Structural Variations in Bacterial Cell Wall Peptidoglycans Studied with *Streptomyces* F_1 *endo-N*-Acetylmuramidase*

Emilio Muñoz,† Jean-Marie Ghuysen, Mélina Leyh-Bouille, Jean-François Petit,‡ and Regina Tinelli

ABSTRACT: An improved procedure for the purification of the *Streptomyces* F_1 β-1,4 *endo-N*-acetylmuramidase is described. F_1 enzyme digests cell walls from bacteria of the most important gram-positive genera. Its mechanism of action upon cell walls of *Staphylococcus aureus*, *Micrococcus roseus*, and *Micrococcus lysodeikticus* has been studied.

To all appearances, the affinity of the F_1 enzyme for the glycosidic linkages of *N*-acetylmuramic acid in the polysaccharide chains is greatly enhanced by the peptide substitution of these residues. From an integration of the properties of the F_1 enzyme digests of various cell walls, it appears that tight network peptidoglycans, typified by a high degree of cross-linkings of the polysaccharide chains through peptide units, as they occur in cell walls of *S. aureus* and *M. roseus*, are not encountered in many cell walls from gram-positive bacteria.

The mechanical strength of the peptidoglycan polymer which forms the rigid network of all bacterial cell walls depends on a high degree of cross-linking between peptide and polysaccharide chains. Linear polysaccharide chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues are substituted with peptides which are themselves cross-linked. All of the bacteriolytic enzymes whose mechanism of action has been studied have been shown to fragment the cell wall peptidoglycan, acting on the glycosidic moiety, or on the peptide moiety, or at the junction between the glycosidic and the peptide moieties. Two classes of bacteriolytic glycosidases are known: *endo-N*-acetylglucosaminidases which hydrolyze glycosidic linkages of *N*-acetylglucosamine to *N*-acetylmuramic acid, and *endo-N*-acetylmuramidases which hydrolyze glycosidic linkages of *N*-acetylmuramic acid to *N*-acetylglucosamine. As far as is known, the glycosidases have limited lytic spectra. This may be due to the presence in the cell walls of nonpeptidoglycan compounds which partially or completely abolish the affinity of the enzyme for the sensitive linkages, or to structural variations occurring in the glycan portion of cell wall peptidoglycans. Much remains to be investigated in this field and, in this regard, new studies of the lytic activities of the *Streptomyces albus* G F_1 *endo-N*-acetylmuramidase (Ghuysen and Salton, 1960; Ghuysen, 1960; Salton and Ghuysen, 1960; Dierickx and Ghuysen, 1962) have been undertaken. The results are presented in this paper.

Material and Methods

Analytical Procedures. The methods and the analytical procedures used throughout the present studies have been previously described (Ghuysen *et al.*, 1966; Leyh-Bouille *et al.*, 1966).

Isolation and Purification of the F_1 endo-N-Acetylmuramidase. A crude F_1 enzyme preparation was obtained as described by Petit *et al.* (1966). The method consisted of adsorbing the concentrated *Streptomyces*–enzyme complex on a column of carboxymethylcellulose (CMC) equilibrated with 0.01 M Tris–HCl buffer, pH 8.0. The column was first washed with the same buffer and then the peptidases were eluted with 0.15 or 0.2 M Tris–HCl buffer, pH 8.0. Finally, the F_1 enzyme was eluted employing 0.2 M phosphate buffer, pH 8.0. The crude F_1 preparations thus obtained from several such fractionations were pooled and concentrated by dialysis on solid Carbowax 4000 BDH. A concentrated solution (100 ml) of crude F_1 enzyme, which had been obtained from 115 l. of *Streptomyces* culture filtrate, was dialyzed against a 0.005 M phosphate buffer, pH 8.0, and applied, at 4°, to a 170-ml column of CMC equilibrated with the same buffer. After adsorption, the column was washed with the buffer until the effluent was free of protein. It was then treated with a gradient of increasing phosphate concentration (Figure 1), employing a mixing vessel containing 2000 ml of 0.005 M phosphate buffer, pH 8.0, to which was added a 1 M phosphate buffer, pH 8.0, to maintain a constant volume. The elution of F_1 enzyme began when a 0.2 M concentration was reached in the mixing

* From the Service de Bactériologie, Université de Liège, Liège, Belgium, and Institut Pasteur, Paris, France. *Received June 6, 1966.* This research has been supported in part by a grant (UR-E4-10-2) made by the U. S. Department of Agriculture under Public Law 480.

† Permanent address: Instituto de Biologia Celular, Centro de Investigaciones Biologicas, Madrid, Spain.

‡ Chargé de Recherches au Centre National de la Recherche Scientifique (France). Supported in part by "Les Accords Culturels Franco-Belges." Permanent address: Institut de Biochimie, Faculté des Sciences, Orsay, France.

