Structural Relationship of the Teichuronic Acid and Peptidoglycan of *Bacillus megaterium*†

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ABSTRACT: The structural relationship between the peptidoglycan and the acidic polysaccharide of the cell envelope of *Bacillus megaterium* M46 has been studied. These studies demonstrate that the polysaccharide, which contains glucuronic acid, glucose, and rhamnose (White, P. J., and Gilvarg, C. (1977), *Biochemistry 16* (preceding paper in this issue)), fulfills both the compositional and structural criteria established for teichuronic acids. There is a coordinate release of polysaccharide and peptidoglycan during lysozyme digestion of the cell envelope, as judged by millipore filtration, suggesting a uniform distribution of the teichuronate over the peptidoglycan. Analysis of the digestion products, by chromatography on Sepharose 2B, reveals that during this coordinate release of the two components there is a rapid separation of the bulk (80%) of the peptidoglycan from the polysaccharide. The polysaccharide is released as soluble polysaccharide–peptidoglycan complexes and this solubilization is followed by a slower displacement of peptidoglycan from these complexes. There is a clear difference in the digestibility of the peptidoglycan closely associated with the polysaccharide (20% of total) and the peptidoglycan connecting these polysaccharide–peptidoglycan complexes (remaining 80%). After exhaustive digestion of these complexes with lysozyme, a small amount of peptidoglycan (0.4% of total) remains associated with the polysaccharide. This association is not disrupted by a variety of common denaturants, or by mild acid and mild alkaline hydrolysis. The linkage between the peptidoglycan and polysaccharide is not a phosphodiester bond. β-Elimination studies reveal the lysozyme-resistant peptidoglycan associated with the teichuronic acid to contain a tetrasaccharide. This tetrasaccharide has uncross-linked peptide chains and thus represents a unique portion of the wall.

The cell walls of gram-positive bacteria are made up of peptidoglycan and acidic polysaccharides (teichoic acids and teichuronic acids). An understanding of the role of these polysaccharides and of the assembly of the bacterial cell wall must begin with a knowledge of the structural relationship between these polysaccharides and the peptidoglycan. A continuously covalent cell envelope, sacculus, can be prepared from *Bacillus megaterium* M46 by detergent extraction combined with nucleolytic and proteinolytic digestion. These sacculi retain the original shape of the bacillus and in this organism are constructed of only two polymers: peptidoglycan and a polysaccharide composed of rhamnose, glucose, and glucuronic acid (White and Gilvarg, 1977). Their retention of the shape of the bacillus, combined with their simple composition make the sacculi of *Bacillus megaterium* M46 a useful model for studying the architecture of the bacterial cell wall.

Identification of a polysaccharide as a teichuronic acid must fulfill two criteria: one chemical and the other structural. Firstly, the polymer must contain a uronic acid. This compositional criterion has been met by polymers isolated from several species of bacteria: *Bacillus licheniformis* (Janczera, et al., 1961), *Micrococcus lysodeikticus* (Perkins, 1963), *Bacillus subtilis* (Ellwood and Tempest, 1969), and *Corynebacterium* (Diaz-Maurino and Perkins, 1974). However, the second criterion is for a covalent linkage between the peptidoglycan and the polysaccharide, since otherwise the polymer might simply be regarded as capsular material. The previously reported uronic acid containing polysaccharides were extracted by mild acid hydrolysis and have been reported to be free of other wall polymers. However, linkage to the peptidoglycan has been demonstrated for the uronic acid containing polysaccharide from *Bacillus licheniformis* (Hughes, 1970) when lysozyme was used to release the material from the wall.

The structural relationship between the uronic acid containing polysaccharide and the peptidoglycan of the cell envelope of *Bacillis megaterium* M46 has been investigated by studying the kinetics and reaction products of the lysozyme digestion process.

Materials and Methods

Bacterial Strain and Growth Conditions. All experiments were carried out with *B. megaterium* M46. Conditions for growth and properties of the mutant have been described previously (Fukuda and Gilvarg, 1968; Pitel and Gilvarg, 1970).

