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THE CELL ENVELOPE IN *PROTEUS VULGARIS* P 18

ISOLATION AND CHARACTERIZATION OF THE PEPTIDOGLYCAN COMPONENT

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SUMMARY

Degradation of heat-treated cells of *Proteus vulgaris* P 18 with a protease preparation from a soil *Bacillus subtilis* allowed the isolation of a cell envelope structural entity, 60–70 Å thick, consisting of two electron-dense layers separated by a light one and composed, at least in part, of lipopolysaccharide O-antigen and of peptidoglycan. An enriched preparation, of which 55 % of the dry weight consisted of an intact peptidoglycan, was obtained through the sequential action upon lyophilized cells of hot aqueous sodium dodecyl sulfate and *B. subtilis* protease.

Both Chalaropsis B endo-*N*-acetylmuramidase and Streptomyces DD carboxypeptidase solubilized the enriched peptidoglycan preparation. The Chalaropsis enzyme completely degraded the glycan moiety into peptide-substituted disaccharide units. The DD carboxypeptidase hydrolyzed the D-alanyl-(D)-*meso*-diaminopimelic acid linkages involved in peptide cross-linking. In the intact peptidoglycan, about 35 % of the L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid-(L)-D-alanine residues occurred as uncross-linked monomers, 40 % of them as peptide dimers and 16 % as peptide trimers. It is likely that not all the peptides retained the D-alanine residue at their C-termini. About half of the *N*-acetylmuramic acid residues in the glycan moiety are O-acetylated, a property which is compatible with the high lysozyme resistance exhibited by the *P. vulgaris* peptidoglycan.

INTRODUCTION

All bacterial walls contain a rigid peptidoglycan network composed of glycan strands interconnected through peptide chains[1]. The glycan strands consist of alternating β-1,4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The *N*-acetylmuramic acid residues, or at least some of them, are substituted by L-alanyl-γ-D-glutamyl-L-R_3-D-alanine units, which in turn are cross-linked by bridges extending from the C-terminal D-alanine of one peptide unit either to the ω-amino group of the L-R_3-diamino acid or to the α-carboxyl group of D-glutamic acid of another peptide unit[1].

The cell envelopes of the Gram-negative bacteria are exceedingly complex. As revealed by thin sections, they consist[2,3] of two triple-layered membrane units, the inner plasma membrane and an outer L membrane containing lipopolysaccharides and lipoproteins, and of a series of intermediate layers that are sandwiched between the two former membrane structures. These intermediate layers usually consist of a dense layer, G_2, separated from the L membrane by a transparent layer, G_1, and from the plasma membrane by another transparent layer M (the terminology used throughout this paper is that proposed by DE PETRIS[3]). The peptidoglycan network has been located within the G_2 layer at the inner boundary of the wall part of the cell envelope and a great variety of methods have been used for its isolation. The peptidoglycan of *Escherichia coli* was the first one to be isolated in the form of a "rigid layer"[4]. Granules of lipoprotein were shown to be covalently linked to the peptidoglycan from which they could be removed with proteolytic enzymes[5,6]. The fine primary structure of the peptidoglycan was recently elucidated[7]. Essentially, the *N*-acetylmuramic acid residues in the glycan strands are substituted by L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine units and about 50 % of them are interconnected through direct D-alanyl-(D)-*meso*-diaminopimelic acid linkages.

By using a particulate cell-free extract of E. *coli*, cross-linking between peptide units was shown to be introduced by a penicillin-sensitive transpeptidase[8–10] which transferred the penultimate C-terminal D-alanine residue of a donor pentapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine to the amino group located on the D-carbon of *meso*-diaminopimelic acid of an acceptor peptide of the same composition. Furthermore, penicillin when added at sublethal dose levels to growing Gram-positive bacteria was also shown to reduce the efficiency of the transpeptidation reaction[11–13], thus giving support to the prevaling hypothesis that the transpeptidase in all penicillin-sensitive bacteria was the target of the penicillin molecule. Recently, however, SCHWARTZ *et al.*[14] reported that a reduction of the extent of peptide cross-linking could not be observed in the *in vivo* biosynthesis of the peptidoglycan after short-time exposure of *E. coli* to penicillin. Similary, KATZ AND MARTIN[15] reported that the peptidoglycan from the normal rod-shaped Gram-negative *Proteus mirabilis* and that from its penicillin-induced instable L-form differed very little, if at all, in their extent of peptide cross-linking.

P. vulgaris P 18 and its parent L-form[16–18] which is stable in the absence of penicillin but was originally induced by penicillin, are another system that can provide further information on the lesion caused by penicillin in Gram-negative bacteria. The experiments presented in this paper deal with the topology of the cell envelope of normal *P. vulgaris* P 18 and with the isolation and the characterization of its peptidoglycan component. Use has been made of a protease[19] derived from a strain of *Bacillus subtilis* isolated from soil. This protease specifically degrades some of the *P. vulgaris* cell envelope layers.

MATERIALS AND METHODS

Growth conditions of P. vulgaris

P. vulgaris P 18 was grown on a modified Medill liquid medium[20], in a New Brunswick fermentor (72 l), for 16 h at 37°.

Enzymes

The following enzymes were used: (1) egg-white lysozyme (Armour) and Chalaropsis B endo-*N*-acetylmuramidase[21] (a gift from Dr. N. A. Hash) hydrolyze β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine linkages in endo-position in the glycan moiety of the peptidoglycans[1]; (2) Streptomyces *N*-acetylmuramyl-L-alanine amidase hydrolyzes the bond of the junction between the glycan and the peptide moieties[1]; (3) D-alanyl-D-alanine carboxypeptidase from *Streptomyces albus* G[22,23] hydrolyzes C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid and other C-terminal D-alanyl-D linkages in the peptide moieties of the peptidoglycans; (4) bovine pancreas trypsin, Type I, was purchased from Sigma Co. (St. Louis, Mo., U.S.A.); (5) an exo-β-*N*-acetylglucosaminidase was prepared from pig epididymis according to SANDERSON *et al.*[24]; (6) a protease excreted by a soil *B. subtilis*[19] was prepared as follows. The selected strain was grown for 6 days at 37°, with shaking, in 4-l flasks each containing 2000 ml of the modified Medill medium. The culture filtrate was concentrated about 100-fold and partially desalted by ultrafiltration on UM10 Diaflo membrane using an Amicon apparatus (Meca-Vigor, Paris). This concentrated preparation was used without any further purification.