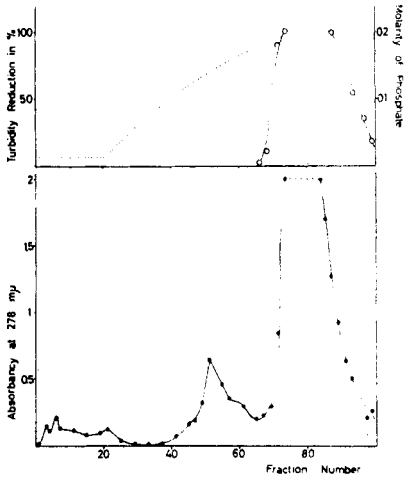

FIGURE 1: Purification of the F_1 *endo-N*-acetylmuramidase on carboxymethylcellulose. A crude F_1 preparation in 0.005 M phosphate buffer, pH 8.0, was applied to a column of CMC (170 ml). The column was first washed with the same buffer, and then a gradient of increasing phosphate concentration was started (tube 20). Aliquots of the fractions (8 ml) were analyzed for absorbancy at 278 mμ and for lytic activity on *B. megaterium* cell walls. Cell walls (500 μg) were incubated 10 min at 37° with 10 μl of each fraction and 10 μl of 0.1 M phosphate buffer, pH 8.0, in a final volume of 500 μl. Turbidity was measured at 550 mμ.

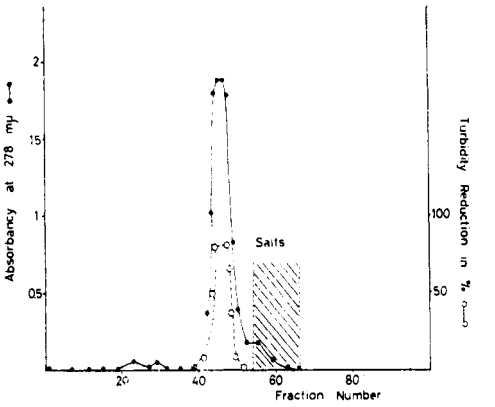

FIGURE 2: Desalting of the F_1 *endo-N*-acetylmuramidase on Sephadex G-50 bead form. The F_1 enzyme already purified by chromatography on CMC as described in Figure 1 was desalted by filtration, in water, on a 600-ml column of Sephadex G-50. Aliquots of the fractions (8 ml) were analyzed as indicated in Figure 1.

vessel (after addition of about 400 ml of 1 M phosphate buffer) and was completed without further increase in the phosphate concentration. The fractions containing the purified F_1 enzyme were pooled and the solution was desalted by filtration on Sephadex G-50 bead form (Figure 2). A column of $V_0 = V_t = 400$ ml was used for desalting 40-ml portions of the enzyme solution. The total yield of pure F_1 *endo-N*-acetylmuramidase was 125 mg, which was dissolved in 130 ml of water. The yield represents about 1.1 mg of enzyme from 1 l. of culture filtrate. The absolute purification of the F_1 enzyme with respect to protein of the starting material cannot be defined. Specific, synthetic or natural, substrates are not available and F_1 is one of about ten different enzymes produced by *S. albus* G which are involved in bacteriolysis.

Results

Physical Properties of the F_1 N-Acetylmuramidase. Purified F_1 enzyme is not salted out from an aqueous solution (1 mg/ml) by solid ammonium sulfate. This property, together with the chromatographic behavior of F_1 enzyme on Sephadex G-50, are indicative of a low molecular weight. Purified F_1 enzyme undergoes denaturation by lyophilization; a loss of 45% of the original activity was observed. Frozen solutions of F_1 enzyme can be stored for months at −20° without damage. F_1 *endo-N*-acetylmuramidase is a basic protein (Figure 3). Three hundred micrograms was submitted to sucrose gradient electrophoresis (Dierickx and Ghuysen, 1962) in 0.1 μ phosphate buffer, pH 6.45, for 67 hr, under 5.8 v/cm. Fractions of 5 ml, each corresponding to 1-cm segment of the column, were collected and the lytic activities upon cell walls of *Micrococcus lysodeikticus*, of *Staphylococcus aureus*, and of *Bacillus megaterium* were measured after adjustment of the pH, respectively, to 3.5, 6, and 8. As shown in Figure 3, the three bacterial cell walls were digested by one enzyme presenting a mobility toward the cathode of 0.46 mm/hr v cm. A mobility of 0.35 mm/hr v cm had been observed with a former F_1 preparation (Dierickx and Ghuysen, 1962). Slight modifications in the apparatus are responsible for this increase of mobility, also observed with egg white lysozyme and methyl orange used as controls.

Bacteriolytic Properties of the F_1 endo-N-Acetylmuramidase. Gram-positive bacteria from the most important genera are sensitive to F_1 enzyme. The optimum pH values for the lytic activities greatly vary according to the bacterial substrates; cell walls of *Sarcina lutea* are most rapidly digested at pH 3.0, those of *B. megaterium* at pH 8.0. Figure 4 illustrates the dependency of the lytic activity of the F_1 enzyme on the pH for several bacterial cell walls. Isolated bacterial cell walls (2000 μg) or 200 μg (dry weight) of living cells were treated with 1–10 μg of F_1 enzyme in a final volume of 500 μl of 0.005–0.02 M buffers of appropriate pH (citrate buffer, pH 3.5–6.5; phosphate buffer, pH 6–8). Turbidity reduction, soluble reducing groups, and soluble acetamido sugars were measured after increasing times of incubation. The lytic properties of the F_1 enzyme, *i.e.*, the optimum pH and the specific activity (expressed in milligrams of substrate digested per hour per milligram of protein and calculated for