Isolation of the Polysaccharide. The polysaccharide was released from the sacculi by lysozyme digestion and isolated as described by White and Gilvarg (1977), with the modifications that Sepharose 6B was chosen as the chromatography matrix instead of Sephadex G200, and the upper limit of the salt gradient for elution from DEAE[1]-cellulose was 1 M ammonium acetate instead of 3 M ammonium acetate.

Limit Lysozyme Digestion of the Polysaccharide. The material isolated as described in the preceding section was redigested with 0.1% (w/v) lysozyme and rechromatographed on Sepharose 6B and DEAE-cellulose. This lysozyme treatment–chromatography cycle was repeated three more times. The peptidoglycan content of this material was substantially reduced by this procedure, as was the reducing sugar content and the anthrone-positive material. There was no detectable

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[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DAP, diaminopimelic acid.

difference between the material prepared by the last two cycles of lysozyme digestion–chromatography.

Analytical Procedures. The amount of free reducing sugar was estimated by the method of Park and Johnson (1949). Glucuronic acid was estimated by the modified carbazole reaction (Bitter and Muir, 1962), glucose was estimated by the procedure described by Spiro (1966), and rhamnose was determined by the methylpentose assay of Dische and Shettles (1948). Standards were assayed to determine the contribution of each sugar in each of the assays, and suitable corrections were applied. Total phosphate was determined by the method of Ames (1966). All samples for radioactivity determination were dissolved in 10 volumes of Bray's scintillation fluid (Bray, 1960) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Electrophoresis was performed in 0.01 M ammonium acetate, pH 6.5, on Baker-flex cellulose at 10 V/cm on a Savant flat-bed electrophoresis apparatus.

Reduction of Reducing Terminal. The reducing terminal of the polysaccharide was identified by reduction with sodium [^{3}H]borohydride and characterized by electrophoresis. Samples (1.0 mL) of the polysaccharide were reacted overnight at 15 °C with 1% (w/v) sodium [^{3}H]borohydride (0.2 mL, 2 Ci/mol) in 0.1 M Tris-HCl buffer, pH 8.4 (Hughes and Thurman, 1970). The polysaccharide was then extensively dialyzed against distilled water. A sample was then hydrolyzed, for 8 h in 4 M HCl at 100 °C, and dried. The hydrolysate was examined by electrophoresis.

β Elimination. The polysaccharide was treated with 4 M NH_3 and kept in a stoppered vessel at 37 °C overnight. Half of the neutralized material was then applied to a Sepharose 2B column (2 × 15 cm) and eluted in ammonium acetate, pH 6.8, I = 0.1. The other half was applied to a Sephadex G-50 column (3 × 40 cm) and eluted in 0.1 M ammonium acetate. A sample of disaccharide–pentapeptide was similarly treated with 4 M NH_3 and chromatographed on Sephadex G-50.

HF Treatment. The polysaccharide was dried, cooled to 0 °C on melting ice, and suspended in 0.2 mL of 50% hydrofluoric acid. The reaction was allowed to proceed for 1 or 36 h and the solution was neutralized with sodium hydroxide. The total reaction mixture was applied directly to a Sepharose 2B column (2 × 15 cm) and eluted in ammonium acetate buffer, pH 6.8, I = 0.1.

Materials. [^{3}H]Diaminopimelic acid was obtained from Amersham/Searle Corp. (280 mCi/mmol). Ribonuclease, deoxyribonuclease, lysozyme, and trypsin were from Worthington Biochemical Corp. α-Amylase (bacterial type IIA) was from Sigma Chemical Co. The disaccharide–pentapeptide was obtained from lysozyme digests of the peptidoglycan released by *Bacillus megaterium* in the presence of penicillin (Mirelman et al., 1974).