Protease unit

A casein solution (1 %, w/v; Hammarsten-Merck) made up in 0.05 M phosphate buffer (pH 7.4) (for *B. subtilis* protease assay) or in 0.1 M phosphate buffer (pH 8) (for trypsin assay) was incubated at 37° in the presence of the relevant enzyme. After increasing times of incubation, 1 ml of a 5 % trichloroacetic acid solution was added. The undegraded casein was removed by filtration through Prat-Dumas No. 7 filters and the absorbance of the filtrate was measured at 280 nm using a 10-mm cuvette. The activities of the preparations were estimated under the conditions required to induce an increase of absorbance of 0.200 or less. One protease unit was defined as the amount of enzyme which catalyzed an increase of 0.001 absorbance unit per min. Under these conditions, the *B. subtilis* protease preparation contained 0.19 unit /ml and the trypsin Type I (Sigma) contained 5 units/mg (Fig. 1).

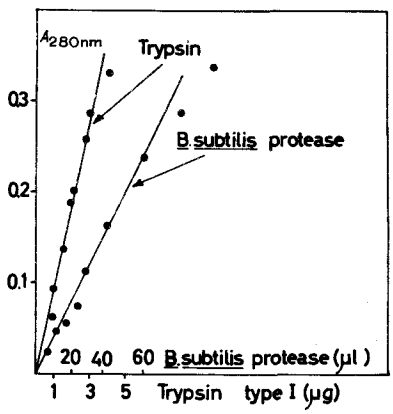


Fig. 1. Caseinolytic activity of trypsin and *B. subtilis* protease. Casein was incubated at 37° with increasing amounts of enzymes (see MATERIALS AND METHODS). After 20 min, undegraded casein was precipitated and filtered, and the absorbance of the supernatant was measured at 280 nm.

Chromatographic solvents

(I) Isopropanol–water–acetic acid (7:2:1, by vol.); (II) methanol–water–12 M HCl–pyridine (40:9:1:5, by vol.); (III) benzyl alcohol–chloroform–methanol–water–ammonia (15:15:15:3:1, by vol.); (IV) isobutyric acid–1 M NH_4OH (5:3, by vol.); (V) 1-butanol–acetic acid–water (3:1:1, by vol.).

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[25,26].

Qualitative identification of diaminopimelic acid in the presence of proteins was carried out by thin layer chromatography on plates of cellulose (83 M 086, Pleuger, Wijnegem, Belgium) of hydrolysates (6 M HCl, 16 h, 105°) of compounds previously oxidized[27] with performic acid. Solvent I was used in the first direction and Solvent II in the second direction.

Quantitative analyses of amino acids (after 16 h of hydrolysis in 6 M HCl, 105°) and of hexosamines (3 M HCl, 95°, 4 h) were also carried out with the help of an Unichrom (Beckman) amino acids analyzer. Separation of muramic acid (between serine and glutamic acid) and separation of *meso*-diaminopimelic acid (between methionine and isoleucine) were carried out on the long column with resin Type AA 15, with buffer at pH 3.2 changed to buffer at pH 4.28 after exactly 1 h 38 min. Separation of basic amino acids and of glucosamine were carried out on the short column with resin Type AA 27 with pH 5.28 buffer.

Quantitative estimation of mono-dinitrophenyl (DNP)-*meso*-diaminopimelic acid was carried out as previously described[7] after separation of the mono-DNP derivative by thin-layer chromatography on plates of Stahl's silica gel G (Merck) with Solvent III, and using mono-DNP-(D)-*meso*-diaminopimelic acid as standard[26].

Identification of 2-keto-3-deoxyoctonate was made according to a modified procedure of WARAVDEKAR AND SASLAW[29]. Samples (350 μl) were mixed with 50 μl of the periodic acid reagent. After 20 min, at room temperature, 100 μl of the arsenite reagent was added and the solutions were thoroughly mixed. 1 min later, 100 μl of the mixture was added to 200 μl of the thiobarbituric acid solution. After 20 min at 100°, absorbance at 535 nm (pink color) was measured.

Model compounds

Peptides L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine and L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine, isolated from *Bacillus megaterium*[7] and from *Clostridium perfringens*[30], respectively, were used. Disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid and β-1,4-*N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid isolated from *Lactobacillus acidophilus* strain 63 AM Gasser[31] were also used.

Electrophoreses

Electrophoreses were carried out on Whatman No. 3 MM paper using an electrorheophor apparatus Pleuger at pH 4 (acetic acid–pyridine–water (9:2:1000, by vol.)).

Gel filtrations

Two columns, connected in series, of Sephadex G-50 fine (700 ml) and Sephadex G-25 fine (500 ml) were used. The gel filtration properties of the compounds were expressed in terms of distribution coefficient $K_D = (V_e - V_o)/V_i$ with V_e = elution volume; $V_o = V_e$ of totally excluded material and $V_i = V_e$ of NaCl $- V_o$.

Serological techniques

Antisera were prepared as previously described[32] by prolonged immunization of rabbits with either heat-treated cells of *P. vulgaris* (2 h in boiling water, under which conditions the flagellar antigens are selectively destroyed) or trichloroacetic acid-extracted lipopolysaccharide O-antigen. Immunodiffusion was performed on agar plates (2 % special Agar Noble) in 0.05 M Veronal buffer (pH 8.6). Agglutination tests were recorded after 2 h of incubation at 37°.

Electron microscopy

(1) Negative staining. A drop of the bacterial suspension was placed on the Formvar support film, left for 2 min and the excess of liquid was removed. A drop of a 2 % potassium phosphotungstate solution (pH 4.5–5) was added. After a few sec, the excess of reagent was removed and the grid was dried. (2) Ultra-thin sections. The bacterial suspension was centrifuged. The pellet was fixed for 1 h with cold 2 % glutaraldehyde solution in 0.05 M phosphate buffer (pH 7.2), post-fixed for 1 h with osmic acid according to the technique of MILLONIG[33], dehydrated in ethanol (using sequentially, for 15 min each, 50 %, 75 %, 90 % and absolute ethanol) and finally embedded in epon. Thin sections (LKB ultra-microtome) were poststained with uranyl acetate and lead citrate[34] and examined in a Philips EM 300 electron microscope. Non lyophilized cells or cell materials were always used.

