Eur. J. Biochem. 38, 301–306 (1973)

The Wall Peptidoglycans of *Neisseria perflava*, *Moraxella glucidolytica*, *Pseudomonas alcaligenes* and *Proteus vulgaris* Strain P18

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(Received May 30, 1973)

The primary structure of the wall peptidoglycans of *Neisseria perflava*, *Moraxella glucidolytica* and *Pseudomonas alcaligenes* is similar to those of the *Enterobacteriaceae Proteus vulgaris* and *Escherichia coli*, suggesting that the peptidoglycans of all gram-negative bacteria contain *meso*-diaminopimelic acid and belong to the same chemotype I. Differences are observed with regard to the thickness of the peptidoglycan layer in the cell envelopes, the extent of peptide crosslinking and the occurrence of *N*,*O*-diacetylmuramic acid residues.

In contrast to the wall peptidoglycans of the gram-positive bacteria which have been extensively studied, the peptidoglycans of very few gram-negative bacteria have been submitted to thorough structural investigations [1,2]. It is known that, in *Escherichia coli* [3,4], the glycan chains are composed of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The *N*-acetylmuramic acid residues are substituted by L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine peptide units and some of these peptides are, in turn, crosslinked through C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkages (peptidoglycan of chemotype I) [1]. The peptidoglycan of *Proteus vulgaris* P18 exhibits the same primary structure [5,6], except that some of the *N*-acetylmuramic acid residues are *O*-acetylated on C6.

In the course of the present study, the peptidoglycans of three taxonomically different gram-negative bacteria belonging to the genera *Neisseria*, *Moraxella* and *Pseudomonas*, were isolated and their structures were compared to that of *P. vulgaris* P18 [6].

MATERIALS AND METHODS

Strains and Growth Conditions

Neisseria perflava was isolated from a throat swab, *Moraxella glucidolytica* from a peritoneal liquid, *Pseudomonas alcaligenes* from a pus wound and *Proteus vulgaris* strain P18 from a coproculture [7]. The bacteria were grown in a New Brunswick fermentor (72 liters) at 37 °C with strong aeration. *Moraxella*, *Neisseria* and *Pseudomonas* were grown on a Merck Nährbouillon Standard I liquid medium for 24 h and *Proteus* was grown either on the Nährbouillon or on a modified Medill liquid medium [8] for 16 h. The cultures of *Neisseria*, *Moraxella* and *Pseudomonas* were sterilized at 120 °C for 30 min. Cells were collected with a Sharpless continuous-flow centrifuge and were lyophilized.

Model Compounds

The following compounds were used: the tetrapeptides L-analyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine and L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine were isolated from walls of *Bacillus megaterium* [4] and of *Clostridium perfringens* [9], respectively. The disaccharides β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid and β-1,4-*N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid were isolated from walls of *Lactobacillus acidophilus* strain 63 AM Gasser [10].

Detection Reagents

Oligosaccharides from peptidoglycans were detected by fluorescence [11]. Amino acids, free amino groups and peptides were detected with ninhydrin (0.5% in isopropyl alcohol).

Chromatography

The following solvents were used: (I) chloroform–methanol–acetic acid (44:5:1, v/v/v), (II) methanol–pyridine–12 N HCl–water (8:1:0.21:1.79, v/v/v/v), (III) benzylalcohol–chloroform–methanol–

water—ammonia (15:15:15:3:1, v/v/v/v/v) and (IV) 1-butanol—acetic acid—water (3:1:1, v/v/v).

Analytical Methods

Reducing groups (Park-Johnson procedure), acetamido sugars (Morgan-Elson reaction), hexosamines (Morgan-Elson reaction after chemical acetylation), amino acids and terminal amino-groups (fluorodinitrobenzene technique) were measured as previously described [12, 13]. Quantitative analysis of amino acids (after 16 h of hydrolysis in 6 N HCl at 105 °C) and of hexosamines (after 4 h of hydrolysis in 3 N HCl at 95 °C) were also carried out with the help of an Unichrom (Beckman) amino acid analyzer. The method described in the Beckman technical manual was slightly modified according to Guire [14]. Separation of amino acids and amino sugars was carried out at 55 °C on one single column (0.9×50 cm) packed with the Beckman resin type AA-15. Two buffers were used in sequence: 0.2 N sodium citrate buffer pH 2.875 and 0.35 N sodium citrate buffer pH 5.25. The buffer change was set at 2 h. Under these conditions, muramic acid, diaminopimelic acid and glucosamine appeared as well-defined single peaks.

