A Teichuronic Acid Containing Rhamnose from Cell Walls of *Bacillus megaterium*†

P. J. White‡ and C. Gilvarg*

ABSTRACT: A cell wall sacculus was isolated from *Bacillus megaterium* M46 (a mutant that requires diaminopimelate plus lysine for growth) by treating stationary-phase bacteria with sodium dodecyl sulfate, nucleases, and trypsin. This sacculus preparation was digested with lysozyme, and soluble high-molecular-weight material (emerging near the void volume on Sephadex G-200) was separated. An acidic polysaccharide (teichuronic acid) was then obtained from the high-molecular-weight fraction by gradient elution from diethylaminoethylcellulose, followed by precipitation with ethanol. Teichuronic acid was also obtained, in lower yield, by extraction of sacculi with trichloroacetic acid, followed by similar purification on Sephadex G-200 and diethylaminoethylcellulose. The teichuronic acid contained glucuronic acid, glucose, and rhamnose in molar proportions of 1.0:1.2:2.1, and these carbohydrates made up approximately 63% (w/w) of the polymer isolated from lysozyme digests. This teichuronic acid also contained 12% (w/w) of peptidoglycan, but only 4% (w/w) of peptidoglycan was present in teichuronic acid isolated after trichloroacetic acid extraction. In the wall itself, teichuronic acid and peptidoglycan occurred in proportions of 1.7:1.0 w/w. The cell-wall sacculus contained 38% (w/w) of these two components and, in addition, poly(β-hydroxybutyrate) (43%, w/w), glycogen (8%, w/w), and protein (5%, w/w). Very little phosphate (about 1%, w/w) was present.

The name "teichuronic acid" was first given to an acidic polysaccharide isolated from cell walls of *Bacillus subtilis* (Janczura et al., 1961). This material was extracted with trichloroacetic acid and was purified by precipitation as a cetylpyridinium salt, followed by chromatography on DEAE[1]-cellulose. Unlike the teichoic acids (Baddiley, 1972), the polysaccharide contained no glycerol or ribitol and very little phosphorus, but was instead made up of equimolar amounts of glucuronic acid and *N*-acetylgalactosamine.

The present paper describes a teichuronic acid from *Bacillus megaterium* strain M46. The polysaccharide was obtained as a relatively undegraded polymer from enzymatic digests of cell walls. It contains three sugar components, glucuronic acid, rhamnose, and glucose, as well as a small amount of peptidoglycan, which may be attached covalently. No polyol and very little phosphorus are present, so that the teichuronic acid is clearly different from the phosphomucopolysaccharide that is a major component of the walls of *B. megaterium* strain KM (Ghuysen et al., 1962).

Experimental Procedure

Organism. B. megaterium M46, which was used throughout, is a mutant that lacks pyruvate-aspartyl-β-semialdehyde condensing enzyme and diaminopimelate decarboxylase (Pitel and Gilvarg, 1970). These organisms require both diaminopimelic acid and lysine for growth. Stock cultures were samples (1 mL) taken from the defined medium (below), while the organisms were in the exponential phase of growth at 32 °C, and each diluted with 0.25 mL of sterile glycerol (50%, v/v in water), and then kept at −60 °C.

Medium. All cultures were grown in medium F (Fukuda and Gilvarg, 1968) containing 0.08% (w/v) glucose plus L-lysine hydrochloride (20 mg/L) and *meso*-diaminopimelic acid (10 mg/L).

Conditions of Growth. Fifty liters of medium at 32 °C in a New Brunswick Model F-130 fermenter was inoculated with a culture (1 L) in the exponential phase of growth at 32 °C. The culture in the fermenter was aerated continuously with an airflow of 5.5 ft^3/min.

Isolation of Sacculi. The turbidities of samples from the fermenter were measured at intervals. When the late exponential phase of growth was reached (about 7 h after inoculation), 20 L of culture was withdrawn, and, when the peak of maximum turbidity had been passed for 2 h (about 11 h after inoculation), another 20 L of culture was drained. A 10% (w/v) solution of sodium dodecyl sulfate (5 L/20 L of culture) was siphoned into each culture over a 30-min period immediately after the culture had been taken from the fermenter. The mixtures were stirred briefly and then were left undisturbed at room temperature for 1 week. Subsequent treatment of both cultures was the same.

About 18 L of clear supernatant liquid was removed from each culture by aspiration, without stirring the sediment. The remainder (about 6 L) from each culture was centrifuged at 15 000*g* for 5 min at 2 °C. The sediment was washed seven times with water (1 L per wash) by centrifuging as above, and then once in 0.1 M Tris buffer, pH 7.6 (1 L). The washed pad was resuspended in Tris buffer (1 L) containing 1 mM magnesium chloride, and toluene (20 mL) was added. The suspension was stirred magnetically at 37 °C, and deoxyribonuclease and ribonuclease (both 50 mg in 10 mL of Tris buffer) were added. After 4 h, trypsin (100 mg in 10 mL of buffer) was added, and incubation was continued overnight.

The solid residue (sacculi) was centrifuged as before, and was washed eight times with water. The sacculi were finally resuspended in water (200 mL for the sacculi from 20 L of culture) and kept at −15 °C. The preparation obtained in this way retained the shape of the original organisms, but contained entrapped granules of poly(β-hydroxybutyrate), which clearly are not part of the cell wall proper.

† From the Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540. *Received July 15, 1976.* Supported by Public Health Service Grant AI 11756.

‡ On leave from the Department of Microbiology, The University of Sheffield, Sheffield, S10 2TN, England.

[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; UV, ultraviolet.