TABLE I: Lytic Activity of the *Streptomyces* F_1 *endo-N*-Acetylmuramidase on Gram-Positive Bacteria.[a]

Cell Walls[b]	Optimum pH	Sp Act.[c]	Soluble Disaccharides (mμmoles/mg of cell walls) Appeared at Completion of the Digestion			Content of the Cell Walls in Dibasic Amino Acid (mμmoles/mg)
			Reducing Power[d]	Morgan–Elson Reaction[e]		
				ε 9500	ε 5700	
S. aureus Copenhagen	5.5	100	366	225	373	470 (Lys)
M. roseus R-27 (IP)	5.5	130	300	180	300	300 (Lys)
M. radiodurans	8	100	375	260	430	660 (L-Orn)
B. megaterium KM	7.5–8	220	216	135	215	450 (DAP)[f]
M. lysodeikticus NCTC 2665	3.5	700	135	120	200	500 (Lys)
Propionibacterium technicum NCIB 5965	7.5–8	30	150	150	250	420 (DAP)
Propionibacterium petersonii NCTC 5962	7–8	10	100	100	155	300 (LL-DAP)
Propionibacterium rubrum NCIB 8901	7–8	25	100	100	155	350 (LL-DAP)
*Bifidobacterium bifidum** Reuter 305a (IP)	5.8	80	166	150	250	200 (DAP), 100 (Lys)
Streptococcus pyogenes A	7–8	25	120	140	230	475 (Lys)
*L. monocytogenes** A 106 (IP)	7.5	100	90	40	65	210 (DAP)
*Clostridium histolyticum** 973 (IP)	4.5	25	65	Trace	Trace	600 (DAP)
Clostridium perfringens BP6K	7.5	27	65	40	65	600 (LL-DAP)
C. anaerobium Prevot 3471	4.5–6	70	65	25	40	640 (LL-DAP)
S. lutea R 262 (IP)	3	300	65	Trace	Trace	300 (Lys)
Mycobacterium avium	—	0				

[a] F_1 enzyme completely or partially solubilizes (MTR = maximum turbidity reduction in per cent) the cell walls of the following bacteria: *Lactobacillus acidophilus* (heated cell walls; pH 4.5–6; MTR 100%), *Corynebacterium fermentans* 3211 (IP) (pH 7–8, MTR 75%), *Micrococcus varians* NCTC 7281 (pH 5.5, MTR 30%), *Micrococcus citreus* R 266 (IP) (pH 3, MTR 50%). F_1 enzyme completely clarifies suspensions of the following bacteria cells: *Micrococcus flavus* 53160 (IP) (pH 5.5), *S. albus* 53124 (IP) (pH 6–6.5), *Bacillus subtilis* A 33 (IP) (pH 7.5–8), *Bacillus cereus* A 30 (IP) (pH 7.5–8), *Clostridium tetani* Mikhailov 330 (pH 6–8). [b] IP = Pasteur Institute, Paris. All cell walls prepared by the authors, except these of *L. monocytogenes*, were trypsin treated.*: these cell walls were heated 10 min at 100° in order to prevent autolysis. Cell walls of *P. petersonii* and of *P. rubrum* were a gift from Dr. E. Work, those of *S. pyogenes*, from Drs. S. S. Barkulis and H. Heymann, those of *M. radiodurans*, from Dr. C. Dean, and those of *M. avium*, from Dr. P. Jolles. [c] Expressed as milligrams of cell walls digested per milligram of enzyme per hour. Data calculated on a basis of 50% turbidity reduction. All cell walls listed, except *M. avium*, are entirely solubilized by F_1 enzyme. [d] Determined employing the Park–Johnson (1949) procedure and the data are expressed relative to β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid (1.5 moles of disaccharide is equivalent to 1 mole of *N*-acetylglucosamine). [e] The color development was carried out after 30 min of heating in 1% borate (Ghuysen *et al.*, 1966) and the data are expressed relative to free disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid (ε 9500) or relative to peptide cross-linked disaccharide (ε 5700). [f] DAP, diaminopimelic acid

a 50% turbidity reduction), and the properties of the cell wall digests obtained after complete degradation, are summarized in Table I.

The endo-N-Acetylmuramidase Activity of the F_1 Enzyme. A. DIGESTION OF CELL WALLS OF *S. aureus* COPENHAGEN. The kinetics of the digestion carried out with F_1 enzyme in 0.02 M citrate buffer, pH 5.5 (ratio enzyme:substrate = 3:2000), are identical with these previously observed employing the *Streptomyces* 32 (Ghuysen and Strominger, 1963a) or the *Chalaropsis* B (Tipper *et al.*, 1964) *endo-N*-acetylmuramidases (Figure 5). The cell wall solubilization is the result of the hydrolysis of a single type of glycosidic bond; it requires the splitting of all sensitive linkages and eventually produces 550 mμmoles of reducing groups per milligram of cell walls (relative to *N*-acetylglucos-