The isomers of diaminopimelic acid were identified as described by Bricas *et al.* [15]. LL- and *meso*-diaminopimelic acid were separated by chromatography (after hydrolysis in 6 N HCl for 16 h at 120 °C) on Whatman no. 1 paper, in solvent II [16]. Didinitrophenylated DD- and *meso*-diaminopimelic acid were separated and monodinitrophenyl-*meso*-diaminopimelic acid was isolated by chromatography on silica-gel (G) thin-layer plates in solvent III [17]. Disaccharides and *O*-acetyldisaccharides were separated by chromatography on Whatman no. 1 paper in solvent IV.

Preparation of the Wall Peptidoglycans

Lyophilized cells of *Proteus* (10 g) were suspended in 1 l of boiling 4% sodium dodecylsulfate solution. The suspension was stirred for 2 h, and kept overnight at room temperature [19]. The colorless gel obtained after repeated washings and centrifugations (30 min at 66000×*g*) was incubated with the *Bacillus subtilis* protease, in 0.05 M phosphate buffer pH 7.4 [20] for 2—3 h at 37 °C. Lyophilized cells of *Neisseria*, *Moraxella* and *Pseudomonas* were first disrupted by ultrasonic treatment for 20 min. After removal of intact cells by centrifugation at 12000×*g* for 5 min, the cell envelopes were isolated by differential centrifugation and treated with boiling 4% sodium dodecylsulfate solution under the same conditions as those described above. All the peptidoglycan materials were treated with trypsin (ratio enzyme-substrate = 1:25, w/w) in 0.1 M phosphate buffer pH 8, for 1 to 2 h at 37 °C, and were washed 5 times with water by centrifugation at 66000×*g* for 30 min.

Enzymic Degradation of Peptidoglycans

Egg-white lysozyme and *Chalaropsis* B enzyme [21] are endo-*N*-acetylmuramidases with hydrolyse β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine linkages. Degradation of the peptidoglycans with lysozyme (grade I Sigma) were performed at 37 °C in 0.06 M phosphate buffer pH 6.2 (ratio, enzyme—substrate = 1:25, w/w). Degradations with *Chalaropsis* B enzyme were performed in 0.02 M acetate buffer pH 4.5 (ratio, enzyme—substrate = 1:100, w/w).

When acting on disaccharide peptide fragments, *Streptomyces* *N*-acetylmuramyl-L-alanine amidase hydrolyses the linkages between the *N*-acetylmuramic acid residues and the peptide units [22]. Degradations were carried out at 37 °C in 0.025 M acetate buffer pH 5.4.

Gel Filtrations

Fractionations were carried out by filtrations in 0.1 M LiCl, on two columns connected in series, of Sephadex G-50 fine (720 ml) and Sephadex G-25 fine (400 ml). The gel filtration properties of the compounds were expressed in terms of distribution coefficients:

$$K_D = \frac{(V_e - V_0)}{V_i}$$

with V_e elution volume, $V_0 = V_e$ of totally excluded material and $V_i = V_e - V_0$ where V_i is the value of V_e for NaCl.

Electrophoreses

Electrophoreses were carried out on Whatman no. 3-mm paper using an electrorheophor apparatus (Pherograph-type), at pH 4 (pyridine—acetic acid—water, 2:9:1000, v/v/v) and at pH 2 (0.5 N formic acid).

RESULTS

Location of the Wall-Peptidoglycan Layer in the Cell Envelopes

The electron micrographs of thin sections (Fig. 1) of intact cells of *Neisseria*, *Pseudomonas* and *Moraxella* showed the multi-layered structure of the cell envelopes. As reported earlier [23], a similar structure was visible in *Proteus* only after brief treatment at high temperature (5 min at 80 °C). According to previous studies, the innermost layer of the cell wall (*i.e.* the G_2 layer according to De Petris' terminology) [24], directly superimposed upon the three-layered cytoplasmic membrane, is the peptidoglycan. The approximate thicknesses of the peptidoglycan layers