RESULTS

Degradation of P. vulgaris P 18 with proteases

Both *B. subtilis* enzyme preparation (MATERIALS AND METHODS) and trypsin had no visible effect on suspensions of living *P. vulgaris* (stationary phase cells) but lysed heat-treated (5 min at 80°) cells. Heated cells were washed twice with water and lyophilized. The rates of clarification of such cell suspensions (150 μg, dry weight, in a final volume of 200 μl) incubated at 37° either in the presence of 0.115 unit of *B. subtilis* protease (in 0.05 M phosphate buffer, pH 7.4) or in the presence of 0.090 unit of trypsin (in 0.1 M phosphate buffer, pH 8) were virtually identical (Fig. 2). At completion of the reactions, however, the residual turbidities represented 5 % of the original value after treatment with the *B. subtilis* enzyme and 16 % after treatment with trypsin. Cell degradations were paralleled in both cases by the liberation of free amino groups which maximally amounted to 2700 nm equivalents after *B. subtilis* protease action and 2100 nm equivalents after trypsin action. No traces of reducing groups of compounds containing 2-keto-2-deoxyoctonate, *meso*-diaminopimelic acid or hexosamines, and of serologically active materials could be detected in the solubilized degraded products.

Electron microscopy of intact and protease-degraded cells of P. vulgaris P 18

Negative staining of non-heated, non lyophilized cells showed the convoluted nature of the cell surface (Fig. 3a) which exhibited deep valleys and grooves separated

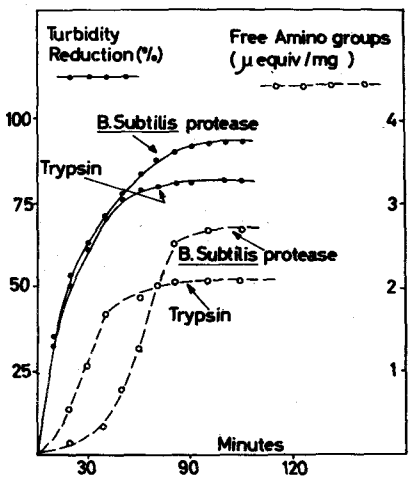


Fig. 2. Kinetics of degradation of heat-treated cells of *P. vulgaris* P 18 by trypsin and by *B. subtilis* protease. Heated and lyophilized cells (see text) (150 μg, dry weight) were incubated at 37° in the appropriate buffer (see text; final volume 200 μl) either in the presence of trypsin (0.09 unit) or in the presence of *B. subtilis* protease (0.115 unit). Solubilization (*i.e.* turbidity reduction) and liberation of soluble amino groups (measured with the fluorodinitrobenzene technique) occurred simultaneously.

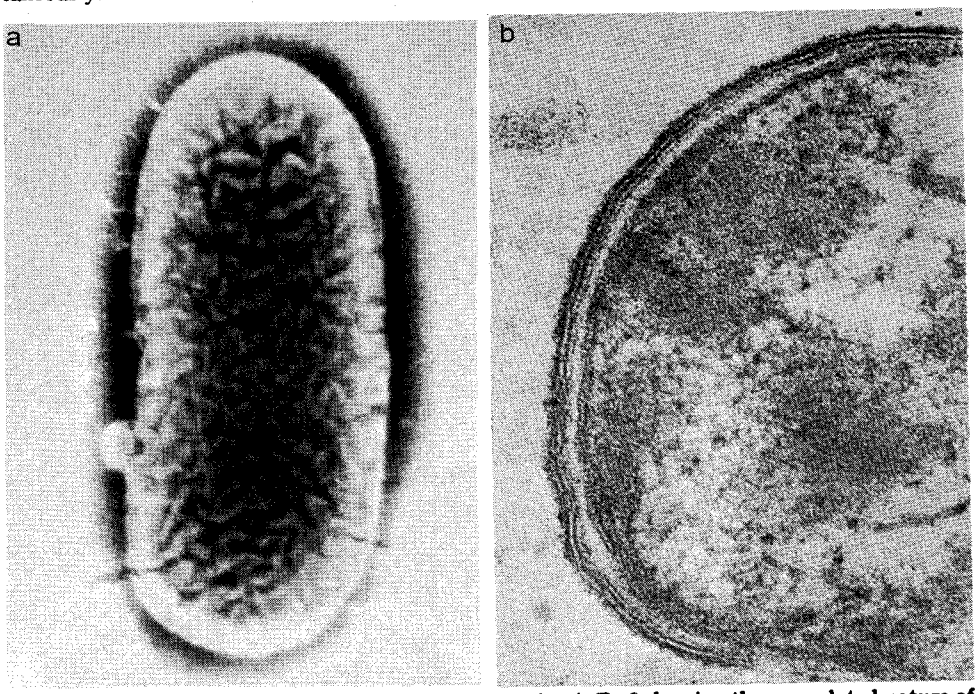


Fig. 3. a. Unfixed and negatively stained cells of *P. vulgaris* P 18 showing the convulated nature of the surface. Magnification × 42000. b. Section of heated cells (5 min at 80°) of *P. vulgaris* P 18 showing the complex multilayered structure of the cell envelope. Magnification × 129500.

by lobules. No fine structure was visible. Sometimes, flagella and pili were observed.

Thin sections of heated cells (5 min 80°) revealed an envelope structure typical of Gram-negative bacteria (Fig. 3b). Proceeding inward from the surface, one recognizes (see INTRODUCTION and ref. 3) the L_1, L_2, and L_3 layers of the outer L membrane, the G_1, G_2 and M layers of the intermediate region and, finally, the triple-layered plasma membrane. The wall part of the envelope (*i.e.* layers L_1 to G_2) was about 160–180 Å and the plasma membrane underlying the M region was about 50–60 Å (Fig. 4).

Survey of thin sections of heat- and trypsin-treated cells showed cytoplasmic material surrounded by a faintly visible membrane and detached at least partially, from the wall part of the cell envelope (Fig. 5). The wall thickness had decreased to 120 Å. Even after prolonged treatments, the cytoplasmic bodies remained trapped within the detached walls.

Treatment of heated cells with the *B. subtilis* enzyme preparation induced the

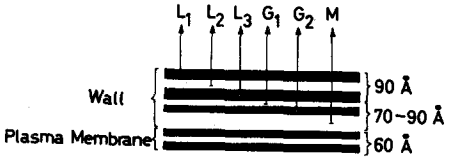


Fig. 4. Schematic representation of the structure of the cell envelope of *P. vulgaris* P 18. The terminology is that used by DE PETRIS[3] to describe the cell envelope of *E. coli*.

