

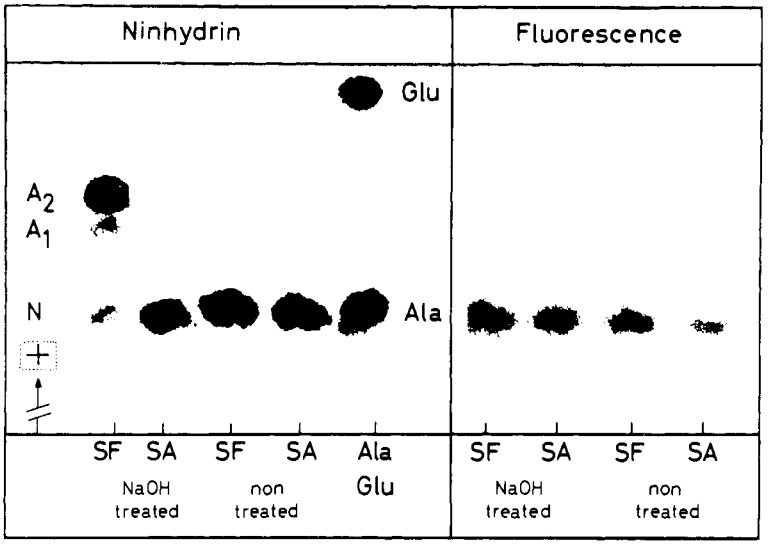

FIGURE 4: Paper electrophoresis at pH 5 of the NaOH-treated disaccharide peptide monomers. The disaccharide-N^{α}-(L-Ala-D-Glu$^{\alpha}$-NH_2)-L-Lys-D-Ala and the acyl peptides from *S. aureus*, *M. roseus*, and *S. pyogenes* are neutral. The *S. faecalis* disaccharide pentapeptide monomer is also neutral. The corresponding acyl peptide (A_2) is acidic. Aliquots were spotted 10 cm from the cathode and electrophoresis was carried out for 2 hr at 20 v/cm. Ninhydrin (peptide moiety) and fluorescence (disaccharide moiety) were used for detection. Abbreviations: SF, disaccharide peptide monomer of *S. faecalis;* SA, disaccharide peptide monomer of *S. aureus*. An alanine standard marks the position of neutrality.

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 Kashelikar, 1966). γ or β substitution will produce ornithine (from a glutaminyl residue) or α,γ-diaminobutyric acid (from an asparaginyl residue). Conversely, isoglutamine and isoasparagine will give rise to γ-aminobutyric 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 phosphite 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 IH. No traces of α,γ-diaminobutyric acid or ornithine were detected. β-Alanine and γ-aminobutyric acid were unequivocally characterized in the forms of dinitrophenyl (DNP) derivatives and were further estimated 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 IG). 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° 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^{ϵ}-(isoasparaginyl)lysyl peptide structure which, as shown in the following paragraph, presents peculiar chemical reactivities.

DEAMIDATION AND INTERCONVERSION OF THE N^{ϵ}-(ISOASPARAGINYL) -LYSYL MOIETY INTO N^{ϵ}-(β- AND α-ASPARTYL)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 K_2CO_3 solution were mixed in a diffusion vial (as used for the ammonia determination) and rotated at 25° for 1–2 hr. It was then found that treatment at 37° for 1 hr of the disaccharide peptide monomer with 10 equiv of NaOH (example, 50 mμmoles 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^{α}-(L-alanyl-D-isoglutaminyl)-L-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