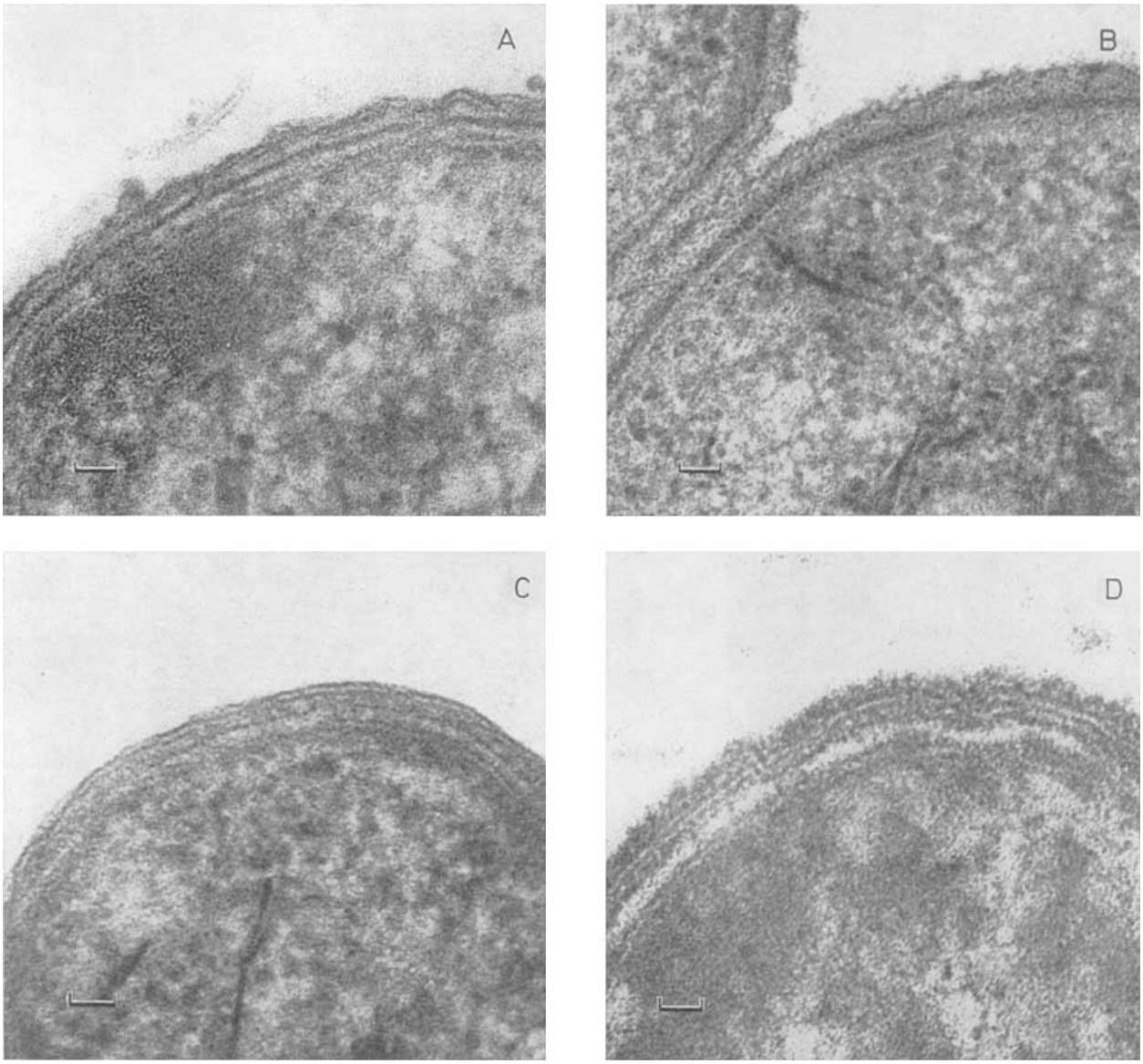

Fig. 1. *Electron micrographs of thin sections of various gram-negative bacteria (magnification ×240000).* (A) *Neisseria perflava,* (B) *Moraxella glucidolytica,* (C) *Pseudomonas alcaligenes* and (D) *Proteus vulgaris* P18 (treated 5 min at 80 °C)

were 10 nm in *Neisseria,* 12 nm in *Moraxella,* 6 nm in *Pseudomonas* and 8 nm in *Proteus.* In *Moraxella,* the thick G_2 electron-dense layer was surrounded by a wavy layer of homogeneous appearance, whereas in the three other organisms, this outermost layer had a typical membrane structure. The isolated peptidoglycan preparations appeared as homogeneous electron-dense layers.