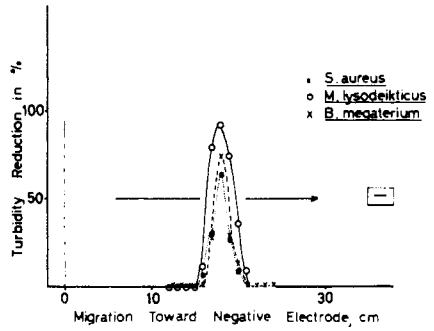

FIGURE 3: Sucrose gradient electrophoresis of purified F_1 *endo-N*-acetylmuramidase. After electrophoresis (for conditions, see text), fractions of 5 ml, representing 1 cm of the column, were collected. To assay F_1 *endo-N*-acetylmuramidase, 100 μl of each fraction was added to 500 μg of cell walls in 500 μl of 0.01 M citrate buffer, pH 3.5 (for *M. lysodeikticus*), or 0.01 M phosphate buffer, pH 6.0 (for *S. aureus*), or 0.02 M phosphate buffer, pH 8.0 (for *B. megaterium*). Turbidity was measured after 30 min (*M. lysodeikticus*), or 60 min (*S. aureus* and *B. megaterium*), at 37°.

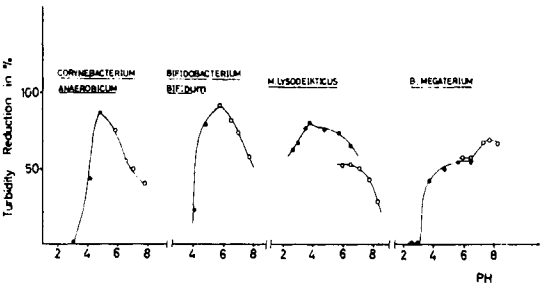

FIGURE 4: Dependency of the optimum pH for the lytic activity of F_1 *endo-N*-acetylmuramidase on the nature of the bacterial substrate. Cell walls (500 μg) were incubated with 2 μg of purified F_1 *endo-N*-acetylmuramidase in a final volume of 500 μl of 0.01 M citrate (black points) or phosphate (open points) buffers. For specific activity of the F_1 enzyme, see Table I.

amine). As observed earlier with the two *endo-N*-acetylmuramidases mentioned above, F_1 enzyme cleaves all the glycosidic linkages of *N,O*-diacetylmuramic acid as well as those of *N*-acetylmuramic acid. After filtration of the products of lysis on Sephadex G-50, a high molecular weight fraction was obtained which consisted of a mixture of soluble petidoglycan and soluble peptidoglycan–teichoic acid complexes (Ghuysen and Strominger, 1963a; Ghuysen *et al.*, 1965a). This fraction was treated with the *Streptomyces* *N*-acetylmuramyl-L-alanine amidase and the new digest was filtered on CMC H^+ form. The peptide components were retained on the resin and the material containing the reducing groups came through in the water eluate. Paper chromatography of this latter fraction yielded the two disaccharides β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid and β-1,4-*N*-acetylglucosaminyl-*N,O*-diacetylmuramic acid, which were characterized as previously described (Ghuysen and Strominger, 1963b; Tipper *et al.*, 1965).

B. DIGESTION OF CELL WALLS OF *M. roseus* R-27. Kinetics of the digestion of cell walls of *M. roseus* with F_1 enzyme in 0.02 M citrate buffer, pH 5.5 (ratio enzyme:substrate = 2:1000), have the same characteristics as these observed with cell walls of *S. aureus* (Figure 5), except that 450 mμmoles of reducing groups relative to *N*-acetylglucosamine is eventually liberated and that 75 mμmoles of terminal amino groups of alanine, native in the intact cell walls (instead of the glycine in *S. aureus*), appear in the digestion products of each milligram of *M. roseus* cell walls. Also as is observed with cell walls of *S. aureus*, the reducing groups appeared in *M. roseus* cell wall digests behave as free disaccharides only after further treatment with *N*-acetylmuramyl-L-alanine amidase; 50 mg of cell walls of *M. roseus* was digested for 15 hr at 37° with 100 μg of F_1 enzyme in 12.5 ml of 0.02 M citrate buffer, pH 5.5. The digest was then treated with the amidase, which induced an increase of 280 mμmoles of terminal amino groups of L-alanine. As shown in Figure 6, this increase of free terminal amino alanine is accompanied by an increase in the color developed in the Morgan–Elson reaction (after 30 min of heating in borate), without, however, modification of the reducing power of the digest. The new digest was filtered in water on a column of Sephadex G-25 bead form (2 × 41 cm). All the reducing groups and some of the peptide compounds were recovered in a low molecular weight fraction. The reducing material was purified first by filtration in water through a column of Amberlite CG 50 (H^+ form) (1.5 × 40 cm) and next by adsorption on a column of Ecteola-cellulose (OH^- form) (2 × 40 cm). Elution from Ecteola was carried out with 0.3 M LiCl and the purified reducing material was finally desalted on a column of Sephadex G-25 bead form. It was identified as disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid.