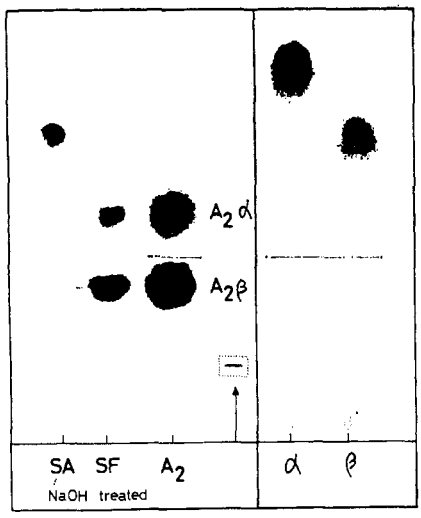

FIGURE 5: Paper electrophoresis in 0.2 N acetic acid of NaOH-treated disaccharide peptide monomers, fraction A_2, and synthetic N^{α}-(α- and -β-aspartyl)lysyl peptides. Aliquots were spotted at 10 cm from the anode and electrophoresis was carried out for 2 hr at 20 v/cm. Ninhydrin (peptide moiety) and fluorescence (disaccharide moiety, not shown) were used for detection. Neutrality is at the level $A_2\beta$. Abbreviations: SA, disaccharide peptide monomer of *S. aureus;* SF, disaccharide peptide monomer of *S. faecalis;* A_2, see Figure 4; α, N^{α}(α-Asp)-Lys-Ile-Val-Gly; β, N^{α}-(α-Asp)-Lys-Ile-Val-Gly. The four untreated disaccharide peptide monomers from *S. aureus*, *M. roseus*, *S. pyogenes*, and *S. faecalis* and the three acyl peptides from the NaOH-treated *S. aureus*, *M. roseus*, and *S. pyogenes* disaccharide peptide monomers are basic and migrate identically. The *S. faecalis* acyl peptide dissociates into two components ($A_2\beta$ and $A_2\alpha$).

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-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-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 A_2 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 A_1), slightly less acidic than the main compound A_2, was detected. Compounds N, A_1, and A_2 from *S. faecalis* were obtained by preparative paper electrophoresis and further purified by gel filtration on Sephadex G-25 in water ($V_0 + V_i$ = 160 ml) (elution volumes, 105 ml for A_1 and A_2; 135 ml for N). The acyl peptide A_2 had no acetamido sugars 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*-acetylglucosamine of the original disaccharide peptide monomer (estimated after enzymatic reacetylation of the glucosamine in an HCl hydrolysate). The A_1 compound was probably a trace of the original disaccharide peptide monomer which had only undergone deamidation of its isoasparaginyl residue. This compound will not be discussed further. On further paper electrophoresis in 0.2 N acetic acid (Figure 5), the acyl peptide A_2 from *S. faecalis* dissociated into two components: compound $A_2\beta$ which is now neutral and compound $A_2\alpha$ which is still acidic. Preparative electrophoresis of fraction A_2 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 $A_2\beta$ and 20% of $A_2\alpha$.

Structure of the *S. faecalis* Acyl Peptides. Compounds A_2, $A_2\beta$, and $A_2\alpha$ 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^{ϵ}-lysine peptide in compound A_2 and its absence in compounds $A_2\alpha$ and $A_2\beta$. The two striking differences are the presence of only one amide ammonia and the complete absence of *N*-acetylhexosamine residues in the three acyl peptide compounds. After Edman degradation of compound $A_2\alpha$, N-terminal aspartic acid disappeared and was quantitatively replaced by N^{ϵ}-terminal lysine, conclusively showing that it is an N^{ϵ}-(α-aspartyl)lysyl peptide. Conversely, compound $A_2\beta$ is the N^{ϵ}-(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 A_2 is a mixture of two acyl peptides, $A_2\alpha$ and $A_2\beta$. The yield of the Edman degradation also confirms the ratio $A_2\beta$:$A_2\alpha$ of 4:1 found by paper electrophoresis in 0.2 N acetic acid. From the foregoing, the structure N^{ϵ}-(β-aspartyl)-N^{α}-(acyl-L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine can be assigned to the main compound in fraction $A_2\beta$ and the structure N^{ϵ}-(α-aspartyl)-N^{α}-(acyl-L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine to the main compound in fraction $A_2\alpha$.