Isolation of Teichuronic Acid. (1) Lysozyme Digestion. Washed stationary phase sacculi (1.54 g dry weight in 150 mL of water) were mixed in a 500-mL flask with 30 mL of 1.0 M ammonium acetate (pH 6.5), 7.5 mL of 0.9% NaCl, and water to 292 mL. The suspension was brought to 32 °C in a water bath and then 8 mL of a solution of lysozyme (10 mg/mL in water) was added. This mixture was incubated at 32 °C for 1 h with occasional swirling. The suspension then was centrifuged at 11 000*g* for 10 min at room temperature, and the supernatant liquid was removed and kept. The pad was washed once in water and then resuspended in water. A sample of this suspension (1 mL) was dried at 105 °C to constant weight.

A column (5 × 90 cm) of Sephadex G-200 was prepared (at room temperature) for upward elution, in 0.1 M ammonium acetate, pH 6.4. Part of the lysozyme digest (48 mL) was eluted through the column with 0.1 M ammonium acetate, pH 6.4, from a constant head device, while fractions (about 20 mL) were collected automatically at a flow rate of about 50 mL/h. Selected fractions were assayed for uronic acid and neutral sugar (with anthrone reagent), and fractions containing uronic acid were pooled. These fractions (210 mL) represented the effluent emerging from the column between 600 and 810 mL after the start of sample application (Figure 1). The void volume of the column (determined with blue dextran) was 580 mL.

Attempts to chromatograph a more concentrated lysozyme digest on this column were unsuccessful. A digest (300 mL, prepared as above) was freeze-dried and taken up in 50 mL of water, to give a turbid and viscous solution. After passage through the column, the uronic acid emerged as a very broad (720 mL) and unsymmetrical peak.

A column of DEAE-cellulose (1.1 × 20 cm) was poured at room temperature in 0.1 M ammonium acetate, pH 6.4. Uronic acid containing material from the Sephadex column (252 μmol of uronic acid in 240 mL) was run through the DEAE-cellulose column, which then was eluted with about 100 mL of 0.1 M ammonium acetate and next with a gradient of ammonium acetate, pH 6.4, throughout. The lower mixing vessel contained 400 mL of 0.1 M ammonium acetate and the upper vessel (fitted with a constant head device) contained 3 M ammonium acetate. Fractions (5 mL) were collected automatically during gradient elution, with a flow rate of about 30 mL/h. Selected fractions were assayed for uronic acid, neutral sugar (with anthrone reagent), and rhamnose, and those containing uronic acid were pooled.

The pooled fractions (67 mL) were concentrated by rotary evaporation and made up with water to 25 mL. Ethanol (75 mL) was added with stirring, and the heavy white precipitate was left to settle at 2 °C for 3 days. The precipitate was collected by centrifuging, washed with ethanol, acetone, and ether, and then dried over silica gel before weighing.

(2) Cl_3AcOH Extraction. Washed stationary-phase sacculi (360 mg in 40 mL of water) were mixed in a 500-mL flask with 40 mL of 0.1 M phosphate buffer, pH 6.8, 4 mL of 0.9% (w/v) NaCl, and water to 157 mL. This suspension (plus 3 mL of toluene) was stirred magnetically at 32 °C and 3 mL of a solution of α-amylase (10 mg/mL in phosphate buffer) was added. The suspension was stirred overnight at 32 °C and then centrifuged at room temperature, and the supernatant liquid was removed. The pad was washed twice in water (about 150 mL for each wash) and then resuspended in water to 40 mL.

To 38 mL of this suspension was added an equal volume of 10% (w/v) Cl_3AcOH, and the mixture was stirred at 32 °C overnight. The solid was centrifuged and the supernatant liquid (first Cl_3AcOH extract) was kept. The solid was resuspended in 50 mL of 5% (w/v) Cl_3AcOH and again incubated overnight at 32 °C. The extraction with Cl_3AcOH was repeated twice more, and the pad after four extractions was washed twice with water and kept as a suspension in water (40 mL) at −15 °C.

The first Cl_3AcOH extract (65 mL) was extracted four times with ether (70 mL for each extraction). Air was bubbled through the residual aqueous solution for a few minutes to remove ether and then the solution was freeze-dried. The solid was taken up in 0.1 M ammonium acetate, pH 6.5, and part was treated (as described above) by Sephadex G-200 chromatography. The high-molecular-weight fractions containing uronic acid were pooled, and subjected to gradient elution from DEAE-cellulose as above, followed by ethanol precipitation from the concentrated fractions that contained rhamnose and uronic acid.

Estimations. Neutral sugar was measured by modified anthrone reagent methods. In different experiments, either the procedure of Roe (1955) or that of Herbert et al. (1971) was used. Standard curves with both methods were measured for glucose, galactose, mannose, rhamnose, and glucuronic acid (each in the range 0–1 μmol per assay tube) and for glucose in the presence of equimolar amounts of both rhamnose and glucuronic acid. The phenol–sulfuric acid method of Dubois et al. (1956) was also used occasionally for assay of neutral sugar; various standard curves were made as above, all with 1 mL of 5% (w/v) phenol per assay tube.

Glucose and galactose were measured after acid hydrolysis (2 N HCl at 100 °C for 1 h) of polysaccharides. The hydrolysates were neutralized with 2 N NaOH and phosphate buffer, pH 7, before assays with Glucostat or Galactostat kits (Worthington Biochemical Corp., Freehold, N.J.).

Rhamnose was measured by the method of Dische and Shettles (1948).

Uronic acids were measured by the modified carbazole assay of Bitter and Muir (1962). Standard curves were measured for glucuronic acid (0–0.2 μmol per assay tube) and for glucuronic acid plus one- and twofold molar proportions of both glucose and rhamnose. Glucuronic acid was also characterized by the specific reaction with mannose (Dische, 1947) but this method was not used routinely for assays.

Hexosamines were assayed by the method of Levvy and McAllen (1959) in which glucosamine and muramic acid give approximately the same yield of colored reaction product per mole. Before assay, test materials were hydrolyzed (4 N HCl at 100 °C for 4 h) and acid was removed by repeated evaporation.