Results

Release of Polysaccharide from the Cell Envelope. Continuously covalent cell envelopes, sacculi, were prepared from *Bacillus megaterium* M46 by detergent extraction combined with enzymatic digestion. These sacculi are composed of only two polymers: peptidoglycan and an acidic polysaccharide (White and Gilvarg, 1977). The kinetics of the lysozyme digestion of these sacculi and the nature of the reaction products have been studied. The time courses of the solubilization of peptidoglycan and polysaccharide, as judged by millipore filtration, are shown in Figure 1. In both instances, after a barely perceptible lag, there is a rapid conversion of the bulk of the sacculus to nonfilterable material, followed by a slower phase in which the process is brought to near completion. The solubilization of the teichuronate and the peptidoglycan proceeds coordinately, as can be seen by the unchanging ratio of these components, in the filtrate, throughout the process. A likely explanation for the biphasic nature of the digestion would be differential sensitivity of longitudinal and septal wall to lysozyme digestion.

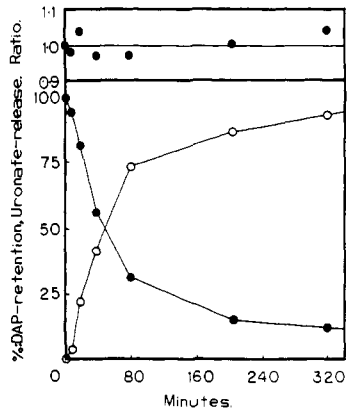

FIGURE 1: The coordinate release of teichuronate and peptidoglycan during lysozyme digestion of the sacculi. Sacculi were prepared as described by White and Gilvarg (1977) from cells grown in the presence of [^{3}H]DAP. The sacculi at a concentration of 2 × 10^7 cells (15 × 10^3 cpm)/mL were digested with lysozyme at a concentration of 10 μg of enzyme/mL, in 0.1 M ammonium acetate at 37 °C. Samples (0.2 mL) were removed at the appropriate time intervals and diluted with 20 volumes of ice-cold water. The sample was then passed immediately over 0.45-μm millipore filters. The time course followed the release of glucuronate into the filtrate (○) and the retention of [^{3}H]DAP by the filter (●). The proportion of the teichuronate to peptidoglycan released into the filtrate is shown at the top of the figure.

In order to gain some insight into the size and homogeneity of the fragments released from the sacculi, the experiment was repeated using, in three separate instances, 0.45, 0.22, and 0.11 μm millipore filters. A fivefold higher concentration of lysozyme was also used. At the higher lysozyme concentrations, the lag was not evident, although the digestion of the sacculi was still pronouncedly biphasic. The fast process had a half-life of 10 min and the slow process had a half-life of approximately 500 min. The profiles for the release of material from the sacculi were identical with the three different sizes of filter, Figure 2. It is apparent from these profiles that 85–90% of the cell wall was rapidly cleaved into fragments smaller than 500 Å in radius. The coordinate release of peptidoglycan and polysaccharide suggests that the polysaccharide is uniformly distributed over the cell wall. The lysozyme digestion process was further analyzed by chromatography on Sepharose 2B. Samples were removed from the digest at various time intervals and precipitated in 75% (v/v) ice-cold ethanol, 0.1% barium acetate. This procedure yields all of the teichuronate and 90% of the [^{3}H]diaminopimelic acid (peptidoglycan). The precipitated material was resuspended in ammonium acetate solution and clarified immediately prior to chromatography. The elution profiles of uronic acid containing material and peptidoglycan are shown in Figure 3. The solubilized uronic acid containing material was eluted at the exclusion limit of Sepharose 2B, and the peptidoglycan was distributed across the elution profile. In early samples, the bulk of the peptidoglycan fragments was distributed in two peaks, one at the exclusion limit and the other in the low-molecular-weight region. As the digestion progressed, there was a displacement of the peptidoglycan from the peak at the exclusion limit to a lower molecular weight region. The shape of the teichuronic acid peak was not altered during lysozyme digestion. During the