CONCLUSION. The main component of the *S. faecalis* disaccharide peptide monomer preparation must, therefore, have the sequence β-1,4-*N*-acetylglucosaminyl *N*-acetylmuramyl-N^{α}-(L-Ala-D-Glu$^{\alpha}$-$CONH_2$)-N^{ϵ}-(D-Asp$^{\alpha}$-$CONH_2$)-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^{ϵ}-lysine residue (see Table I). Disaccharide peptide oligomer 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_F 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_F value as a sample of authentic lactic acid (R_F 0.85). This value was different from that of *N*-acetylmuramic acid (streak, R_F 0.6–0.9) or pyruvic acid (R_F 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*-acetylglucosamine = 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*-acetylglucosamine can be specifically estimated after enzymatic reacetylation of an HCl hydrolysate, using the 7-min Morgan–Elson reaction. Exo-β-*N*-acetylglucosaminidase cleaves the compound into *N*-acetylglucosamine and a compound migrating in solvent XIII with R_F 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 mμ, 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*-acetylglucosamine residue at the nonreducing end, glycosidically β linked to a reducing neutral compound. When freed from *N*-acetylglucosamine, 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 glucosamine content or by determining its total hexosamine content after $NaBH_4$ reduction (for conditions of reduction, see Leyh-Bouille *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 μl) of the borate solution were heated in boiling water for 7 and 30 min. The borate and water solutions (125 μl) were then mixed with 500 μl of the Morgan–Elson reagent (Ghuysen *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*-acetylglucosaminyl-β-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.

TABLE II: Molar Extinction Coefficients in the Morgan–Elson Procedure.

Compound[a]	No Borate	Time at 100° in 1% Borate: 7 min	30 min
β-1,4-X-Y	0	3,500	9,500
β-1,4-X-Chrmg (*i.e.*, *S. faecalis* fraction N)	0	8,000	10,500
Chrmg arising from Y, calculated after exo-β-GlcNacase hydrolysis of compound N	40,000	20,000	
Y	0	19,000	

[a] X, *N*-acetylglucosamine; Y, *N*-acetylmuramic acid; Chrmg, chromogenic compound.

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-β-*N*-acetylglucosaminidase (final volume: 25 μl of 0.01 M citrate buffer, pH 4.2). Some aliquots were placed at 37° for 20 min in the presence of 600 μl of Ehrlich's reagent (Ghuysen *et al.*, 1966). Others were first mixed with a borate solution (final volume: 125 μl containing 1% borate), exposed to 100° for 7 min, and then treated with 500 μl of the Ehrlich's reagent as above. Since *N*-acetylglucosamine 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 (Ghuysen *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.