C. DIGESTION OF CELL WALLS OF *M. lysodeikticus* NCTC 2665. It has been shown that treatment of cell walls of *M. lysodeikticus* with lysozyme (ratio enzyme:substrate = 1:100) induces a progressive liberation of reducing groups which eventually are equivalent to 570 mμmoles/mg of *N*-acetylglucosamine (Leyh-Bouille *et al.*, 1966). The solubilization of the cell walls, however, is already complete when 110 mμmoles only of reducing groups has been produced. Kinetics of the digestion of the same cell walls with F_1 enzyme in 0.01 M citrate buffer, pH 3.5 (ratio enzyme:substrate = 1:2000) (Figure 5), show that the rate of liberation of the reducing groups is biphasic. Indeed, a 40% turbidity reduction of the cell wall suspension is paralleled by the appearance of about 180 mμmoles/mg of reducing groups, Then, as the clarification proceeds to completion, the number of free reducing

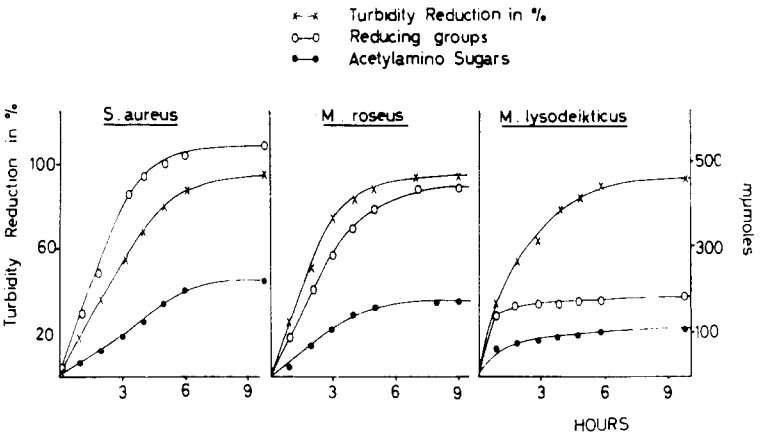

FIGURE 5: Hydrolysis of cell walls of *S. aureus*, *M. roseus*, *and M. lysodeikticus* by the F_1 *endo-N*-acetylmuramidase. Aliquots of 2000 μg of cell walls were incubated with 1 μg (*M. lysodeikticus*), 3 μg (*S. aureus*), and 4 μg (*M. roseus*) of F_1 *endo-N*-acetylmuramidase in 500 μl of 0.02 M citrate buffer, pH 5.5 (*S. aureus* and *M. roseus*), or 0.01 M citrate buffer, pH 3.5 (*M. lysodeikticus*). After incubation, each mixture was centrifuged and the supernatant was analyzed for soluble reducing groups and acetamido sugars. Data are expressed as millimicromoles of *N*-acetylglucosamine (reducing groups) and as millimicromoles of disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid (Morgan–Elson reaction, employing a molar extinction coefficient of 9500 after 30 min of heating in borate), solubilized per milligram of cell walls.

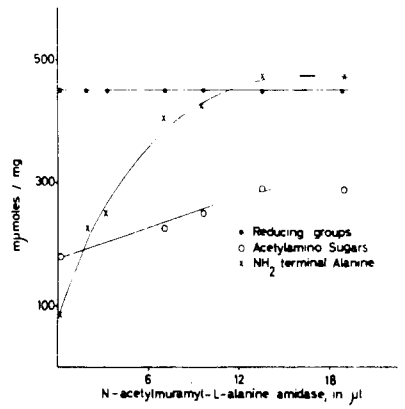

FIGURE 6: Influence of the peptide substitution of the disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid on the color development in the Morgan–Elson reaction. Aliquots (25 μl) of F_1 digested cell walls of *M. roseus* R-27 (100 μg in 0.02 M citrate buffer, pH 5.5) were added with a *Streptomyces* *N*-acetylmuramyl-L-alanine amidase (2–24 μl) and incubated at 37° for 2 hr in a final volume of 40 μl. Data for reducing groups and acetylamino sugar are expressed as indicated in Figure 5. Data for terminal amino group alanine are also expressed as millimicromoles per milligram of cell walls.

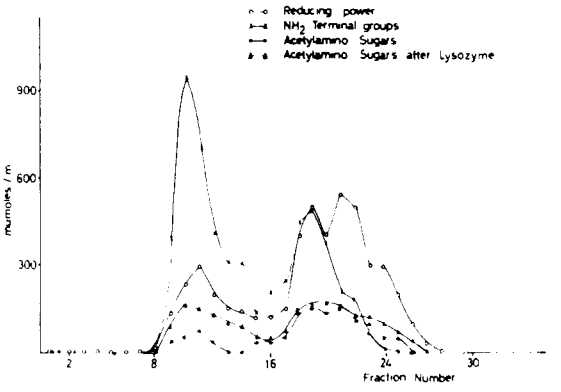

FIGURE 7: Filtration of Sephadex G-50 medium of the F_1 *endo-N*-acetylmuramidase digest of *M. lysodeikticus* cell walls. Reducing groups (standard *N*-acetylglucosamine), acetylamino sugar before and after treatment with lysozyme (standard, disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, Morgan–Elson procedure, 30 min of heating, ϵ 9500), and terminal amino groups (standard, alanine) are expressed as millimicromoles per milliliter of each fraction. Aliquots of 100 μl of each fraction (5 ml) were added with 25 μl of 0.1 M phosphate buffer, pH 6.5, and were incubated for 18 hr, at 37°, with 20 μg of lysozyme.