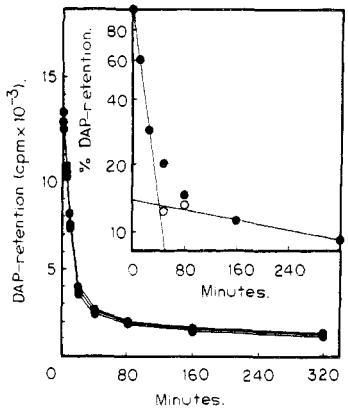

FIGURE 2: The absence of intermediately-sized wall fragments during lysozyme digestion of the sacculi. The digestion was carried out at 37 °C with a sacculi concentration of 2×10^7 cells/mL, lysozyme concentration 50 μg/mL in 0.1 M ammonium acetate. Samples, 0.1 mL, were taken, and the time course followed the retention of [^{3}H]DAP on 0.45, 0.22, and 0.11 μm millipore filters. The semilog plot of the time-course data reveals two first-order processes. The first has a half-life of 10 min and accounts for 85–90% of the [^{3}H]DAP and the second has a half-life of approximately 500 min. The open circles represent the amount of [^{3}H]DAP not accounted for by the faster processes.

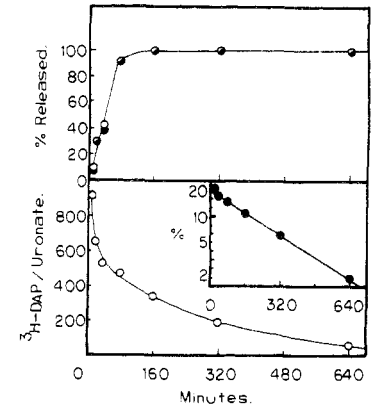

FIGURE 4: The release of a defined polysaccharide-peptidoglycan complex from the sacculi and the subsequent displacement of peptidoglycan from this solubilized complex during lysozyme digestion. The upper time course shows the polysaccharide (●) and peptidoglycan (○) released during lysozyme digestion, obtained as the total uronate and [^{3}H]DAP, respectively, over the elution profiles in Figure 3. The lower time course shows the ratio of [^{3}H]DAP to uronate (cpm/μmol) in the peak fractions of the polysaccharide in the elution profiles in Figure 3. The inset shows a semilog plot of this [^{3}H]DAP to uronate ratio, expressed as a percentage relative to the value of the ratio in the intact wall, as a function of time.

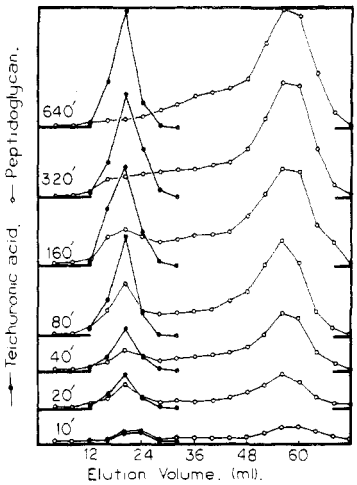

FIGURE 3: The size distribution of the peptidoglycan released from the sacculi during lysozyme digestion. Sacculi (2×10^7 cells/mL) containing [^{3}H]DAP were digested with 50 μg/mL lysozyme at 37 °C. A series of samples (2–5 mL) was removed from the digest at various time intervals and made 75% (v/v) ethanol, 0.1% (w/v) barium acetate, and cooled to 0 °C. The precipitated material was resuspended in 4 mL of 0.1 M ammonium acetate and centrifuged to remove insoluble material, and the supernatant was chromatographed on Sepharose 2B. Elution was performed in 0.1 M ammonium acetate. The distribution of the polysaccharide (●) and peptidoglycan (○) was monitored.