Chemical Composition of the Peptidoglycans

The preparations obtained from *Moraxella* and *Neisseria* were pure peptidoglycan materials. They consisted of muramic acid, glucosamine, alanine, *meso*-diaminopimelic acid and glutamic acid occurring in the molar ratio, 1:1:2:1:1 (Table 1). The peptidoglycans of *Pseudomonas* and *Proteus* had the same chemical composition but they represented only about 50%, dry weight, of the final preparations. Small amounts of non-peptidoglycan amino acids were also present in these latter preparations. Based on these estimations, the peptidoglycan layers represented about 2.5%, dry weight, of the cells of *Moraxella,* 0.6% of the cells of *Neisseria,* 0.44% of the cells of *Pseudomonas* and 0.28% of the cells of *Proteus.*

Table 1. *Chemical composition of the peptidoglycans isolated from* Neisseria, Moraxella, Pseudomonas *and* Proteus

Constituent	*Neisseria*		*Moraxella*		*Pseudomonas*		*Proteus*	
	Content	Molar ratio	Content	Molar ratio	Content	Molar ratio	Content	Molar ratio
	nmol/mg		nmol/mg		nmol/mg		nmol/mg	
Glutamic acid	980	1	850	0.9	404	1	560	1
Alanine	1900	1.9	1560	1.8	880	2.3	920	1.75
meso-Diaminopimelic acid	990	1	950	1	380	1	530	1
Glucosamine	848	0.8	800	0.8	462	1.1	620	1.1
Muramic acid	720	0.75	820	0.8	430	1.1	590	1.1

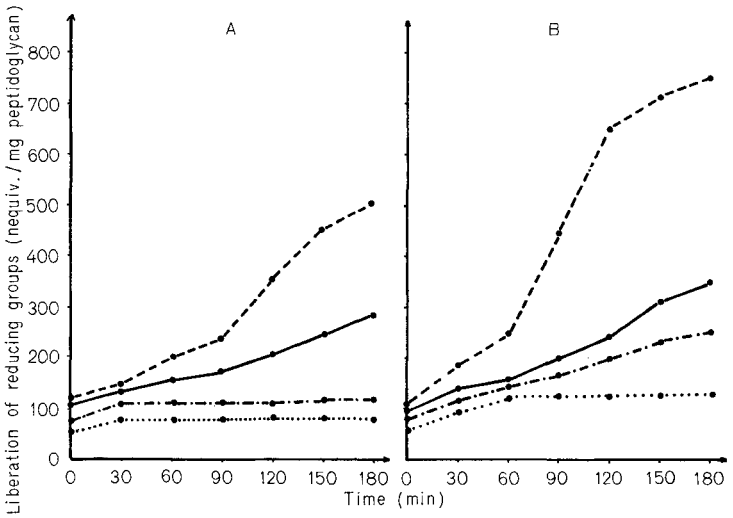

Fig. 2. *Effect of egg-white lysozyme on peptidoglycans of* Neisseria, Moraxella, Pseudomonas *and* Proteus. (A) Liberation of reducing groups (for conditions, see text). (B) Same experiment after prior treatment of the peptidoglycans by 0.02 N NaOH for 1 h at 37 °C. *Neisseria* (●——●), *Moraxella* (●– – –●), *Pseudomonas* (●· · · ·●), and *Proteus* (●–·–·–●)

Degradation of the Peptidoglycans into Disaccharide-Peptide Units

Chalaropsis B endo-*N*-acetylmuramidase completely solubilized the peptidoglycan preparations. The amounts of disaccharide-peptide units in the degraded peptidoglycan preparations, expressed in nequiv. per mg dry weight were: 950 for *Neisseria*, 900 for *Moraxella*, 360 for *Pseudomonas* and 580 for *Proteus*. In contrast to the *Charopsis* B enzyme, egg-white lysozyme had a very weak lytic effect and exposed reducing groups which represented only 25% to 60% of the theoretical amounts (Fig. 2). Prior treatment with 0.02 N NaOH for 1 h at 37 °C (under which conditions *O*-acetyl groups, if present, should be hydrolyzed) [18] considerably increased the lysozyme sensitivity of the peptidoglycan of *Moraxella*, but had very little effect on that of the other peptidoglycans (Fig. 2).