groups very slowly increases. Cell walls (100 mg) were digested with 100 μg of F_1 enzyme for 20 hr at 37°, in 0.01 M citrate buffer, pH 3.5. The digest contained 200 mμmoles/mg of reducing groups. It was filtered in water on a column of Sephadex G-50 medium (2 × 40 cm) and yielded a high molecular weight fraction (elution volume, 40–90 ml) and a low molecular weight fraction (elution volume, 90–140 ml) (Figure 7). This latter fraction contained all the acet-

amido sugars responsible for the positive Morgan–Elson reaction given by the cell wall digest, 70% of the reducing groups present in the digest, 65% of the total hexosamines, 32% of the terminal amino groups, and no glucose. An aliquot of each of the tubes was treated with lysozyme. An increase of the sensitivity to the Morgan–Elson reaction was observed mainly with the high molecular weight fraction. The low molecular weight fraction was passed over a column of CMC (H^+ form) (4 × 23 cm) in water; 55% of the reducing groups and of the total hexosamines present in the original cell wall digest came through in the water eluate. Filtration of this reducing material on Sephadex G-25 bead form (1.5 × 95 cm), in water, gave rise to several fractions: fraction 1 (elution volume 95–115 ml) was disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid; fraction 2 was rich in tetrasaccharide *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramyl-(β?)-1,4-*N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid (Leyh-Bouille *et al.*, 1966) but still contained a small amount of oligosaccharide–peptide compounds. This fraction 2 was submitted to paper electrophoresis in 1 N acetic acid solution (Whatman 3MM paper, 40 × 40 cm), for 3 hr, under 20 v/cm. The neutral tetrasaccharide was extracted from the paper with water and submitted to a last filtration on a Sephadex G-25 bead form column. The free oligosaccharides present in the F_1 digest of the cell walls were estimated by submitting that fraction of the digest which had not been retained on the CMC H^+ column to a direct paper chromatography in 1-butanol–acetic acid–water (3:1:1). Free disaccharide (R_F 0.39, 90 mμmoles/mg of cell walls) and free tetrasaccharide (R_F 0.25, 55 mμmoles/mg of cell walls) were detected on marker strips and eluted with water. The quantitative estimates were made after HCl hydrolysis of the eluates. Free disaccharide and free tetrasaccharide, respectively, represent 20 and 25% of the total hexosamine content of the cell walls.

D. CHARACTERIZATION OF THE FREE OLIGOSACCHARIDES LIBERATED FROM BACTERIAL CELL WALLS BY F_1 *endo-N*-ACETYLMURAMIDASE (for techniques, see Leyh-Bouille *et al.*, 1966). Disaccharides from *S. aureus* and from *M. lysodeikticus* and tetrasaccharide from *M. lysodeikticus* were characterized by paper cochromatography with the corresponding saccharides previously isolated. HCl hydrolysis yielded glucosamine and muramic acid in equimolar amounts and no amino acid. $NaBH_4$ destroyed 50% of the total hexosamines in disaccharides and 25% in tetrasaccharide without modification of the glucosamine content; all muramic acid in disaccharides and 50% of it in the tetrasaccharide disappeared. *exo*-β-*N*-Acetylglucosaminidase liberated one free *N*-acetylglucosamine from two residues of total hexosamines in disaccharides and from four residues of total hexosamines in tetrasaccharide. Disaccharide from *M. roseus* was similarly characterized. Moreover, 1 mole of this reduced disaccharide consumed, within 5–10 min, 1 mole of periodate with production of 0.97 mole of formaldehyde and, slowly, a second mole of periodate with parallel destruction of glucosamine.

E. ACTION OF F_1 *endo-N*-ACETYLMURAMIDASE ON *M. lysodeikticus* TETRASACCHARIDE AND ON *M. roseus* POLYSACCHARIDE. F_1 *endo-N*-acetylmuramidase has very little activity on the peptide-free tetrasaccharide from *M. lysodeikticus* and on the polysaccharide moiety from *M. roseus* peptidoglycan (Petit *et al.*, 1966). However, using a ratio enzyme:substrate of 1:10 (w/w), about 20% of glycosidic linkages was hydrolyzed within 20 hr. Hydrolysis of the tetrasaccharide only occurred at acidic pH values. Optimum pH is about 3.5 (0.01 M citrate buffer). No hydrolysis was observed at pH 5 or higher, as was previously observed (Dierickx and Ghuysen, 1962).

Discussion

F_1 enzyme acts as a β-1,4-*endo-N*-acetylmuramidase on cell walls of *M. lysodeikticus*, as had been observed with former enzyme preparations (Salton and Ghuysen, 1960), and on cell walls of *S. aureus* and *M. roseus*. This mechanism of action designates F_1 enzyme as an egg white lysozymelike enzyme. However, F_1 enzyme has a more powerful lytic action than egg white lysozyme. Employing cell walls of *M. lysodeikticus* as substrates, F_1 enzyme and lysozyme have specific activities expressed in milligrams of cell walls digested per milligram of protein, per hour, equal to 700 and 250, respectively, both working in optimal conditions. F_1 enzyme lyses bacteria of the most important gram-positive genera or digest the corresponding cell walls. In contrast to this, many bacteria or isolated bacterial cell walls from *Streptococcus* sp, *Staphylococcus* sp, *Clostridium* sp, *Corynebacterium* sp, *Bifidobacterium* sp, *Listeria* sp, and *Propionibacterium* sp are resistant to lysozyme unless special treatments are applied. Like lysozyme and other *endo-N*-acetylmuramidases, however, F_1 enzyme does not act on *Mycobacterium* sp or on gram-negative bacteria. The high lipoprotein content of the cell walls probably prevents the enzyme from reaching the sensitive linkages in the peptidoglycan.