initial phase of this digestion, there is a coordinate release of peptidoglycan and uronic acid containing polysaccharide from the sacculus, Figure 4 (upper profile). This release is followed by a secondary displacement of the peptidoglycan from association with the solubilized polysaccharide, Figure 4 (lower profile). Analysis of the time course of this displacement suggests that the polysaccharide is freed from the cell wall as a complex containing perhaps as much as 70% of the coreleased peptidoglycan. Most of this peptidoglycan is rapidly displaced from the soluble complex. However, as can be seen in the inset in Figure 4, 20% of the peptidoglycan is much less easily displaced from association with the polysaccharide. This displacement proceeds with a half-life of approximately 200 min. By 640 min, the peptidoglycan to polysaccharide ratio has been reduced to $1/15$ that of the intact wall. This ratio of peptidoglycan to polysaccharide is further reduced by exhaustive digestion with lysozyme and reaches a limit of 0.4% (see below).

It is apparent from the above time courses that the peptidoglycan that is between the polysaccharide molecules is more easily digested than the peptidoglycan that is more closely associated with the polysaccharide. Even after extensive redigestion with lysozyme, there remained a small portion of the peptidoglycan in association with the polysaccharide. It was of obvious interest to examine the peptidoglycan associated with the polysaccharide and to investigate the nature of the linkage.

Nature of the Peptidoglycan Associated with the Polysaccharide. The uronic acid containing polysaccharide was prepared as described by White and Gilvarg (1977). This material has approximately 10% peptidoglycan associated with it. The material was redigested with lysozyme and rechromatographed. The amount of peptidoglycan associated with the polysaccharide was reduced by this further lysozyme digestion. However, it was not possible to reduce the proportion of peptidoglycan below 0.4% by this procedure alone. The peptidoglycan associated with the polysaccharide after extensive lysozyme digestion coeluted with the polysaccharide in the presence of 8 M urea, 0.2% sodium dodecyl sulfate, and 0.2% Triton X-100.

The teichuronic acid extracted from *Bacillus licheniformis* has been shown to be linked to the peptidoglycan by a phosphodiester bond. As this is also the linkage between teichoic acids and the peptidoglycan, it was important to investigate the possibility that the uronic acid containing polysaccharide of *Bacillus megaterium* M46 was linked to the peptidoglycan by a phosphodiester bond. Determination of the total phosphate of the polysaccharide-peptidoglycan complex revealed that there was less than 1 nmol of phosphate for every 8 μmol of glucuronate (5 mg of teichuronate). This is a stoichiometry of ten teichuronic acid molecules per phosphate. This is based on a molecular weight of 480 000 for the teichuronic acid (Ivatt, R. J., and Gilvarg, C., unpublished results). The absence of a phosphodiester bridge between the polysaccharide and the peptidoglycan fragments was confirmed by exposure of the

polysaccharide-peptidoglycan complex to 50% aqueous hydrofluoric acid at 0 °C. This procedure has been shown to cleave phosphodiester linkages (Lipkin et al., 1969). The neutralized digestion products were chromatographed on Sepharose 2B in ammonium acetate. No detectable separation of peptidoglycan and polysaccharide was observed after 1 h of hydrolysis, demonstrating the absence of a phosphodiester linkage between the polysaccharide and peptidoglycan. Prolonged digestion with hydrofluoric acid, for 36 h, led to partial degradation of the polysaccharide. However, the ratio of peptidoglycan to polysaccharide remained fairly constant over the elution profile, demonstrating that random cleavage of the polysaccharide had occurred in the absence of any specific cleavage of the peptidoglycan-polysaccharide linkage. In the absence of enzymes capable of specifically degrading the polysaccharide, we have not been able to explore this linkage beyond eliminating a phosphodiester bridge. This linkage is stable to prolonged exposure to dilute alkali (4 M ammonia, 24 h, 37 °C) and dilute acid (10% Cl_3AcOH, 24 h, 37 °C), and at this stage in the investigation is presumed to be a glycosidic linkage.