Fractionation of the Degraded Peptidoglycans

Filtration of the *Chalaropsis*-B-degraded peptidoglycans, on two linked Sephadex G-50 to G-25 columns, in 0.1 M LiCl, yielded four fractions (X, T, D and M) from the peptidoglycan of *Pseudomonas* and three fractions (T, D and M) from the peptidoglycans of *Neisseria*, *Moraxella* and *Proteus* (Fig. 3). All the fractions were separately desalted by filtration in water on Sephadex G-15 columns.

The various fractions were homogeneous and indistinguishable from one another by paper electrophoresis at pH 4. Their chemical compositions were identical to those of the original peptidoglycan preparations. Estimation of terminal amino groups revealed that 93 to 98% of the *meso*-diaminopimelic acid residues in fractions M, 46 to 50% in fractions D, 28 to 35% in fractions T and 20% in fraction X (from *Pseudomonas*) had one amino group free. These determinations, together with the K_D values (Fig. 2) allowed to identify the fractions M ($K_D = 0.55$) as disaccharide peptide monomers, fractions D ($K_D = 0.35$) as bisdisaccharide peptide dimers, fractions T ($K_D = 0.20$) as trisdisaccharide peptide trimers and fraction X (from *Pseudomonas*; $K_D = 0$) probably as a tetramer. Finally, the electrophoretic migrations in 0.2 M formic acid (under which condi-

tions disaccharide-peptide compounds of various sizes can be separated), confirmed the monomeric and oligomeric structures of the various fractions. The proportions of disaccharide peptide monomer and oligomers present in the various degraded peptidoglygans greatly varied depending upon the bacterial species (Table 2).

Fractions M, D and T showed heterogeneity by chromatography on silica-gel thin-layer plates in solvent IV (Fig. 4). Fractions N gave rise to sub-fractions M_1 and M_2 and fractions D to sub-fractions D_1, D_2 and D_3. Again, these sub-fractions had the same chemical compositions as the undegraded peptidoglycans. Under these conditions, fractions T and X could not be separated into individual sub-fractions.

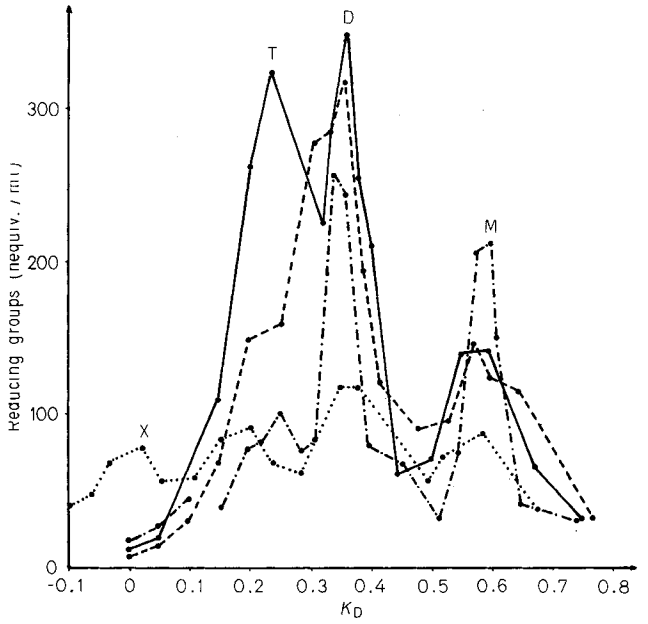

Fig. 3. *Filtration on Sephadex G-50 to G-25, in 0.1* M *LiCl, of the* Chalaropsis-*B-degraded peptidoglycans from* Neisseria, Moraxella, Pseudomonas *and* Proteus. Results are expressed in nequiv. reducing groups per ml. Fractions were of 4 ml. For conditions, see text. *Neisseria* (●——●), *Moraxella* (●----●), *Pseudomonas* (●·····●), and *Proteus* (●-·-·-●)

Structure of the Disaccharide Units

The disaccharide peptide monomers, M_1 and M_2, were degraded into free disaccharides and free peptide units with the help of the *Streptomyces N*-acetylmuramyl-L-alanine amidase. The molar absorption coefficient of the free disaccharides (Morgan-Elson reaction; 30 min of heating in 1% borate) suggested that the glycoside linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid were β-1,4 [11]. The free disaccharides from fractions M_1 and fractions M_2 had R_F values of 0.56 and 0.36, respectively (paper chromatography in solvent IV). These R_F values were those of authentic β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid and β-1,4-*N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid, respectively. When treated with 0.02 N NaOH for 1 h