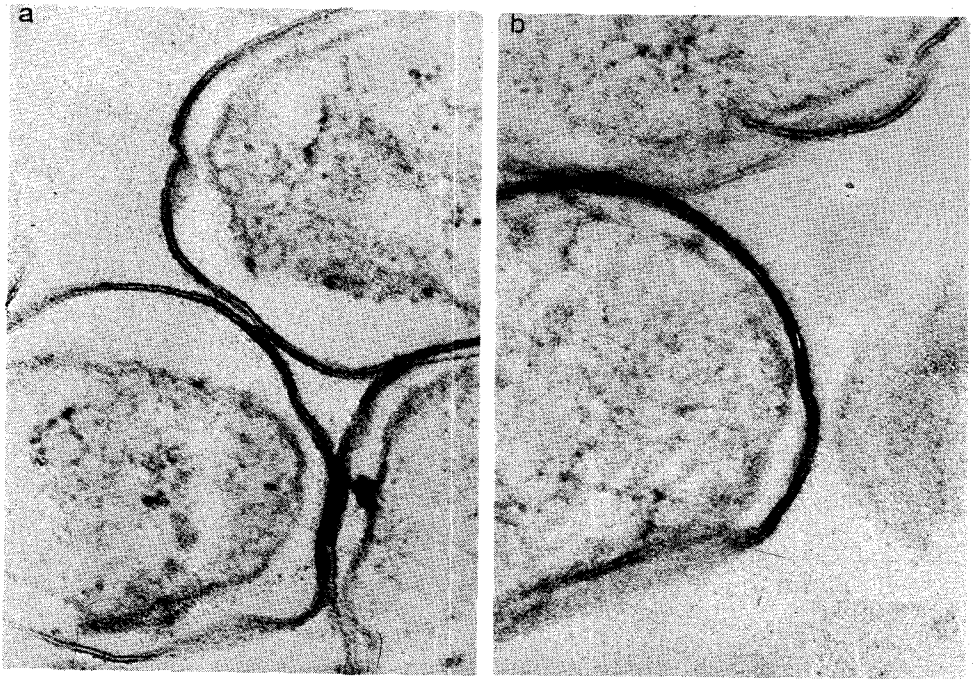


Fig. 5. Heated cells (see Fig. 3b) of *P. vulgaris* P 18 after prolonged treatment with trypsin. Thin section micrographs. Conditions of treatment are those which insured maximal degradation (see text and Fig. 2). a. Magnification × 56000. b. Magnification × 105000.

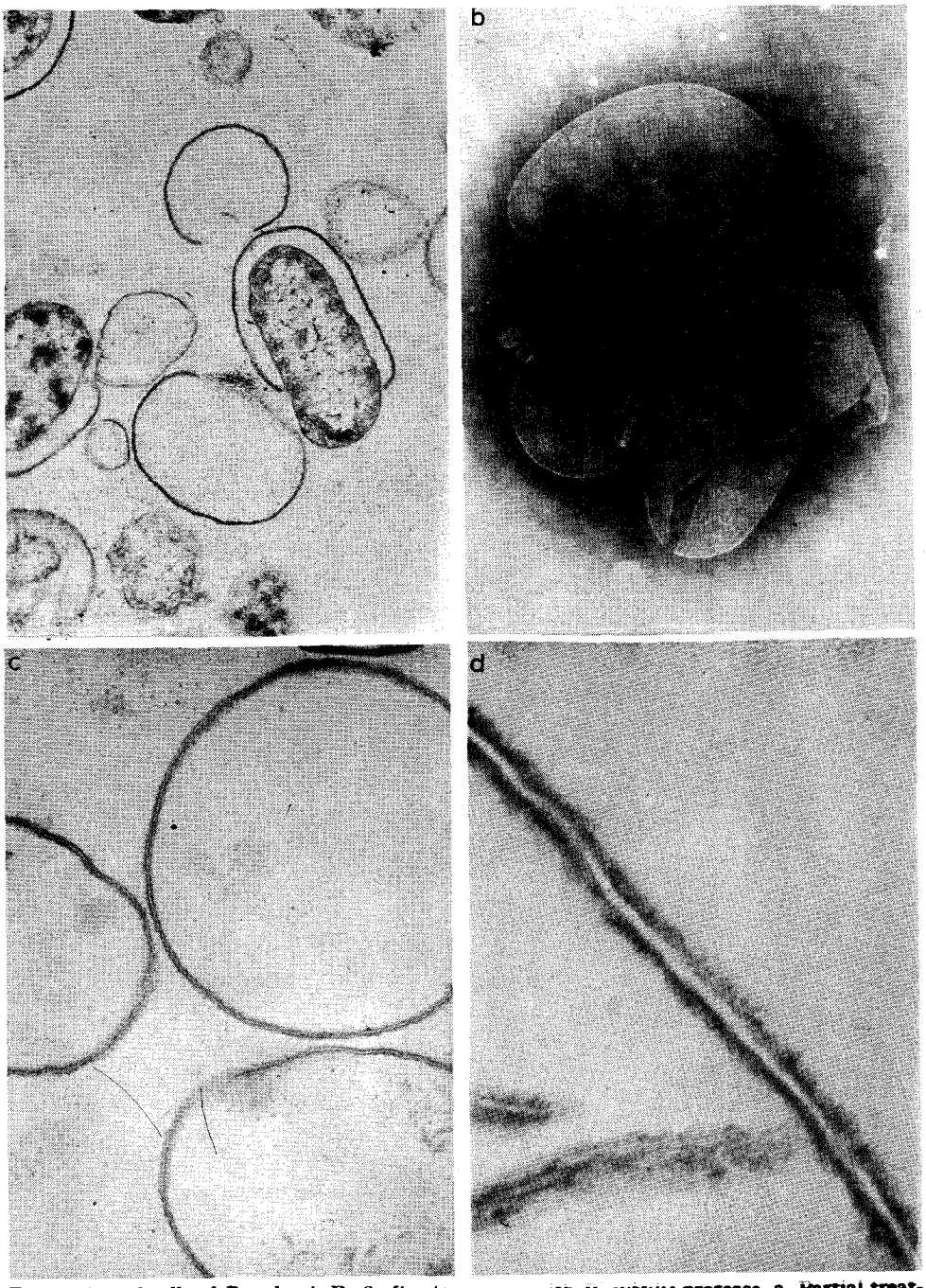


Fig. 6. Heated cells of *P. vulgaris* P 18 after treatment with *B. subtilis* protease. a. Partial treatment with *B. subtilis* protease. Thin section micrographs. Magnification × 28000. b. Thourough treatment with *B. subtilis* protease. Negative staining. Conditions of treatment are those which insured maximal degradation (see text and Fig. 2). Magnification × 42000. c. Thorough treatment with *B. subtilis* protease. Thin section micrographs. Magnification × 56000. d. Magnification × 290500.