F_1 enzyme is free of peptidases. The terminal amino groups present in the F_1 digests are native to the cell walls. These groups are present in the same amounts in similar digests obtained by means of other *endo-N*-acetylmuramidases (lysozyme for cell walls of *M. lysodeikticus; Streptomyces* 32 enzyme or *Chalaropsis* B enzyme for cell walls of *S. aureus*). Terminal amino groups can also be determined by direct labeling of the cell walls with fluorodinitrobenzene. Using this technique, it has been shown that the cell walls contain these amino groups found in the F_1 digests.

For each milligram of cell walls of *M. roseus* digested with F_1 enzyme, 450 mμmoles of reducing groups, relative to *N*-acetylglucosamine, is produced. These groups have been identified as belonging to β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid units. Since the color yield in the reducing power determination of 1 mole of this disaccharide is equivalent to 1.5 moles of *N*-acetylhexosamine, the polysaccharide moiety of the peptidoglycan must have been split by

F_1 enzyme into 300 mμmoles of disaccharide units. The same conclusion is reached when the disaccharides in the F_1 digest are estimated by the Morgan–Elson reaction (after 30 min of heating in borate) employing a molar extinction coefficient of 9500, provided that the disaccharide units have been freed with the help of the *N*-acetylmuramyl-L-alanine amidase. The cross-linking of the disaccharide units through complex peptides prevents a full development of the color in the Morgan–Elson reaction (Figure 6) and in order to estimate the disaccharide units in a F_1 digest of the cell walls which has not been treated with *N*-acetylmuramyl-L-alanine amidase, a molar extinction coefficient of 5700 must be applied, as can be calculated from Figure 6. It should be noted (E. Muñoz, unpublished observations) that the disaccharide–tetrapeptide monomer (Petit *et al.*, 1966) has in the Morgan–Elson reaction, when the reaction is carried out under identical conditions, a molar extinction coefficient of about 8000, *i.e.*, lower than that of free disaccharide (9500; Ghuysen *et al.*, 1966) but higher than that of cross-linked disaccharide–peptide. It has been demonstrated that the peptide portion of 1 mg of *M. roseus* cell walls contains 300 mμmoles of peptide subunits cross-linked by $(L\text{-Ala})_3$-L-Thr bridges (Petit *et al.*, 1966). This number of peptide subunits is equal to the number of disaccharide units appearing as a consequence of the F_1 enzyme treatment. It can therefore be concluded that each disaccharide unit of the polysaccharide chains is substituted by peptide subunit, the latter being cross-linked through additional amino acids.

A similar structure has been proposed for the cell wall peptidoglycan of *S. aureus* (Ghuysen *et al.*, 1965b; Petit *et al.*, 1966). Cell walls (1 mg) contain 470 mμmoles of peptide subunits bridged by pentaglycines. F_1 enzyme once again cleaves virtually all of the glycosidic linkages of *N*-acetylmuramic acid, yielding disaccharide units (60% of them are, in this case, *O*-acetylated). The disaccharides were liberated through the action of the *N*-acetylmuramyl-L-alanine amidase and were actually isolated and characterized. The properties of the F_1 digest of the cell walls are compatible with these results. The reducing power is equivalent to 550 mμmoles/mg of cell walls of *N*-acetylglucosamine or to 366 mμmoles of disaccharide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, and the color development in the Morgan–Elson reaction corresponds to 373 mμmoles of this disaccharide on the basis of ϵ 5700. The ratio disaccharide:peptide subunit is here 0.78:1 but it must be noted that there are about 50 mμmoles of N-terminal alanine in the peptidoglycan portion of 1 mg of *S. aureus* cell walls (Ghuysen *et al.*, 1965b) and that therefore some of the peptide subunits seem not to be linked to the polysaccharide chains.

Contrary to what is observed with cell walls of *S. aureus* and *M. roseus*, the hydrolysis of only part of the *N*-acetylmuramic linkages is sufficient to induce the complete solubilization of the cell walls of *M. lysodeikticus*, irrespective of the *endo*-*N*-acetylmuramidase, F_1 enzyme, or lysozyme, which is used. It has been demonstrated (Leyh-Bouille *et al.*, 1966) that the polysaccharide chains of *M. lysodeikticus* cell walls closely resemble those of *S. aureus* and *M. roseus*, except, however, in one aspect: in *M. lysodeikticus*, about 50% of the *N*-acetylmuramic acid residues are not substituted by peptides. This finding has been confirmed in the course of the present studies. Free disaccharides and free tetrasaccharides, which altogether represent about 45% of the total hexosamine content of the cell walls, have been isolated from F_1 digests without the intervening action of any *N*-acetylmuramyl-L-alanine amidase. From the foregoing, it can be concluded that the fragility of the *M. lysodeikticus* cell walls toward the action of glycosidases is related to their low degree of peptide substitution. It is indeed conceivable that if only a few *N*-acetylmuramic acid residues serve as branching points for cross-linkings, the whole peptidoglycan network can be broken into pieces through the hydrolysis of a small number of glycosidic bonds. Also to all appearances, this low degree of peptide substitution in the polysaccharide moiety of *M. lysodeikticus* cell walls is responsible for its incomplete splitting through the action of the F_1 *endo*-*N*-acetylmuramidase. Conversely, the high affinity of the F_1 enzyme for the glycosidic linkages of *N*-acetylmuramic acid in *M. roseus* and *S. aureus* cell walls must depend on the substitution of these residues by peptide subunits. In accord with this hypothesis, both the peptide-free tetrasaccharide isolated from *M. lysodeikticus* cell walls or the carbohydrate fraction isolated from *M. roseus* cell walls are very incompletely hydrolyzed by very large amounts of F_1 enzyme and after prolonged incubations.