Table 2. *Proportion of peptide subunits occurring in the form of monomer, dimer, trimer and tetramer in the* Chalaropsis-*B-degraded peptidoglycans of* Neisseria, Moraxella, Pseudomonas *and* Proteus

Form of subunit	Peptidoglycans of			
	Neisseria	Moraxella	Pseudomonas	Proteus
	%	%	%	%
Monomer	21	31	28	22
Dimer	21	53	32	48
Trimer	58	16	24	20
Tetramer	0	0	16	0

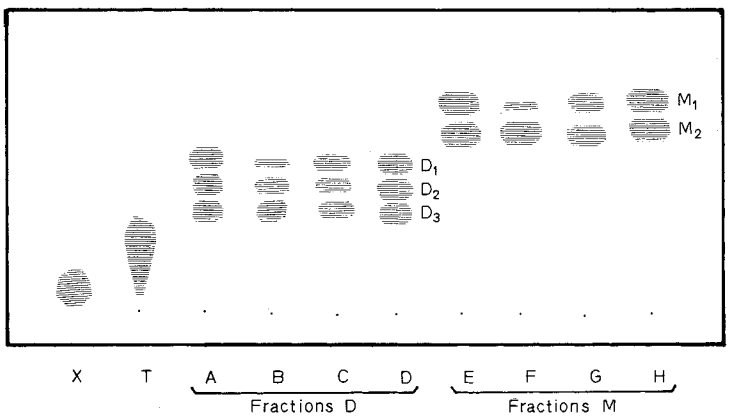

Fig. 4. *Silica-gel thin-layer chromatography in solvent IV (two runs) of the various fractions obtained by filtration on Sephadex G-50 to G-25 columns (see Fig. 3).* X = Fraction X from *Pseudomonas*; T = trimers; A—D = dimers from *Neisseria* (A), *Moraxella* (B), *Pseudomonas* (C), and *Proteus* (D); E—H = monomers from *Neisseria* (E), *Moraxella* (F), *Pseudomonas* (G), and *Proteus* (H)

at 37 °C, the *O*-acetyldisaccharide from fractions M was converted, at least partially, into *O*-acetyl-free disaccharide.

The bisdisaccharide peptide dimers D_1, D_2 and D_3 were also treated with the *Streptomyces* amidase. The two disaccharide residues present in the dimers D_1 appeared to be *O*-acetylated whereas those of dimers D_3 were free of *O*-acetyl substituents. In dimer D_2, one disaccharide residue was *O*-acetylated and the other was not.

Identification of the Peptide Monomers

The peptide monomers obtained by action of the amidase upon the fractions M were indistinguishable by paper electrophoresis at pH 4 from the standard peptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine (Materials and Methods) and were more anionic than the peptide L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine, demonstrating that the carboxyl groups of glutamic acid and *meso*-diaminopimelic acid were not amidated.

CONCLUSIONS

Insofar as can be judged, the wall peptidoglycans of the four taxonomically different gram-negative bacteria: *Moraxella, Neisseria, Pseudomonas* and *Proteus* have identical primary structures (*meso*-diaminopimelic-acid-containing peptidoglycans of chemotype I; see Introduction) [1] and contain *O*-acetyl substituents on some of their *N*-acetylmuramic acid residues. This structure is also that found in *E. coli* except that in this latter organism the glycan strands are not substituted by *O*-acetyl groups. *O*-Acetyl groups occur in the peptidoglycans of some gram-positive bacteria (*Staphylococcus aureus* and *Lactobacillus acidophilus*) [1, 10].

The peptidoglycans represent 2.5% of the dry weight of the cells of *Moraxella* but less than 1% of the cells of *Neisseria, Pseudomonas* and *Proteus*. The thicknesses of the G_2 peptidoglycan layers in the cell envelopes also vary according to the bacteria. In addition, differences were observed with regard to the extent of peptide cross linking. The peptidoglycan of *Neisseria* was especially highly cross linked, 58% of the peptide units occurring as trimers, 21% as dimers and 21% as uncross-linked monomers. Oligomers higher than dimers had not been detected in the peptidoglycan of *E. coli* [3, 4].

This work was supported in part by grants (to J.M.G.) from the *Fonds de la Recherche Fondamentale Collective*, Brussels (No 1000), by the *Centre National de la Recherche Scientifique*, France, and by the *Institut National de la Santé et de la Recherche Médicale*, France.

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