extrusion through the walls of the cytoplasmic bodies (Fig. 6). Eventually the cytoplasmic bodies were completely degraded. Simultaneously, outer wall layers were eliminated. At completion of the reaction (Figs. 6b–6d), the detached walls were almost completely free of cytoplasmic debris and they were reduced to structural entities, 60–70 Å thick, consisting of two electron dense layers, each about 15–20 Å thick, separated by a light one.

Chemical and serological analyses of the cell materials obtained by centrifugation at 60000 × *g* after either trypsin or *B. subtilis* protease treatments showed that both consisted, at least partially, of lipopolysaccharide O-antigen (presence of 2-keto-3-deoxyoctonate and of serologically active compounds) and of peptidoglycan (presence of muramic acid and of *meso*-diaminopimelic acid).

Use of B. subtilis protease to obtain wall peptidoglycan from P. vulgaris P 18

We tried to isolate the peptidoglycan from cells previously freed of the lipopolysaccharide O-antigen by extraction with hot sodium dodecyl sulfate[5] solution. Preliminary experiments showed that prior isolation of the cell envelopes was not necessary, hence the procedure was directly applied to lyophilized bacteria. Bacteria (10 g) were suspended in water (100 ml) and added dropwise, with stirring, to 1 l of boiling 4 % sodium dodecyl sulfate solution. The suspension was stirred for another two h during which time it slowly cooled and then kept overnight at room temperature. The suspension was centrifuged for 30 min at 66000 × *g* and the pellet was washed 5 times with water and lyophilized. The rates of clarification of the sodium dodecyl sulfate-treated cells (150 μg, dry weight, in a final volume of 200 μl), incubated at 37° either in the presence of *B. subtilis* protease (in 0.05 M phosphate buffer, pH 7.4) or in the presence of trypsin (in 0.1 M phosphate buffer, pH 8) are shown in Fig. 7. A maximal turbidity reduction of 72 % of the original value with a parallel liberation of 1250 nequivalents of amino groups per mg (dry weight) were obtained with the help of 0.350 unit of trypsin. Higher enzyme concentrations and/or prolonged incubations

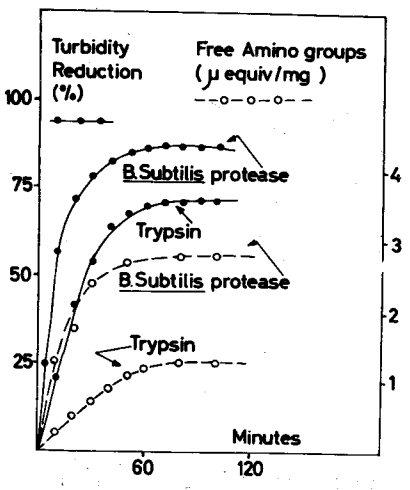


Fig. 7. Kinetics of degradation of heat- and sodium dodecyl sulfate-treated cells of *P. vulgaris* P 18 by trypsin and by *B. subtilis* protease. Trypsin (0.350 unit) and *B. subtilis* protease (0.143 unit) were used for 150 μg of envelope material. (For other conditions, see Fig. 2.)

did not increase the degradation. In contrast, a maximal turbidity reduction of 88 % with a parallel liberation of 2800 nequivalents of amino groups, per mg, was obtained with only 0.143 unit of *B. subtilis* protease.

Large amounts of sodium dodecyl sulfate-treated cells were separately degraded with trypsin and with *B. subtilis* enzyme under the conditions described above. The residual, non solubilized materials were washed and analyzed (Table I). About 55 %, dry weight, of the preparation obtained after *B. subtilis* enzyme treatment consisted of a peptidoglycan compound in which muramic acid, glucosamine, alanine, glutamic acid and *meso*-diaminopimelic acid occurred in the molar ratios of 1:1:1.75:1:1. This preparation also contained about 0.14 mole of aspartic acid per mole of *meso*-diaminopimelic acid. All the other amino acids were either absent or present in trace amounts. In the preparation obtained after sodium dodecyl sulfate treatment alone as well as in that obtained after further treatment with trypsin, large amounts of proteins were still present and the peptidoglycan component only represented 15 % and 35 %, respectively, of the relevant preparations.

TABLE I

CHEMICAL COMPOSITION OF *P. vulgaris* P 18 CELL ENVELOPE OBTAINED AFTER TREATMENT OF HEATED CELLS WITH HOT SODIUM DODECYL SULFATE (PREPARATION P 18, SODIUM DODECYL SULFATE) FOLLOWED BY TREATMENT WITH TRYPSIN (PREPARATION P 18, SODIUM DODECYL SULFATE, TRYPSIN) OR BY TREATMENT WITH *B. subtilis* PROTEASE (PREPARATION P 18 SODIUM DODECYL SULFATE, *B. subtilis*).

Results are exposed in nmoles/mg dry weight of cell envelope material.