Diaminopimelic acid was measured by the method of Gilvarg (1958). After acid hydrolysis (6 N HCl for 18 h at 105 °C) of test samples and repeated evaporation of the acid, the diaminopimelate was separated from brown materials (in samples that had contained much polysaccharide) by its absorption onto a column (0.8 × 15 cm) of Dowex 50-X2 (H^+ form, in water). The diaminopimelate was eluted from the resin with 3 N ammonium hydroxide, which was removed by evaporation before the assay.

Phosphorus was measured as described by Ames (1966). All samples (standard and test) were ashed with magnesium nitrate before assay to convert organic phosphorus to inorganic orthophosphate.

Poly(β-hydroxybutyrate) was assayed (in chloroform extracts made in various ways described later) by the method of Law and Slepecky (1961).

Protein was determined by the method of Lowry et al.

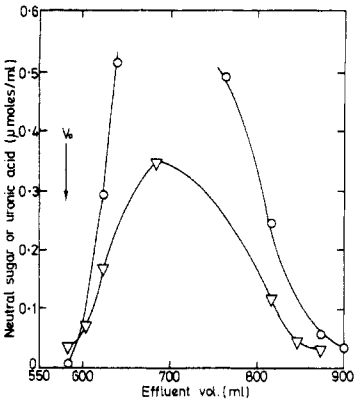

FIGURE 1: Elution of high-molecular-weight material in a lysozyme digest of stationary-phase sacculi through a column of Sephadex G-200. Operating details are given in the text. (○) Neutral sugar (measured with anthrone reagent in terms of a glucose standard); (▽) uronic acid. V_0 is the void volume of the column.

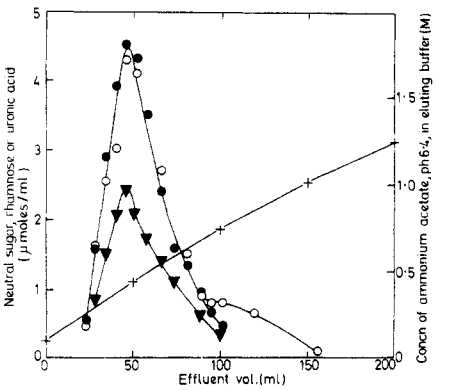

FIGURE 2: Gradient elution of high-molecular-weight material (from a lysozyme digest, after Sephadex G-200) off a DEAE-cellulose column. Procedure is described in the text. (○) Neutral sugar (measured with anthrone reagent in terms of a glucose standard and corrected for the presence of rhamnose); (●) rhamnose; (▼) uronic acid; (+) concentration of ammonium acetate. A peak of neutral high-molecular-weight material emerged from the column before the gradient was started (not shown).

(1951) with bovine albumin as standard. Sacculi were heated at 100 °C for 10 min in 0.5 N NaOH to dissolve protein from the insoluble peptidoglycan, which was removed by centrifuging before assay. The standards were similarly treated with alkali.

The dry weights of sacculi (or other materials) were measured by heating an aqueous suspension (usually 1 mL) for 3 h (or longer if necessary) at 105 °C until constant weight was obtained.

Paper Chromatography. Descending chromatograms were run on Whatman No. 1 paper at room temperature with the following principal solvents (proportions are parts by volume): (A) ethyl acetate–acetic acid–water (9:2:2); (B) butanol–acetic acid–water (63:10:27); (C) butanol–pyridine–water (6:4:3). Spots were revealed by the following methods (described by Bailey, 1969): silver nitrate–NaOH (general carbohydrate reagent); *p*-anisidine (aldoses); aniline phthalate (aldoses); orcinol (ketoses); bromophenol blue (uronic acids); ninhydrin (amino compounds).

Electrophoresis. Teichuronic acid (1 μg in 2 μL of water) was placed as a line at the middle of a strip of cellulose acetate. Electrophoreses at pH 3.0 and 6.9, followed by staining with Alcian Blue, were done as described by Herd (1968).

Hydrolysates of teichuronic acid were examined by high-voltage electrophoresis on Whatman No. 1 paper at pH 6.4 in pyridine–acetic acid buffer (70 V/cm, 120 mA, for 30 min) in a Savant flat-plate apparatus (Model FP22A) with water cooling. Spots were revealed with *p*-anisidine.

Tests with Concanavalin A. Precipitin tests in gels were made as described by Goldstein (1972).

Chemicals. Ribonuclease R was bought from Worthington Biochemical Corp.; deoxyribonuclease II (bovine pancreas) was from Calbiochem; trypsin was from Schwarz/Mann Laboratories. Lysozyme ("salt free") was from Worthington Biochemical Corp.; α-amylase (bacterial type IIA) was from Sigma Chemical Co., as was concanavalin A.

Results

Both late exponential phase and stationary-phase sacculi contained similar amounts of uronic acid (0.37 μmol/mg of both preparations) and neutral sugar (1.1 and 0.93 μmol/mg, respectively) which were measured in terms of a glucose standard by the method of Roe (1955). Lysozyme solubilized 50% (w/w) of the late exponential phase sacculi, and 54% (w/w) of the stationary-phase material. The insoluble residues seemed microscopically to consist entirely of granules, and must very largely be poly(β-hydroxybutyrate) (see later). Approximately 90% of the neutral sugar of the preparation appeared in the lysozyme digest, and all of the uronic acid was dissolved. The late exponential phase sacculi have not been examined further.

Isolation of Teichuronic Acid Released by Lysozyme. When the lysozyme digest was eluted through Sephadex G-200, 85% of the anthrone-positive material and 94% of the uronic acid put onto the column appeared in a single peak, which will be called "high-molecular-weight material" (Figure 1). Most of the phosphorus and diaminopimelic acid from the digest emerged later from the column as a single peak which did not overlap the peak of uronic acid plus neutral sugar.