Several groups of bacterial cell walls emerge from the data of Table I. Cell walls of *Propionibacterium* sp, *Bifidobacterium bifidum*, and *Streptococcus pyogenes* behave, with respect to the sensitivity to F_1 enzyme, as cell walls of *M. lysodeikticus*. Cell walls of *Clostridium* sp, *Corynebacterium anaerobium*, or *Listeria monocytogenes*, and *S. lutea* give rise, under the action of F_1 enzyme, to a still smaller number of reducing groups. The polysaccharide moieties of some cell wall peptidoglycans may have unusual features; the presence of *O*-acetyl groups has been demonstrated in cell walls of *S. aureus* (Ghuysen and Strominger, 1963a) and of certain strains of *M. lysodeikticus* (Brumfitt, 1959); 1,3 glycosidic linkages might occur in *Streptococcus* cell walls (Barkulis *et al.*, 1964). It cannot be precluded that, in some cases, peculiarities in the polysaccharide chains would be responsible for a limited hydrolytic action of the F_1 enzyme. However, even if this be the case, the hydrolysis of a small number of glycosidic links in all of the bacterial cell walls just mentioned induces the solubilization of the peptidoglycan, *a property typical of M. lysodeikticus cell walls*. Therefore, it seems plausible that, with a few possible exceptions, the polysaccharide chains in all cell wall peptidoglycans have the same basic structural properties but that the degree of the peptide cross-linkings between the polysaccharide chains varies extensively from one type of bacterial cell wall to another. Therefore, an unexpected result of the present study is the

observation that tight networks, typified by *S. aureus* and *M. roseus* cell wall peptidoglycans and in which all of the *N*-acetylmuramic acid residues have cross-linked peptide substituents, are not commonly encountered among gram-positive bacteria; cell walls of *B. megaterium* and of *Micrococcus radiodurans* are the only ones of which at least part of the peptidoglycan may have similar types of structure (Table I).

When bacterial cell walls contain equivalent amounts of muramic acid, glutamic acid, and dibasic amino acid, as frequently occurs, a low order of peptide branching on the *N*-acetylmuramic acid residues must be paralleled, as previously pointed out (Salton, 1961; Leyh-Bouille *et al.*, 1966), by peptide moieties having structures much more complicated than these of *S. aureus* or *M. roseus* cell walls. There is here an area of research which is almost unexplored. This study of the numerous variations which must occur in the peptide portions of the bacterial cell walls will probably provide an explanation for the dependence of the optimum pH of the F_1 enzyme activity on the nature of the bacteria or of the bacterial cell walls which are used as substrates.

References

Barkulis, S. S., Smith, C., Boltralik, J. J., and Heymann, H. (1964), *J. Biol. Chem. 239*, 4027.

Brumfitt, W. (1959), *Brit. J. Exptl. Pathol. 40*, 441.

Dierickx, L., and Ghuysen, J. M. (1962), *Biochim. Biophys. Acta 58*, 7.

Ghuysen, J. M. (1960), *Biochim. Biophys. Acta 40*, 473.

Ghuysen, J. M., and Salton, M. R. J. (1960), *Biochim. Biophys. Acta 40*, 462.

Ghuysen, J. M., and Strominger, J. L. (1963a), *Biochemistry 2*, 1110.

Ghuysen, J. M., and Strominger, J. L. (1963b), *Biochemistry 2*, 1119.

Ghuysen, J. M., Tipper, D. J., Birge, C. H., and Strominger, J. L. (1965b), *Biochemistry 4*, 2245.

Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1965a), *Biochemistry 4*, 474.

Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1966), *Methods Enzymol. 8* (in press).

Leyh-Bouille, M., Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1966), *Biochemistry 5*, 3079 (this issue; preceding paper).

Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.

Petit, J. F., Muñoz, E., and Ghuysen, J. M. (1966), *Biochemistry 5*, 2764.

Salton, M. R. J. (1961), Proceeding of the 2nd Symposium on Fleming's Lysozyme, Milan, Vol. II, p I/Xii.

Salton, M. R. J., and Ghuysen, J. M. (1960), *Biochim. Biophys. Acta 45*, 355.

Tipper, D. J., Ghuysen, J. M., and Strominger, J. L. (1965), *Biochemistry 4*, 468.

Tipper, D. J., Strominger, J. L., and Ghuysen, J. M. (1964), *Science 146*, 781.