Amino acid	*P 18, sodium dodecyl sulfate*	*P 18, sodium dodecyl sulfate, trypsin*	*P 18, sodium dodecyl sulfate, B. subtilis*
Lysine	140	90	40
Histidine	46	30	0
Arginine	102	30	0
Aspartic acid	200	150	80
Threonine	85	55	0
Serine	90	40	40
Glutamic acid	335	520	560
Proline	+	0	0
Glycine	155	155	60
Alanine	355	800	920
Cysteine	0	0	0
Valine	+	+	0
Methionine	0	0	0
Isoleucine	140	120	40
Leucine	210	210	60
Tyrosine	+	0	0
Phenylalanine	+	0	0
meso-Diaminopimelic acid	150	335	530
Glucosamine	190	420	620
Muramic acid	120	120	580

Enzymatic degradations of P. vulgaris P 18 peptidoglycan

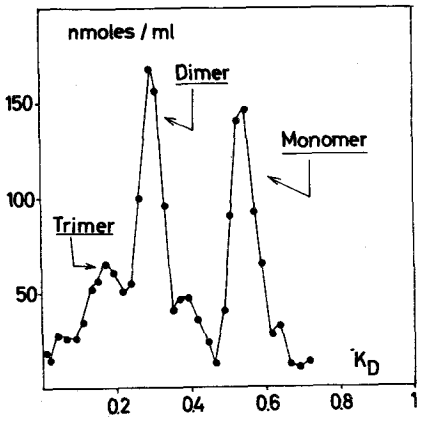
Egg white lysozyme had a very weak lytic effect on the peptidoglycan preparation obtained after sequential treatments of the cells with sodium dodecyl sulfate and *B. subtilis* protease. At a substrate to enzyme ratio of 25:1 (in 0.06 M phosphate

buffer, pH 6.2), lysozyme induced a maximal turbidity decrease of 62 % and a maximal liberation of 0.08 equivalent of reducing groups (standard: *N*-acetylglucosamine) per disaccharide peptide unit. In contrast to lysozyme, Chalaropsis B endo-*N*-acetylmuramidase (10 μg of enzyme per mg of substrate, in 200 μl of 0.02 M acetate buffer, pH 4.5, 1 h at 37°) induced a complete solubilization of the preparation and the liberation per mg of 480 nmoles of β-1,4 *N*-acetylglucosaminyl-*N*-acetylmuramyl peptide-substituted disaccharide units: that is, the glycan moiety was virtually completely hydrolyzed into disaccharide fragments. Analyses of the products of Chalaropsis B degradation showed that terminal amino groups of L-alanine only occurred in trace amounts and that about 50 % of the *meso*-diaminopimelic acid residues had one amino group free, thus demonstrating that all the *N*-acetylmuramic acid residues in the glycan were peptide-substituted and that, on the average, the extent of cross-linking within the peptide moiety was that of a peptide dimer. The involvement of *meso*-diaminopimelic acid in peptide cross-linking was further demonstrated through the action of the Streptomyces DD carboxypeptidase (MATERIALS AND METHODS). Under its action (2.5 μg of enzyme for 100 μg of substrate, in 50 μl of 0.02 M Tris–HCl buffer, pH 7.5, supplemented with 0.002 M $MgCl_2$, at 37°), the intact peptidoglycan preparation also underwent complete solubilization as a result of the hydrolysis of D-alanyl-(D)-*meso*-diaminopimelic acid linkages. At the end of the process (1 h), all the *meso*-diaminopimelic acid residues had one amino group free.

Fractionation of P. vulgaris P 18 peptidoglycan

The peptidoglycan preparation (12.5 μmoles expressed as *meso*-diaminopimelic acid residues) obtained through sequential treatments with sodium dodecyl sulfate and *B. subtilis* protease (Table I), was degraded into disaccharide peptide fragments with the help of the Chalaropsis B endo-*N*-acetylmuramidase. Filtration of the degraded products in 0.1 M LiCl on columns of Sephadex G-50 and G-25 (MATERIALS AND METHODS) yielded three fractions (Fig. 8) of which the K_D values were those expected for a disaccharide peptide monomer M ($K_D = 0.55$; yield: 35 % in terms of either *meso*-diaminopimelic acid residues or reducing groups), for a bisdisaccharide peptide dimer D ($K_D = 0.30$; yield: 40 %) and for a trisdisaccharide peptide trimer T ($K_D = 0.18$; yield: 16 %). The fractions were separately desalted by filtration in water on the same Sephadex G-50, G-25 columns. They were found to be homogeneous, and indistinguishable from each other, by paper electrophoresis at pH 4. Fractions M, D and T had identical amino acid and hexosamine composition; glucosamine, muramic acid, alanine (L + D), glutamic acid and *meso*-diaminopimelic acid occurred in the molar ratio: 1:1:1.75:1:1. Estimation of terminal amino groups, however, showed that 98 % of the *meso*-diaminopimelic acid residues in Fraction M, 47 % of them in Fraction D and 32 % in Fraction T, had one amino group free, thus establishing the monomeric, dimeric and trimeric structures of the relevant fractions.

Fractions M, D and T showed heterogeneity by silica gel thin-layer chromatography in Solvent IV (Fig. 9). Fraction M could be separated, under these conditions, into Subfractions M_1 and M_2 and fraction D into Subfractions D_1, D_2 and D_3. Again these individual subfractions had identical chemical compositions. Fraction T could not be separated into subfractions because of the slow chromatographic migration of the compounds.



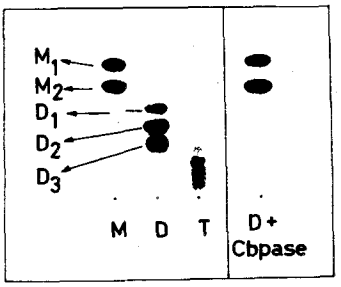


Fig. 8. Filtration on Sephadex G-50, G-25, in 0.1 M LiCl, of *P. vulgaris* peptidoglycan degraded by Chalaropsis B endo-*N*-acetylmuramidase. Results are expressed in nmoles of disaccharide units per ml.

Fig. 9. Silica gel thin layer chromatography in Solvent IV (two runs of Fraction M (*i.e.* Monomer of Fig. 8), Fraction D (*i.e.* Dimer of Fig. 8) and Fraction T (*i.e.* Trimer of Fig. 8)), and of Fraction D after treatment with *S. albus* G DD carboxypeptidase (Cbpase). Detection: ninhydrin.