The nature of the peptidoglycan associated with the polysaccharide has been explored. The degree of cross-linkage of the peptidoglycan was determined by the method of Fordham and Gilvarg (1974). The polysaccharide-peptidoglycan complex was treated with nitrous acid, hydrolyzed to the free amino acids, and analyzed by ion-exchange chromatography. More than 95% of the radioactivity, originally incorporated into the peptidoglycan as diaminopimelic acid, was now retained by the anion-exchange resin. Therefore, less than 5% (3 ± 2%) of this diaminopimelic acid was protected from modification, and thus was not involved in peptide cross-linkage. This value is very low in comparison to the average for the total peptidoglycan, 50–55%, and thus represents a unique section of the wall.

The reducing sugar content of the polysaccharide-peptidoglycan complex was found to be very low—less than one reducing sugar per 500 glucuronates, by the method of Park and Johnson (1949). It was of obvious diagnostic value to know both the number and identity of the reducing terminals. The peptidoglycan-polysaccharide complex was reduced with tritiated sodium borohydride. After hydrolysis of the sample and the removal of HCl, the bulk of the radioactivity, 92%, was retained by a cation-exchange resin and eluted by 4 M ammonium hydroxide. This material comigrated on electrophoresis with a muramic acid standard, which had been reduced with tritiated sodium borohydride under the same conditions as the polysaccharide. It is a significant finding that less than 8% of the reducing sugar content was contributed by sugars from the polysaccharide, indicating that virtually every teichuronic acid chain contained peptidoglycan. The reducing terminals were also investigated by β-elimination studies. Under the standard conditions for β elimination, only muramic acid molecules with reducing groups lose their peptide chains. When the polysaccharide-peptidoglycan complex was exposed to β-elimination conditions (4 M NH_3, 37 °C), some of the [^{3}H]DAP was no longer excluded from Sephadex G-50. The time course for this displacement is shown in Figure 5. A limiting value of 52% was obtained for the amount of [^{3}H]DAP released during β elimination. This value was obtained from a double-reciprocal plot of the time-course data, which is shown in the inset of Figure 5. In contrast to the [^{3}H]DAP associated with the polysaccharide, which is excluded from Sephadex G-50, the [^{3}H]DAP-containing fragment released during β elimination has a k_d of 0.86. This apparently low-molecular-weight material, when analyzed by thin-layer chromatography (*tert*-butyl alcohol:acetic acid:water 5:2:3), comigrated with the peptide chain β eliminated from a disaccharide-pentapeptide sample (R_f 0.55) and was distinguishable from the parent compound (R_f 0.42). This finding, that half of the muramic acid molecules lose their peptide chains, confirms the suggestion that a tetrasaccharide-dipeptide fragment of the peptidoglycan is associated with each polysaccharide molecule.

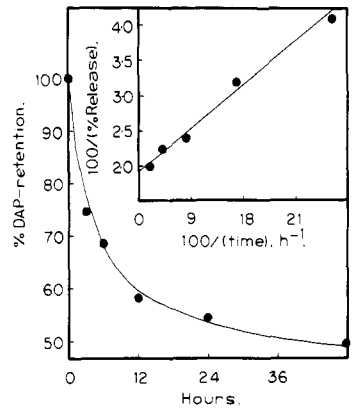

FIGURE 5: The release of peptide chains from the lysozyme-resistant polysaccharide-peptidoglycan complex during β elimination. The lysozyme-resistant polysaccharide-peptidoglycan complex (5×10^4 cpm) was exposed to 4 M ammonia at 37 °C. Aliquots were removed during the treatment and were chromatographed on Sephadex G-50 in 0.1 M ammonium acetate. The fraction of radioactivity excluded from Sephadex G-50 was plotted as a function of time. The double-reciprocal plot reveals a limiting value, for the release of [^{3}H]DAP from the complex, of 52%.