When the high-molecular-weight material was eluted through a column of DEAE-cellulose with 0.1 M ammonium acetate, 35% of the applied anthrone-positive material emerged at once from the column. This material proved to be a polymer of glucose (see below); no uronic acid or rhamnose emerged at this stage. Gradient elution of the column led to the appearance of a sharp peak that contained most of the remaining anthrone-positive material (glucose and rhamnose) and all of the uronic acid put onto the column (Figure 2). There was some tailing of anthrone-positive material other than rhamnose behind the main peak, and this material was discarded.

The pooled fractions from the main peak contained (μmol): glucose, 170; rhamnose, 176; and glucuronic acid, 95. If these three components were indeed parts of the ammonium salt of an acidic polymer, then at least 72 mg of the polymer salt should be present in the pooled fractions. In fact, when these fractions were concentrated and the teichuronic acid was precipitated with ethanol, 78 mg of solid was obtained which contained (μmol): glucose, 125; rhamnose, 164; glucuronic acid, 75. The isolation procedure, therefore, preserves most of the uronic acid and rhamnose of the sacculus as precipitated teichuronic acid.

Isolation of Teichuronic Acid Released by Cl_3AcOH. The first extraction with Cl_3AcOH removed 78% of the uronic acid from the α-amylase-treated sacculi, and a further 9% was removed by the second, 7% by the third, and none by the fourth. No uronic acid was detected in the solid residue after this last extraction. Only material from the first Cl_3AcOH extract was purified further.

On elution of part of this extract (20 μmol of uronic acid)

through Sephadex G-200, about half of the uronic acid emerged as a distinct peak of high-molecular-weight material between 565 and 705 mL of effluent volume. This suggests that extraction with Cl_3AcOH caused degradation of part of the teichuronic acid. The pooled high-molecular-weight fractions contained (μmol): glucose, 19; rhamnose, 24; glucuronic acid, 12. Collection of fractions was stopped after the high-molecular-weight material had emerged from the column, and so it was not established when the remaining uronic acid was eluted.

All of the high-molecular-weight material was subjected to gradient elution from DEAE-cellulose. A sharp peak containing glucose, rhamnose, and glucuronic acid was eluted with 0.4–0.5 M ammonium acetate. The pooled fractions making up this peak contained (μmol): glucose, 13; rhamnose, 23; glucuronic acid, 11. Thus, almost all of the uronic acid and rhamnose put onto the column was recovered as a single peak, but some glucose was lost. Possibly this glucose was a neutral polymer that had resisted α-amylase treatment and had been eluted from the DEAE-cellulose column before the gradient was started—these fractions were not assayed. When the peak fractions were concentrated and the teichuronic acid was precipitated with ethanol, 7.8 mg of solid was obtained. The expected amount of teichuronic acid precipitate (calculated as before) was at least 7.6 mg.

Composition of the Teichuronic Acid. The teichuronic acid (0.6 mg) isolated from a lysozyme digest was hydrolyzed with 2 N HCl (0.6 mL) in a sealed tube at 100 °C for 2 h. This hydrolysate was dried under diminished pressure at 40 °C and the residue was taken up in water (about 3 mL) and dried again. After repeating this drying twice more, the residue was taken up in water (50 μL) and samples (5 μL) were chromatographed in solvent B. Aniline phthalate spray (viewed under UV) revealed three main spots: (1) R_{Glc} 2.1, (2) R_{Glc} 1.04, (3) R_{Glc} 0.78. Spot 1 was yellow-brown, and had a dull yellow-brown fluorescence under UV. In these respects and in its mobility spot, 1 resembled a rhamnose marker. Spot 2 was greenish-brown and under UV had a yellow fluorescence; a glucose marker had the same appearance. Spot 3 was light brown (but became green-brown after 24 h) and was dull brown under UV. A glucuronic acid marker had similar appearances but a slightly different R_{Glc}, 0.72. Other fainter spots near the glucuronic acid position were seen and will be considered later.

Major Carbohydrate Components. The presence of glucose was confirmed by chromatography in solvents A and C, and by specific enzymatic assay. To obtain maximum release of glucose, samples of teichuronic acid were heated with acid at 100 °C for various times (Figure 3). Hydrolysis for only 30 min with 2 N acid liberated most glucose, but this measure of free glucose could be an underestimate of the glucose actually present in the polymer because of the difficulty of completely hydrolyzing biuronic acids (Green, 1957), such as glucuronic acid-β-1,4-glucose.

The presence of a methylpentose in the teichuronic acid was confirmed by a positive result in the assay of Dische and Shettles (1948). Fucose also reacts in this assay, but chromatography in solvents A and C confirmed that rhamnose and not fucose was present after acid hydrolysis. Since the colorimetric assay is done by heating the sample at 100 °C in 30 N H_2SO_4, all the rhamnose ought to be liberated and measured. Assays with known amounts of glucose plus rhamnose showed that rhamnose could be accurately estimated in the presence of a twofold molar excess of glucose.

The presence of one or more uronic acids in the teichuronic

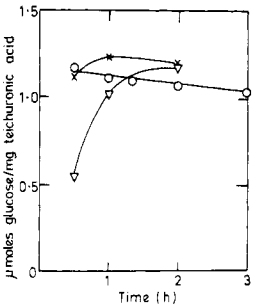

FIGURE 3: Release of free glucose by acid hydrolysis of teichuronic acid. Purified teichuronic acid (from a lysozyme digest) was heated for various times at 100 °C with: (○) 2 N HCl; (X) 2 N H_2SO_4; (▽) 1 N H_2SO_4. The acid was neutralized with NaOH and phosphate buffer, pH 7, and free glucose was assayed with Glucostat reagents.

acid was suggested by the acidic nature of the polymer, and was confirmed by a positive result in the colorimetric assay (Bitter and Muir, 1962). The standard curves for this assay were made with samples of glucuronic acid alone plus 1 or 2 molar proportions of glucose, since glucose contributed slightly to the color developed in the assay; rhamnose did not interfere. Chromatography and electrophoresis of hydrolysates (see below) indicated that at least part of the uronic acid was glucuronate. The colorimetric assay of Dische (1947), in which only glucuronic acid is supposed to give a colored product with mannose, was used to try to show that all the uronic acid was glucuronate. Standard solutions of glucuronic acid gave a positive result, whereas galacturonic acid or alginic acid (poly(mannuronic acid)) did not. However, glucose plus rhamnose (both equimolar with glucuronate) interfered seriously in the assay of glucuronic acid standards by this method, which could not therefore be satisfactorily applied directly to the teichuronic acid. Instead, uronic acid was isolated and examined after acid hydrolysis of the polymer.

