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Polymer Length of Teichuronic Acid Released from Cell Walls of *Micrococcus luteus*

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Teichuronic acid released from its phosphodiester linkage to peptidoglycan in the cell walls of *Micrococcus luteus* by mild acid treatment is resolved into a ladderlike series of bands by electrophoresis on polyacrylamide gels in the presence of borate. Each band of the ladder differs from its nearest neighbor by one disaccharide repeat unit, →4)-2-acetamido-2-deoxy-β-D-mannopyranuronosyl-(1→6)-α-D-glucopyranosyl-(1–. Acid-fragmented teichuronic acid, after conversion to the phenylamine derivative, was fractionated by preparative-scale molecular sieve column chromatography, which produced a series of elution peaks. Fast-atom-bombardment mass spectrometry of the smallest member of the series determined its molecular weight and established its identity as the phenylamine derivative of one disaccharide repeat unit of teichuronic acid. Homologous fractions of the same series were used to index the ladder of bands obtained by polyacrylamide gel electrophoresis from samples containing a more extensive distribution of polymer lengths. Nearly native teichuronic acid consists of polymers with a broad range of molecular sizes ranging from 20 to 55 disaccharide units. The most abundant species are those which have 25 to 40 repeat units. Prolonged treatment of teichuronic acid with the acid conditions used to release it from peptidoglycan causes gradual fragmentation of the teichuronic acid.

The cell walls of *Micrococcus luteus* consist of teichuronic acid covalently attached to peptidoglycan (4, 10, 12, 20, 25). The teichuronic acid of this bacterium is a polymer of alternating residues of D-glucose and *N*-acetyl-D-mannosaminuronic acid (ManNAcA), which, together, constitute a disaccharide repeat unit with the structure →4)-2-acetamido-2-deoxy-β-D-mannopyranuronosyl-(1→6)-α-D-glucopyranosyl-(1– (9, 14).

The covalent attachment of teichuronic acid to peptidoglycan is through a phosphodiester (10, 17). It is generally accepted that phosphate is esterified to the C-6 hydroxyl of a portion of the muramic acid residues of peptidoglycan, although some of the phosphate may be esterified to the C-6 position of glucosamine residues (15, 22). The second ester of phosphate serves as the point of attachment of the reducing end residue of the teichuronic acid which has been reported to be *N*-acetylglucosamine (10) or glucose (20, 21). We recently determined that the reducing end residue is *N*-acetylglucosamine (7), in accordance with the sequence of residues predicted by the order of incorporation of saccharides during teichuronic acid biosynthesis (13, 26).

The length of teichuronic acid polymer chains has not been established, although compositional analysis of cell walls gave a ratio of 10 mol of glucose per mol of phosphate (4). In vitro studies of teichuronic acid biosynthesis indicated that the degree of glucose incorporation relative to the reducing terminal residue *N*-acetylglucosamine was about 40 (27). Nasir-ud-Din and Jeanloz (20) also reported a chain length of 40 disaccharide residues.

We used the method developed for fractionation of hyaluronate oligo- and polysaccharides (19, 29) to determine the size distribution of teichuronic acid polymers. Application of these methods demonstrated that the conditions of mild acid hydrolysis, normally used to cleave the phosphodiester and release teichuronic acid from cell walls, simultaneously caused gradual fragmentation of teichuronic acid.

MATERIALS AND METHODS

Polyacrylamide gel electrophoresis. Slab gels (135 or 270 by 164 by 0.75 mm) were prepared with acrylamide (15 g), bisacrylamide (0.44 g), ammonium persulfate (40 mg), and *N,N,N',N'*-tetramethylethylenediamine (TEMED) (40 μl) in 100 ml of Tris-borate buffer (0.2 M Tris base, 0.2 M boric acid, 2.0 mM EDTA) (19). Samples were mixed with 0.2 volumes of Tris-borate buffer containing 2.0 M sucrose to increase sample density. Portions (1 to 5 μl) of the resulting mixtures, containing 1 to 3 μg of teichuronic acid, were loaded into sample wells with a 10-μl Hamilton syringe. The tracking dye was bromphenol blue (1 mg/ml). Electrophoresis was performed at 10 to 15 mA per gel until the tracking dye was 2 to 4 cm from the bottom of the gel. The apparatus was cooled with circulating tap water (10°C).

The gel-staining procedure was adapted from methods described previously (18, 19). Following electrophoresis and removal from the glass plate sandwich, each gel was gently agitated in an aqueous solution of alcian blue (1 mg/ml) for 30 min. The dye solution was removed by aspiration, and the gel was washed several times in deionized water and sometimes allowed to destain in water overnight. The gel was treated for 7 min in 3.4 mM potassium dichromate–3.2 mM nitric acid and again washed several times in deionized water. The gel was soaked with gentle agitation in 12 mM silver nitrate under bright illumination (30 to 40 cm from a 100-W incandescent light bulb) for 25 min. Following aspiration of the silver solution and several quick washes with deionized water, the image was developed on the gel by three very brief treatments with 0.28 M sodium carbonate containing 6 mM formaldehyde. The first treatment solution turned brown almost immediately and was removed as quickly as possible. The second treatment solution was also removed almost as soon as it had covered the gel. The third

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treatment solution was allowed to remain in contact with the gel while the image intensified, but was quickly removed as soon as the background of the gel began to darken. Immediately, the gel was flooded with 0.1 M acetic acid to stop the development process.