Characterization of the peptidoglycan fragments from P. vulgaris P 18

The disaccharide units. Fraction M was degraded into free disaccharide and free peptide units with the help of the Streptomyces *N*-acetyl-muramyl-L-alanine amidase (in 0.025 M acetate buffer, pH 5.4). Color yields in the reducing power determination, kinetics of the color development in the Morgan–Elson reaction (with increasing times of heating in 1 % borate) and determination of the molar extinction coefficient, after 30 min of heating, indicated that the free disaccharide was a 1:4, and not a 1:6, linked *N*-acetylglucosaminyl-*N*-acetylmuramic acid unit. Pig epididymis exo-β-*N*-acetylglucosaminidase, an enzyme specific for β-glycosidic linkages, converted the disaccharide into free *N*-acetylhexosamine residues (on the basis of the color development in the Morgan–Elson reaction after 7 min of heating in 1 % borate). Paper chromatography, using Solvent V, of the amidase-treated Fraction M, however, yielded two disaccharide fragments which exhibited R_F identical to authentic β-1,4 *N*-acetylglucosaminyl-*N*-acetylmuramic acid ($R_F = 0.36$) and β-1,4 *N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid ($R_F = 0.57$), respectively. Moreover, sequential treatments of Fraction M with amidase and with exo-β-*N*-acetylglucosaminidase yielded *N*-acetylglucosamine ($R_F = 0.38$), *N*-acetylmuramic acid ($R_F = 0.60$) and *N*,*O*-diacetylmuramic acid ($R_F = 0.76$). Finally, Fractions M_1, M_2 and Fractions D_1, D_2 and D_3 were separately treated with amidase. Fractions M_1 and D_1 gave rise to the *O*-acetyldisaccharide, Fractions M_2 and D_3 to disaccharide and Fraction D_2 to both disaccharide and *O*-acetyldisaccharide.

The peptide moiety. After treatment of Fraction M with amidase, the free peptide was found by paper electrophoresis at pH 4 to be indistinguishable from L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine. Both were anionic. Under the same conditions, the amidated peptide L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine was cationic. After treatment of Fraction D with the *S. albus* G DD carboxypeptidase (an enzyme which specifically hydrolyzes D-alanyl-(D)-*meso*-

diaminopimelic acid linkages) Compounds D_1, D_2 and D_3 disappeared (Fig. 9) and gave rise to the disaccharide peptides M_1 and M_2.

Evidently, the above results are only compatible with the presence in the Chalaropsis-degraded peptidoglycan of two disaccharide peptide monomers M_1 and M_2, of three bisdisaccharide peptide dimers D_1, D_2 and D_3, and of several trisdisaccharide peptide trimers. The two disaccharide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine peptide monomers differ from each other by the presence of an acetyl group on the *N*-acetylmuramic acid residue of one of them, *i.e.* the M_1 monomer. The three disaccharide peptide trimers, in which two peptide monomers are interlinked through D-alanyl-(D)-*meso*-diaminopimelic acid linkages, differ from each other by the number of disaccharide and O-acetylated disaccharide substituents (two disaccharides in D_3; one disaccharide and one *O*-acetyldisaccharide in D_2; two *O*-acetyldisaccharides in D_1).

DISCUSSION

The cell envelope of *P. vulgaris* P 18 showed in thin sections the multilayered structure and organization that had been previously found in several other Gram-negative bacteria[2,3]. The inner plasma membrane 50–60 Å and an outer L membrane 90 Å are separated from each other by a series of layers which can be tentatively identified as the G_1 (light), G_2 (dense) and M (light) layers (Figs. 3b and 4), previously described in *Escherichia coli*[3]. Incubation of heat-treated Gram-negative bacteria with proteolytic enzymes such as papain, trypsin, pronase, chymotrypsin and pepsin[2,3,5,6,35] constantly resulted in the detachment of the wall part of the cell envelope from the cytoplasmic body without any apparent damage to the outer L membrane. Fine analyses revealed that the peptidoglycan-containing G_2 layer was simultaneously detached from the outer L membrane with the parallel obliteration of the G_1 region, and from the cytoplasmic membrane probably through the hydrolysis of the adhesion sites within the M region. The G_2 layer then appeared as a dense line 15–20 Å, still following the contours of the plasma membrane. The true structural protein which is hydrolyzed is, most likely, that lipoprotein which is covalently linked to the peptidoglycan. In *E. coli*, it was shown[6] that a lysylarginine dipeptide extends from some of the diaminopimelic acid residues of the peptidoglycan to the lipoprotein component and that, on the average, one lipoprotein molecule is linked to every tenth repeating peptide unit in the peptidoglycan. The lysylarginine linkage and the arginyllipoprotein linkage were shown to be specifically hydrolyzed by trypsin and pronase, respectively[6].

With respect to the action of trypsin, heated cells of *P. vulgaris* behaved as the other Gram-negative bacteria. Degraded cytoplasmic bodies were dissociated from, but remained trapped in the interior of the outer cell envelopes (Fig. 5). The enzyme complex excreted by *B. subtilis* had no visible lytic effect on living cells of *P. vulgaris*. On heat-treated cells it had a more powerful degrading action than trypsin both with respect to the extent of lysis and to the amount of amino groups released (Figs. 2 and 7). No traces of reducing groups and of hexosamines- and *meso*-diaminopimelic acid-containing compounds were detected in the solubilized degraded products. 2-Keto-3-deoxyoctonate and serologically active compounds were solely found in the non-solubilized residual cell material. Finally, analyses of the peptidoglycan preparation

obtained after action of the *B. subtilis* enzyme on heat–sodium dodecyl sulfate-treated cells (Table I) indicated that the peptidoglycan compound had not been damaged. Hence the enzyme preparation had no detectable effect on both the lipopolysaccharide O-antigen and the peptidoglycan components of the cell envelope and, to all appearances, its lytic action was solely due to one or several proteases.

Electron microscopic studies revealed that after thorough action of *B. subtilis* protease(s) upon heated cells of P. *vulgaris*, the only organelle left apparently intact was a structure 60–70 Å consisting of two dense layers separated by a light one. From the foregoing, one of the two dense layers could thus be tentatively identified as the peptidoglycan, *i.e.* the G_2 layer, and the other as the O-antigen which consequently, should be the L_3 layer of the outer L membrane or at least part of it. Following this conclusion, both M and L_2 layers would be the targets of the *B. subtilis* protease(s). The 60–70 Å entities observed after thorough action of the *B. subtilis* protease(s) presented gaps or spots of discontinuity (Fig. 6) as if each of these entities had been opened or torn in one, perhaps specifically located region. It may be that these gaps were actually produced by the *B. subtilis* protease within the peptidoglycan network (possibly in conjunction with prior treatment by heat). It may also be hypothesized that the protease elimination of one or several structural proteins from the wall, particularly impaired the mechanical strength of those regions involved in wall biosynthesis and expansion (in which autolytic enzymes are usually located), so that the unprotected, nascent peptidoglycan readily underwent physical break-down.