Teichuronic acid (4 mg) was heated in 2 N HCl (4 mL) at 100 °C for 1 h. The dried hydrolysate was taken up in water, adjusted to pH 9 with dilute ammonia solution, and made up to 5 mL. Part (4 mL) of this solution was washed through a column (0.6 × 8 cm) of Dowex 1-X2 (formate form) with water at pH 9. The effluent contained free glucose and rhamnose (Table I). The column was eluted with 2 N HCl (50 mL) and the effluent was dried and then examined by paper chromatography in solvent A. A strong spot with mobility similar to glucuronic acid was found, and very faint spots in the glucose and rhamnose positions.

Samples of the redissolved HCl eluate were assayed for anthrone-positive material (Herbert et al., 1971), rhamnose, free glucose, and uronic acid (Table I). Assay by the method of Bitter and Muir (1962) indicated 0.6 μmol of uronic acid/mL and the method of Dische (1947) indicated 0.8 μmol of glucuronic acid/mL; in both assays, the standard curves were prepared from mixtures containing equimolar glucuronic acid and rhamnose. The glucuronic acid eluted from the column by HCl was accompanied by rhamnose (presumably as a glucuronic acid $\xrightarrow{1,2}$rhamnose biuronic acid) and free glucose (assayed with Glucostat). The color yield in the anthrone assay of this eluate was, however, somewhat greater than would be expected for the measured amounts of the identified components that it contained.

Glucuronolactone was identified in acid hydrolysates of teichuronic acid by its unusual chromatographic behavior; in solvent A it ran ahead of rhamnose, and in solvent B behind rhamnose. Material from the glucuronolactone position on a

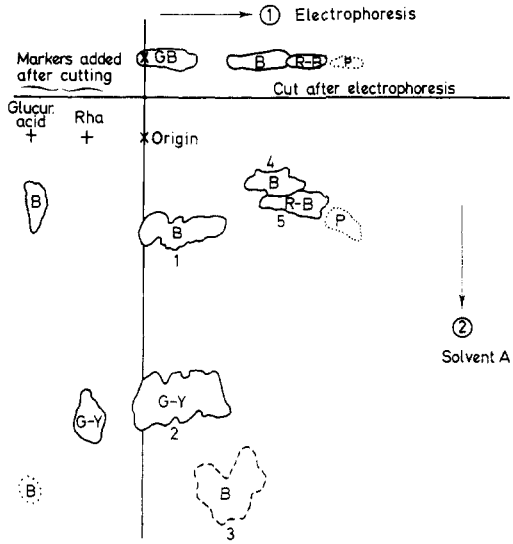

FIGURE 4: Electrophoresis and chromatography of an acid hydrolysate of teichuronic acid (purified from a lysozyme digest). The hydrolysate (from 2 mg of teichuronic acid heated 1 h at 100 °C in 2 N HCl) was dried and taken up in 200 μL of water. This material (10 μL) was placed at the two origins (X) and electrophoresed (see Experimental Procedure). A strip was cut from the dried sheet and sprayed with *p*-anisidine reagent. Markers were added (+) to the remaining unsprayed paper, which then was run as a descending chromatogram with solvent A. Finally, the paper was dried and sprayed with *p*-anisidine reagent. Relative intensities of spots (viewed by daylight) are roughly indicated by their outlines: (—) strong; (- - -) weak; (· · ·) very faint. Colors were: (B) brown; (RB) red-brown; (GB) green-brown; (GY) green-yellow; (P) pink. The spots from the hydrolysate were tentatively identified as: (1) glucose; (2) rhamnose; (3) glucuronolactone; (4 and 5) one glucuronic acid, the other glucuronic acid-rhamnose.

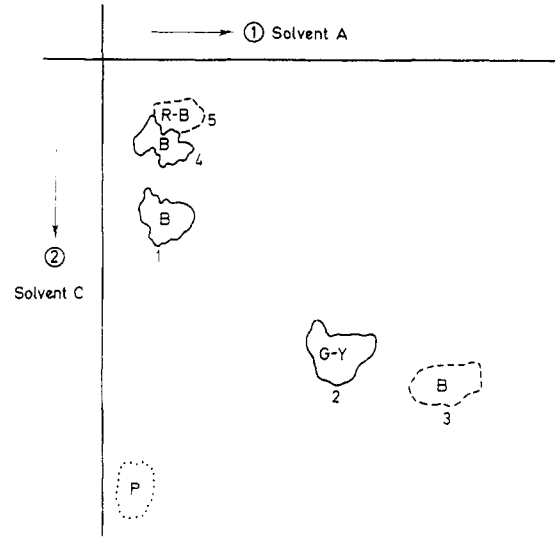

FIGURE 5: Two dimensional paper chromatogram of an acid hydrolysate of teichuronic acid. The hydrolysate (10 μL, prepared as described in Figure 4) was chromatographed first in solvent A, and then the paper was dried before solvent C was run at right angles. The paper was sprayed with *p*-anisidine reagent. Intensities and colors as in Figure 4. Spots were tentatively identified as: (1) glucose; (2) rhamnose; (3) glucuronolactone; (4 and 5) one glucuronic acid, the other glucuronic acid-rhamnose.