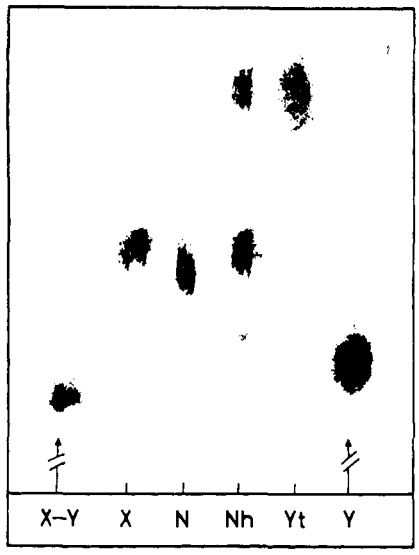

FIGURE 6: Paper chromatography in solvent XIII of the chromogenic compound arising from *N*-acetylmuramic acid and of related compounds. Abbreviations: X, *N*-acetylglucosamine (R_F 0.5); Y, *N*-acetylmuramic acid (R_F 0.38); X-Y, *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid (R_F 0.35); N, compound N or *N*-acetylglucosaminyl-β-1,4-prochromogen (R_F 0.48); Nh, compound N after hydrolysis by exo-β-*N*-acetylglucosaminidase; Yt, NaOH-treated *N*-acetylmuramic acid (R_F 0.65). Detection was made using Salton'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*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide but not from muramic acid or from the isomeric β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine disaccharide. Moreover, glucosamine and *N*-acetylglucosamine 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*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide units with the help of either the indigenous wall autolysin (Shockman *et al.*, 1967) or the *Streptomyces* F_1 endo-*N*-acetylmuramidase. It is thus composed of alternating *N*-acetylglucosamine 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^{α}-(L-alanyl-D-isoglutaminyl)-L-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, 1966; Tipper *et al.*, 1967).

The *S. faecalis* peptide cross-links consist of single D-isoasparaginyl residues extending from the C-terminal D-alanine of one peptide subunit to the N^{ϵ}-lysine of another. The proposed foregoing structure rests upon isolation from cell walls of the disaccharide pentapeptide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine. The location of the D-isoasparaginyl residue as a substituent of the N^{ϵ}-lysine in this disaccharide pentapeptide monomer emerges from a series of comprehensive determinations: (1) presence of two amide ammonias per monomer unit; (2) disappearance through Edman degradation of the terminal amino groups of aspartic acid without consequent exposure of any other terminal amino group (which is compatible with a peptide bond involving the β-carboxyl group of aspartic acid; (3) production of β-alanine through dehydration reduction 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) deamidation of the isoasparagine residue followed by interconversion into a mixture of N^{ϵ}-(β-aspartyl)lysyl and N^{ϵ}-(α-aspartyl)lysyl derivatives; and (6) confirmation by Edman degradation of the presence of the β and α links in the two former peptides.