Discussion

The cell envelope of *Bacillus megaterium* M46 retains the shape of the bacillus, and because of its simple composition provides a useful model for studying the synthesis and assembly of the bacterial cell wall. The structural relationship of the peptidoglycan and teichuronic acid components of the *Bacillus megaterium* cell envelope has been approached by studying the kinetics and reaction products of the lysozyme-digestion process. The cell envelope contains two types of peptidoglycan: those regions rapidly reduced by lysozyme to fragments not retained by millipore filtration, and those regions more slowly disintegrated. The rapidly degraded regions account for almost 90% of the bulk of the cell envelope. The half-life of this rapid degradation is the same whether judged by the retention of material on millipore filters or by the release of polysaccharide. Use of the differently sized millipore filters failed to detect a pool of material of intermediate size, i.e., between 0.45 and 0.11 μm in diameter. This lack of intermediately sized material was emphasized by the finding, in a separate experiment, that the polysaccharide and peptidoglycan released during this initial phase also passes through a 0.05-μm diameter millipore filter and is indicative of a processive mechanism of digestion, where the lysozyme molecules associate with the cell wall and digest a local region, rather than dissociating and randomly reassociating between successively cleaved peptidoglycan bonds. The polysaccharide has been shown to be a large rod-shaped molecule (Ivatt, R. J., and Gilvarg, C, unpublished results), with a molecular weight of 480 000 and having a hydrodynamic radius of 500 Å. Polysaccharide-peptidoglycan complexes that are less than 0.11 μm in diameter can be expected to represent discrete polysaccharide molecules with associated peptidoglycan. Analysis of the reaction products of lysozyme digestion, by chromatography on Sepharose 2B,

demonstrates the existence of discrete polysaccharide–peptidoglycan complexes containing 20% (w/w) peptidoglycan, and that, after extensive exposure to lysozyme, this proportion is slowly reduced. A limit of 0.4% (w/w) was reached by exhaustive lysozyme digestion. There is therefore a strong indication that the regions of peptidoglycan between the polysaccharide molecules are more readily digested than those regions more closely associated with the polysaccharide. As nearly 90% of the polysaccharide and peptidoglycan are coordinately released by a simple first-order process, (Figures 1 and 4), we conclude that the bulk of the polysaccharide molecules are uniformly distributed over the surface of the peptidoglycan. If the polysaccharide were not uniformly distributed over the peptidoglycan, one would expect to see a preferential release of one of the polymers during the initial phase of digestion.

The peptidoglycan associated with the polysaccharide is eluted at the exclusion limit of Sepharose 2B and thus has a Stoke's radius of 500 Å. The peptidoglycan does not have a large intrinsic size, as demonstrated by the lack of peptide cross-links and the occurrence of half of the muramic acid molecules at reducing terminals. Therefore, the peptidoglycan is not eluted at the exclusion limit of Sepharose 2B as a highly polymerized impurity.

The finding that the reducing groups were predominantly, 92%, muramic acid (i.e., contributed by peptidoglycan) is strong evidence that the reducing terminal of the polysaccharide is linked to the peptidoglycan. The linkage is not by a phosphodiester bridge and is presumed to be by a direct glycosidic bond. The presence of common denaturants, urea and detergents, which disrupt noncovalent bonds, does not separate the polysaccharide and the peptidoglycan, neither do a variety of ionic and pH conditions. Two more factors favor a covalent rather than a noncovalent linkage. Firstly, there is a simple stoichiometry of one peptide chain from the peptidoglycan per molecule of polysaccharide, after extensive digestion with lysozyme and β elimination, and, secondly, the stability of the association. Conditions which cause degradation of the polysaccharide do not cause dissociation of the peptidoglycan and polysaccharide, suggesting that the polysaccharide–peptidoglycan bond is more stable than the covalent bonds within the polysaccharide to acid and alkali hydrolysis. We therefore conclude from these results that after extensive digestion with the enzyme lysozyme, the polysaccharide is covalently linked to a tetrasaccharide unit of the peptidoglycan having uncross-linked peptide chains, presumably by a glycosidic bond. The polysaccharide contains uronic acid and is covalently bound to the peptidoglycan; it thus fulfills both the compositional and structural criteria established for teichuronic acids. This teichuronic acid is the major component of the cell envelope of *Bacillus megaterium* M46 and its roles in the structure and function of the cell envelope are currently under investigation.

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