TABLE I: Separation on Dowex-1 of Carbohydrate Components of Teichuronic Acid after Acid Hydrolysis.[a]

Solution	Component	μmol/mg of teichuronic acid hydrolyzed
pH 9 eluate	Free glucose	1.13
	Rhamnose	1.62
2 N HCl eluate	Free glucose	0.10
	Excess anthrone-positive material[b]	0.35
	Rhamnose	0.44
	Glucuronic acid	0.96
Totals (μmol/mg of teichuronic acid)	glucuronic acid, 0.96 glucose, 1.2 rhamnose, 2.1 other anthrone-positive material, 0.35	

[a] Teichuronic acid was hydrolyzed at 100 °C for 1 h in 2 N HCl, dried, and taken up at pH 9 as described in the text. [b] Excess anthrone-positive material other than rhamnose, free glucose, and glucuronate (expressed as glucose).

chromatogram after running in solvent A was eluted with water, dried, taken up in water, and rechromatographed in solvents A and B. With each solvent, a strong spot in the glucuronic acid position was seen (*p*-anisidine spray), as well as a weaker spot in the glucuronolactone position. At pH 9, glucuronolactone was retained by Dowex-1.

Hence, when the teichuronic acid is hydrolyzed for 1 h at 100 °C in 2 N acid, glucuronic acid is released from the polymer as its lactone and (probably) as biuronic acid. Whether nonlactonized glucuronic acid is also set free is uncertain because this compound and the biuronic acid have very similar chromatographic mobilities in the solvents used. Polysaccharides are not generally found to contain two different uronic acids. Glucuronic acid is definitely present in the teichuronic acid, and so it is unlikely (provided that the teichuronic acid is not really two distinct carbohydrate polymers) that another uronic acid is present.

No other major carbohydrate component appears to be present in the teichuronic acid. After acid hydrolysis, samples were examined by electrophoresis on paper in one dimension, followed by chromatography in the second dimension (Figure 4). Two dimensional paper chromatography was also used (Figure 5). One-dimensional paper chromatograms of the hydrolyzed teichuronic acid were also run with solvents A, B, and C and treated with the various reagents for detection that are described under Experimental Procedure.

Some other materials in the hydrolyzed teichuronic acid were seen as faint spots on paper chromatograms, but have not been identified. A spot with bluish fluorescence under UV (invisible by daylight) was present (R_{Glc} 1.7) on chromatograms run in solvent A and then merely dried without spraying. A weak spot in the same position appeared when the chromatograms were treated with ninhydrin, but *p*-anisidine gave no reaction. Another spot (very dark brown in daylight) near the origin (R_{Glc} 0.5 in solvent A) was found on chromatograms treated with *p*-anisidine, but only appeared when the chromatogram had been kept at room temperature for 24 h after the spraying and heating.

Peptidoglycan Components. After elution from DEAE-cellulose and ethanol precipitation, the teichuronic acid did contain diaminopimelic acid (Table II). It has therefore been supposed that a fragment of peptidoglycan remains attached to the teichuronic acid isolated after lysozyme digestion of sacculi. Attempts to measure hexosamine in the teichuronic acid were unsatisfactory because of the formation of interfering colored breakdown products when the polymer was hydrolyzed under the rather severe conditions needed to liberate hexosamine monomers (4 N acid for 4 h at 100 °C). The assays did

TABLE II: Assays of Components of Teichuronic Acid from *Bacillus megaterium* M46.

Component	Teichuronic acid isolated by: Lysozyme digestion (μmol/mg)	Lysozyme digestion (μg/mg)[a]	Cl_3AcOH extraction (μmol/mg)	Cl_3AcOH extraction (μg/mg)[a]
Glucuronic acid	0.9	173[b]	1.0	190[b]
Rhamnose	1.9	280	2.2	320
Glucose (after acid hydrolysis)	1.1	178	1.2	194
Diaminopimelate (peptidoglycan)	0.13	117[c]	0.04	39[c]
Phosphate	0.03	3	*d*	
Anthrone-positive material other than glucuronic acid plus rhamnose (as glucose)	1.9	308	2.0	320
Phenol-sulfuric acid positive material other than glucuronic acid plus rhamnose (as glucose)	1.5	243	*e*	
Total proportion of teichuronic acid accounted for (as identified compounds)	75% (w/w)		75% (w/w)	

[a] Calculated as μmol/mg of teichuronic acid X (mol wt of monomer − 18) to allow for loss of water on condensation into polymer. [b] As ammonium salt. [c] Assuming 2 mol of *N*-acetylhexosamine, 1 mol of glutamate, and 2.5 mol of alanine per mol of diaminopimelate. [d] Not measured with purified material. The Cl_3AcOH extract of sacculi contained only 0.07 μmol of phosphate/mol of glucuronate. [e] Not measured.

establish that the teichuronic acid contained no more hexosamine than might be expected (as part of a peptidoglycan polymer unit) from the amount of diaminopimelate that was present.

Since an attachment between peptidoglycan and teichuronic acid might possibly occur at a muramic acid 6-phosphate residue, the teichuronic acid was isolated from working solutions (i.e., ammonium acetate, pH 6.4) that contained no added phosphorus. The very low content of phosphorus in the polymer indicated that teichoic acid was absent (Table II).

In earlier studies, teichuronic acids have generally been isolated from Cl_3AcOH extracts of cell walls. Extraction with Cl_3AcOH and lysozyme digestion were therefore compared in terms of yields of teichuronic acid and nature of the product. It was hoped that Cl_3AcOH extraction might liberate the teichuronic acid free of peptidoglycan by breaking the links between the two polymers.

Repeated extraction with Cl_3AcOH at 32 °C did remove almost all of the uronic acid and rhamnose from sacculi. The insoluble residue after extraction still retained the shape of the organisms, and still contained poly(β-hydroxybutyrate) granules. However, of the uronic acid in the first extract with Cl_3AcOH, only 50% was present as high-molecular-weight material. After further purification (by DEAE-cellulose chromatography and precipitation with ethanol), this high-molecular-weight teichuronic acid was not free of diaminopimelate, though only about one-third as much was present per mg of polymer as was present in the teichuronic acid liberated by lysozyme (Table II).