D-Isoasparagine 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

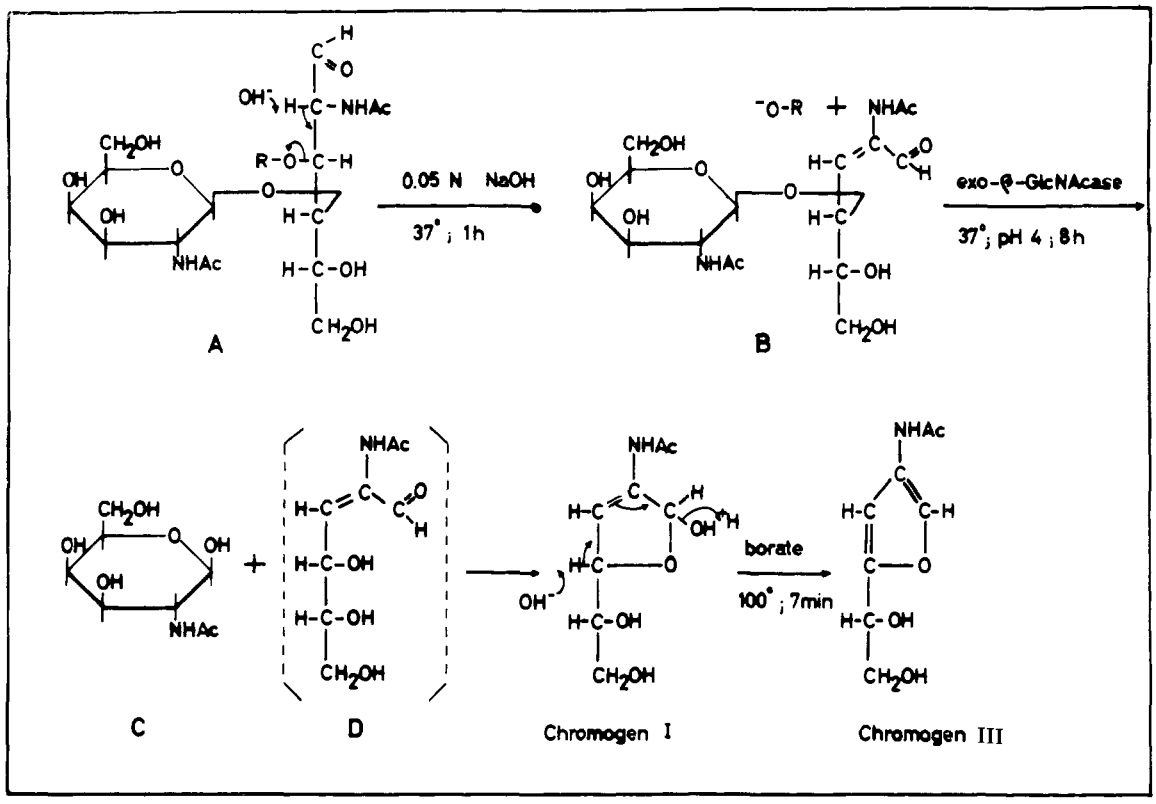

FIGURE 7: Proposed reaction sequence for disaccharide peptide giving rise to Morgan–Elson chromogen(s) *via* a prochromogen glycoside. (A) *N*-Acetylglucosaminyl-β-1,4-*N*-acetylmuramyl peptide. R represents the lactoyl peptide residue. (B) Prochromogen glycoside or compound N. (C) *N*-Acetylglucosamine. (D) Intermediate prochromogen.

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^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-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-isoasparaginyl linkages in the peptide moiety. Evidence for the opening of these D-alanyl-D-isoasparaginyl 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-isoasparaginyl 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-isoasparaginyl 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-isoasparaginyl linkages and thus is, 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 strenghtened 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* autolysin, 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 walls components is not known.

Deamidation and Interconversion of the N^{ϵ}-Isoasparaginyllysyl Peptide. The base-catalyzed deamidation and interconversion of the N^{ϵ}-isoasparaginyllysyl peptide yield 4 moles of the N^{ϵ}-(β-aspartyl)lysyl derivative/mole of the N^{ϵ}-(α-aspartyl)lysyl derivative. The succinimide derivative N^{ϵ}-(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 (Swallow and Abraham, 1958; Ikawa, 1964). It seems plausible that the transformation, with the concomitant loss of 1 mole of ammonia, of the N^{ϵ}-(isoasparaginyl)lysyl portion of the *S. faecalis* 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^{ϵ}-(isoasparaginyl)lysyl structure. The mechanism of this reaction might be related to the well-known imide formation from an amide ester (*i.e.*, cyclization of *N*-acylasparagine or *N*-acylisoasparagine esters; Sondheimer 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 alkali treatment of these imides results in the ring opening in both possible ways.

The Base-Catalyzed Lactyl Elimination from N-Acetylmuramic Acid. The *S. faecalis* 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*-acetylmuramyl-L-alanine. An acyl peptide (most likely a lactoyl peptide) and a neutral still-reducing disaccharide are produced. The *N*-acetylmuramic 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. Exo-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 acetic 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 prochromogen 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 prochromogen glycoside (as observed with compound N) may arise from the allylic position of the 1,4 glycosidic bond. The prochromogen liberated by exo-β-*N*-acetylglucosaminidase action is probably 2,3-dihydro-*N*-acetylglucosamine (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*-acetylglucosamine is heated under alkaline conditions. During the course of its enzymatic liberation, free prochromogen could then cyclize into a Δ^3-dihydrofuran 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°.

Acknowledgments

We thank Dr. Pierre Lefrancier, Institut de Biochimie, Université de Paris, Orsay, for performing the dehydration reduction; Miss Claudine Willems, Service de Génétique Humaine and Service de Chimie Médicale, Liège; Dr. M. Prince Brigham and Mr. Earl Pearson of Temple University for performing the amino acid autoanalyses; Dr. Charles Panos, Research Laboratories, Albert Einstein Medical Center, Philadelphia, for the infrared spectral analysis.

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