The *B. subtilis* protease, whatsoever its precise mechanism of action, appears to be a useful tool for the isolation of the peptidoglycan from Gram-negative bacteria. The primary structure of the *P. vulgaris* peptidoglycan isolated in the course of the present study, appears to be similar to that of *E. coli* (see INTRODUCTION), except that in *P. vulgaris*, both *N*-acetylmuramic acid and *N,O*-diacetylmuramic acid residues occur in the glycan moiety and that the extent of peptide cross-linking appears to be higher. In *P. vulgaris*, about 35 % of the peptide units occur as uncross-linked monomers, 40 % as dimers and 16 % as trimers. No peptide trimers were detected in walls of *E. coli*. The structure of the peptide units isolated in the course of the present study was not fully established. The data, however, are consistent with the sequence L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine, previously demonstrated in *E. coli*[7]. As in *E. coli*, some of the peptide monomers and oligomers in *P. vulgaris* have probably not retained the C-terminal D-alanine residue (Table I). By contrast to the peptidoglycan of *E. coli* which is readily degraded by lysozyme, that of *P. vulgaris* is highly resistant to this endo-*N*-acetylmuramidase. The glycan in *P. mirabilis* also exhibits similar "lysozyme resistance"[15]. This property, at least in *P. vulgaris*, is probably due to the occurrence of *O*-acetyl substituents in the glycan strands. It could also be due to the nature and structure of other wall components which are linked to the peptidoglycan moiety. Analytical data of Table I suggest, for example, that in contrast to *E. coli*, a lysylarginine dipeptide is probably not involved in the linkage between the peptidoglycan and the lipoprotein in *P. vulgaris*. Aspartic acid might be part of the bridging.

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REFERENCES

1 J. M. GHUYSEN, *Bacteriol. Rev.*, 32 (1968) 425.
2 A. M. GLAUERT AND M. J. THORNLEY, *Ann. Rev. Microbiol.*, 23 (1969) 159.
3 S. DE PETRIS, *J. Ultrastruct. Res.*, 19 (1967) 45.
4 W. WEIDEL AND H. PELZER, *Advan. Enzymol.*, 26 (1964) 193.
5 V. BRAUN AND K. REHN, *European J. Biochem.*, 10 (1969) 426.
6 V. BRAUN AND U. SIEGLIN, *European J. Biochem.*, 13 (1970) 336.
7 J. VAN HEIJENOORT, L. ELBAZ, P. DEZELEE, J. F. PETIT, E. BRICAS AND J. M. GHUYSEN, *Biochemistry*, 8 (1968) 207.
8 K. IZAKI, M. MATSUHASHI AND J. L. STROMINGER, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 656.
9 Y. ARAKI, R. SHIRAI, A. SHIMADA, N. ISHIMOTO AND E. ITO, *Biochem. Biophys. Res. Commun.*, 23 (1966) 466.
10 J. L. STROMINGER, in TH. BUCKER AND H. SUS, *Inhibitor Tools in Cell Research, 20. Coll. Ges. Biol. Chem., 1969*, Springer Berlin, 1969, p. 187.
11 D. J. TIPPER AND J. L. STROMINGER, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1133.
12 E. M. WISE AND J. T. PARK, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 75.
13 E. MUNOZ, J. M. GHUYSEN, M. LEYH-BOUILLE, J. F. PETIT, H. HEYMANN, E. BRICAS AND P. LEFRANCIER, *Biochemistry*, 5 (1966) 3748.
14 U. SCHWARTZ, A. ASMUS AND H. FRANK, *J. Mol. Biol.*, 41 (1969) 419.
15 W. KATZ AND H. H. MARTIN, *Biochem. Biophys. Res. Commun.*, 39 (1970) 744.
16 R. TULASNE, *Rev. Immunol.*, 15 (1951) 223.
17 R. MINCK, *Rev. Immunol.*, 19 (1955) 86.
18 R. TULASNE, R. MINCK, A. KIRN AND J. KREMBEL, *Ann. Inst. Pasteur*, 99 (1960) 859.
19 J. FLECK AND R. MINCK, *Compt. Rend.*, 267 (1968) 812.
20 R. MINCK, A. KIRN AND M. GALLERON, *Ann. Inst. Pasteur*, 92 (1957) 138.
21 J. N. HASH AND N. V. ROTHLAUF, *J. Biol. Chem.*, 242 (1967) 853.
22 J. M. GHUYSEN, M. LEYH-BOUILLE, R. BONALY, M. NIETO, H. R. PERKINS, K. H. SCHLEIFER AND O. KANDLER, *Biochemistry*, 9 (1970) 2955.
23 M. LEYH-BOUILLE, J. M. GHUYSEN, R. BONALY, M. NIETO, H. R. PERKINS, K. H. SCHLEIFER AND O. KANDLER, *Biochemistry*, 9 (1970) 2961.
24 A. R. SANDERSON, J. L. STROMINGER AND S. G. NATHENSON, *J. Biol. Chem.*, 237 (1962) 3603.
25 J. M. GHUYSEN, D. J. TIPPER AND J. L. STROMINGER, *Methods Enzymol.*, 8 (1966) 685.
26 J. M. GHUYSEN, E. BRICAS, M. LACHE AND M. LEYH-BOUILLE, *Biochemistry*, 7 (1968) 1450.
27 C. E. DENT, *Biochem. J.*, 41 (1947) 240.
28 E. BRICAS, J. M. GHUYSEN AND P. DEZELEE, *Biochemistry*, 6 (1967) 2598.
29 V. S. WARAVDEKAR AND L. D. SASKAW, *J. Biol. Chem.*, 234 (1959)
30 M. LEYH-BOUILLE, R. BONALY, J. M. GHUYSEN, R. TINELLI AND D. J. TIPPER, *Biochemistry*, 9 (1970) 2944.
31 J. COYETTE AND J. M. GHUYSEN, *Biochemistry*, 9 (1970) 2935.
32 R. MINCK, A. KIRN AND J. FLECK, *Ann. Inst. Pasteur*, 101 (1961) 178.
33 G. MILLONIG, *J. Appl. Physiol.*, 32 (1961) 1637.
34 E. S. REYNOLDS, *J. Cell Biol.*, 17 (1963) 208.
35 T. USHIJIMA, *Japan. J. Microbiol.*, 14 (1970) 15.