Properties of the Teichuronic Acid. Electrophoresis. Solutions of the teichuronic acid (2 mg/mL in water) were very slightly opalescent and were slightly viscous. Electrophoresis of the polymer on cellulose acetate strips showed a main band that moved 1.0 cm towards the anode at pH 3.0 and a much fainter band that moved 1.35 cm in the same direction. At pH 6.9, only a single though broader band was seen, that moved 1.0 cm towards the anode.

Failure to Precipitate with Concanavalin A. Polymers that contain numerous glucose (or mannose) residues with free hydroxyl groups at positions 2, 3, 4, and 6 give a precipitate with the protein concanavalin A (Sharon and Lis, 1972). A precipitate was obtained from a solution of glycogen and a precipitin line in agar also was formed by glycogen under conditions described in Experimental Procedures. No precipitate or precipitin line was seen when teichuronic acid was treated similarly. The teichuronic acid did not interfere with nor enhance the positive reactions given by glycogen.

Insensitivity to α-Amylase. Teichuronic acid (isolated from a lysozyme digest of sacculi without prior treatment with α-amylase) was not degraded by α-amylase. Purified teichuronic acid (2 mg) was incubated at 37 °C for 3 h with α-amylase (200 μg) and NaCl (900 μg) in 0.025 M phosphate buffer, pH 6.8 (total volume 2 mL). Part of the mixture (1 mL) was then eluted through a column of Sephadex G-200 (1.1 × 25 cm) with 0.1 M ammonium acetate, pH 6.5; all of the anthrone-positive material and uronic acid put onto the column emerged as a sharp peak near the void volume. The remainder of the mixture was put onto a column of DEAE-cellulose (0.8 × 8 cm); no anthrone-positive material or uronic acid emerged from the column when it was eluted with 0.1 M ammonium acetate, pH 6.5.

Neutral Polysaccharide from Sacculi. The high-molecular-weight material isolated from lysozyme digests of sacculi by chromatography on Sephadex G-200 could be separated into a neutral fraction and an acidic component (teichuronic acid). The neutral polymer comprised 35% of the total anthrone-positive material (measured by the assay of Roe (1955) in terms of a glucose standard) put onto a DEAE-cellulose column, and was obtained free of rhamnose and uronic acid as a solution in 0.1 M ammonium acetate. This was evaporated to dryness under diminished pressure and the residue was taken up in water (5 mL) and eluted with water through a column (1.7 × 26 cm) of Sephadex G-25. All the anthrone-positive material emerged as a single sharp peak at the void volume (18 mL) of the column. After acid hydrolysis (2 N H_2SO_4, 1 h at 100 °C), very nearly the same amount of free glucose (2.0 μmol/mL) was found by enzymatic assay as would be expected if all the anthrone-positive material in the polymer were glucose (2.2 μmol/mL). Thus, the carbohydrate of the neutral polymer seemed to consist only of glucose units; no other carbohydrate was found on paper chromatograms of acid hydrolysates. The neutral polymer contained 0.021 μmol of phosphorus/μmol of glucose, but diaminopimelic acid was barely detectable (not more than 0.009 μmol/μmol of glucose).

The neutral polymer seemed likely to be glycogen, which has previously been found in *B. megaterium* (Barry et al., 1953). An aqueous solution of the neutral polymer (1 mL containing carbohydrate equivalent to 1 μmol of glucose) was mixed with 5 μL of a 1% (w/v) solution of iodine in 5% (w/v) KI. A brown color appeared immediately, and showed an absorption maximum at 500 nm when measured against water containing 5 μL of iodine solution/mL. The teichuronic acid (equivalent to 2.2 μmol of glucose/mL) when tested similarly gave no peak at 500 nm.

Composition of the Sacculi of B. megaterium M46. Sacculi of *B. megaterium* M46 contained teichuronic acid, a glycogen-like polymer, and poly(β-hydroxybutyrate), as well as

TABLE III: Composition of Stationary-Phase Sacculi of *Bacillus megaterium* M46.

Component assayed	μmol/mg of sacculi	μg/mg of sacculi[a]	Nature of polymer and % (w/w) in sacculi
Diaminopimelate	0.16	30	Peptidoglycan 14[b]
Hexosamine	0.24	55	
Glucuronic acid	0.25	44	Teichuronic acid (peptidoglycan-free) 24
Rhamnose	0.74	108	
Glucose[c]	0.51	83	
Inorganic phosphate	0.15	14	1
Glucose[d]	0.50	81	Glycogen 8
β-Hydroxybutyrate	5.0	430	Poly(β-hydroxybutyrate 43
Protein		46	Protein 5
Total proportion of sacculus accounted for is 95% (w/w)			

[a] Calculated as μmol/mg of sacculi X (mol wt of monomer −18) to allow for loss of water on condensation into polymers. [b] Assuming 2 mol of *N*-acetylhexosamine, 1 mol of glutamate, and 2.5 mol of alanine per mol of diaminopimelate. [c] Anthrone-positive material (other than rhamnose plus glucuronate) in α-amylase-treated sacculi. [d] Amount liberated by lysozyme (as neutral high mol wt material), or by α-amylase.

peptidoglycan. These materials together account almost completely for the weight of the sacculus, so that it is unlikely that any other major component is present (Table III).

The diaminopimelic acid (all meso isomer in this strain) was assumed to be present entirely as part of a peptidoglycan made up of: 1 mol of *N*-acetylglucosamine, 1 mol of *N*-acetylmuramic acid, 2–3 mol of alanine, 1 mol of glutamate/mol of diaminopimelate (Rogers and Perkins, 1968). Hence, for every micromole of diaminopimelate, about 900 μg of peptidoglycan should be present (when allowance is made for loss of water upon condensations between the various components of the peptidoglycan). Since the peptidoglycan and the teichuronic acid appear to be covalently linked, these two polymers seem clearly to be components of the cell wall proper. On the other hand, the poly(β-hydroxybutyrate) granules must be material that is trapped inside the network of the peptidoglycan–teichuronic acid complex. When this network was opened, e.g., by breaking with ultrasonication, or by dissolving with lysozyme, then the granules were readily soluble in chloroform at 60 °C, and the poly(β-hydroxybutyrate) could be assayed. However, all attempts to dissolve the granules out of the undamaged sacculi with various organic solvents (chloroform, methylene dichloride, pyridine, dioxan, dimethyl sulfoxide, 2-methoxyethanol) have been unsuccessful.

Whether the glycogen is also trapped material or is really a part of the wall structure has not been conclusively established, but the former possibility is more likely. Sacculi (45 mg) containing 50 μmol of neutral sugar (as glucose, by anthrone assay) were incubated with α-amylase (4 mg) overnight at 37 °C with stirring in 20 mL of 25 mM phosphate buffer, pH 6.8. After this treatment, the solid residue contained 30 μmol of neutral sugar and 15 μmol appeared in the supernatant liquid. After acid hydrolysis of this liquid (2 N HCl, 1 h, 100 °C), 14 μmol of free glucose was found. No uronic acid or rhamnose was released from the sacculi by α-amylase. The amount of glucose released by α-amylase (30% of the anthrone-positive material) is similar to the amount released as neutral high-molecular-weight material by lysozyme. After α-amylase treatment, the sacculi still retained their shape and still contained poly(β-hydroxybutyrate) granules. These remained insoluble in organic solvents, so that the sacculi did not seem to have become more permeable on removal of the glycogen.

Discussion

The method of isolating cell walls used in the present study has the advantage of yielding the peptidoglycan and teichuronic acid virtually quantitatively, and in the shape of the organism. Since only detergents and treatments with enzymes of defined specificities are used, no covalent bonds in the sacculi should be broken. However, the method does have the disadvantage of additionally yielding trapped inclusions, such as poly(β-hydroxybutyrate). Glycogen, which may also be an inclusion, can be removed by α-amylase, but no procedure for dissolving trapped poly(β-hydroxybutyrate) has been found, even though the granules, when free of the walls, are readily soluble in chloroform. Possibly the long chains of polymer that first may be dissolved by chloroform inside the cell wall are unable to escape through the pores of the peptidoglycan, so that a saturated solution accumulates internally and little of the granular material actually dissolves.

The method of isolating teichuronic acid from a lysozyme digest also seems to be advantageous in giving a high yield of the polymer. In contrast, a single extraction of the sacculi with Cl_3AcOH liberated only 78% of the uronic acid. Moreover, only half of this material had high molecular weight.

Peptidoglycan components accompanied the teichuronic acid during its purification. In the cell wall, the teichuronic acid and peptidoglycan may indeed be covalently linked, and when the glycan structure is broken down by lysozyme fragments of the peptidoglycan remain attached to the teichuronic acid.

From its behavior on gel filtration (Sephadex G-200 and Sepharose 4B), the molecular weight of the teichuronic acid seems to be high ($\sim 10^6$). This may be a correct estimate, but some other possibilities need to be considered. The exclusion limits of gel columns are usually established with dextrans that are roughly spherical molecules. Because of its negative charge at neutral pH values, the teichuronic acid may be a linearly extended molecule which might behave on gel filtration like a spherical molecule of much higher molecular weight. Another possibility is that polymer molecules of teichuronic acid may be joined together by lengths of peptidoglycan to give structures larger than the teichuronic acid itself. However, it seems unlikely that many more than two or three teichuronic acid molecules would be attached to a single piece of peptidoglycan. Most of the peptidoglycan of the cell wall (roughly 80%) is liberated by lysozyme without any attached teichuronic acid. If the remaining 20% of the peptidoglycan were liberated in the form of high-molecular-weight fragments, each of which carried numerous relatively small molecules of teichuronic acid, then this would imply that the teichuronic acid was located densely in limited areas, rather than randomly in the cell wall. Without evidence to the contrary, it is more reasonable to suppose that the molecules of teichuronic acid are evenly distributed in the wall and thus will not often be linked together by peptidoglycan when isolated after lysozyme digestion.

When teichuronic acid was extracted by treatment of sacculi with Cl_3AcOH solutions, it was hoped that the links between the teichuronic acid and the peptidoglycan might be broken. In fact, the teichuronic acid obtained as high-molecular-weight material after Cl_3AcOH extraction still contained some peptidoglycan, though only about one-third as much as was present in teichuronic acid isolated after lysozyme digestion of sacculi.

The persistence of the high molecular weight of the teichuronic acid, while peptidoglycan content was decreased, suggests that the teichuronic acid polymer does itself have high molecular weight.[2]

Because the teichuronic acid gave no precipitate with concanavalin A, and because the uronic acid was, after acid hydrolysis of the polymer, associated with rhamnose more than glucose, much of the teichuronic acid may have the structure

$$\text{glucuronic acid} \xrightarrow{1,?} (\text{rhamnose}) \xrightarrow[1 \text{ or } 2]{?} \text{glucose}$$

In its carbohydrate content, the teichuronic acid is similar to the type II capsular polysaccharide of *Pneumococcus* (Sutherland, 1972), though structurally the two polymers may be different. If the teichuronic acid really is covalently linked to the peptidoglycan, it is better regarded as a component of the cell wall than as capsular material. However, it would be interesting to find whether the teichuronic acid is present as an outer layer at the bacterial surface with the peptidoglycan in deeper position.

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[2] More extensive digestion with lysozyme decreases the peptidoglycan content to less than 1% with no apparent change in the molecular weight of the teichuronic acid (Ivatt and Gilvarg, 1977).