Release of teichuronic acid from cell walls. Cell walls prepared by grinding cells of *M. luteus* with glass beads (7) were suspended at 8 mg/ml in 50 mM HCl and treated at 90°C for 5 to 60 min. Following neutralization, cell wall residue was removed by centrifugation, after which the supernatant fraction was dialyzed against water and lyophilized. Short acid treatment times released teichuronic acid in its most nearly native form, whereas longer treatment increased the amount which could be recovered (maximum of about 200 mg from 1 g of cell walls) but decreased its purity. Large-scale samples released by 0- to 30- and 30- to 60-min treatments with acid were essentially devoid of peptidoglycan constituents as determined by proton nuclear magnetic resonance spectroscopy (8), whereas gradually increasing amounts of peptidoglycan were detected in samples released from cell walls by more extensive acid hydrolysis. Samples prepared solely for electrophoresis were analyzed without dialysis or freeze drying.

Fragmentation of teichuronic acid. Teichuronic acid was hydrolyzed in 50 mM HCl at 90°C at a concentration of 12.5 mg of teichuronic acid per ml for various periods up to 2 h. Time course aliquots were neutralized immediately after removal from the reaction mixture.

Formation of phenylamine derivatives of saccharides. Reducing saccharides were derivatized by a modification of the procedure of Wang et al. (30) to introduce a UV-absorbing group. Teichuronic acid, teichuronic acid fragments, and authentic mono- or oligosaccharides (1 to 100 mg) each dissolved in 0.9 ml of 0.33 M potassium phosphate buffer (pH 6.5) were reacted in screw-cap culture tubes with 2.1 ml of a solution of aniline (0.8 ml) and sodium cyanoborohydride (380 mg) in methanol (3.0 ml) at 80°C for 60 min. After cooling, 6 ml of water was added and the solution was extracted four times each with 6 ml of chloroform. Derivatives of authentic oligosaccharides were desalted by passage through a Bio-Gel P-2 column (Bio-Rad Laboratories) developed with deionized water. Phenylamine derivatives of carbohydrates were detected by their A_{240}.

Preparative fractionation of teichuronic acid fragments. A preparation of the phenylamine derivatives of the teichuronic acid fragments (100 mg) was applied in a 1.5-ml volume to a Bio-Gel P-30 column (2.5 by 195 cm) which had been equilibrated with 0.25 M triethylammonium bicarbonate. The flow rate was maintained at 0.4 ml/min by a Buchler polystaltic pump. Fractions were collected over 48 h. After monitoring the fractions collected at 240 nm, appropriate fractions constituting elution peaks were pooled, lyophilized, suspended in a small volume of water, lyophilized again, and recovered in 1.0 ml of water.

HPLC analysis. The high-pressure liquid chromatography (HPLC) system consisted of a Beckman model 421 system controller, two model 114M pumps, model 340 solvent mixer, 20-μl injector, guard column (containing TSK-DEAE-SW guard gel) and TSK-DEAE-2SW column (4.6 by 250 mm), a variable-wavelength detector, and a Hewlett-Packard 3396A integrator. The flow rate was 0.6 ml/min. The anion-exchange column was equilibrated with 15 mM sodium sulfate. At 1 min after injection the system began a 20-min linear ramp to 150 mM sodium sulfate, which was followed immediately by an additional 15-min linear ramp to 250 mM sodium sulfate. Detection was by monitoring A_{240}.

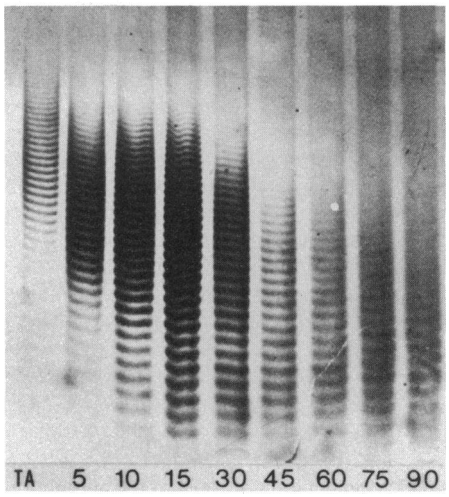

FIG. 1. Release of teichuronic acid from cell walls. Cell walls (8 mg/ml) were treated with 50 mM HCl at 90°C to release teichuronic acid. Aliquots removed at the indicated time intervals (minutes) were neutralized and then centrifuged to remove residual walls. The supernatant solutions were subjected to polyacrylamide gel electrophoresis (135-mm gel). The figure shows the portion of the gel which extended from 45 to 130 mm (as measured from the bottom of the sample well, which is defined as 0 mm). Bands were visualized by fixation with alcian blue followed by photochemical silver staining. TA is a preparation of teichuronic acid recovered from the soluble fraction after a 10-min acid treatment of cell walls.

Mass spectrometry. Fast-atom-bombardment mass spectrometry (5) was performed by using a Kratos MS25 mass spectrometer equipped with an Icon Tech fast-atom bombardment saddle field source using xenon gas. Underivatized saccharides gave weak signals. Therefore, to increase the signal, portions of some samples were converted to the methyl esters by bubbling diazomethane generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald; Aldrich Chemical Co.) through the sample in the protonated form dissolved in 50 μl of dry dimethyl sulfoxide. Alternatively, to effect peracetylation, 1- to 50-μg samples freeze-dried in Reacti-Vials (Pierce Chemical Co.) were reacted for 2 h at 37°C with 50 μl of a freshly prepared mixture of acetic anhydride (1.0 ml), glacial acetic acid (0.05 ml), and 1-methylimidazole (0.10 ml) (3).

RESULTS

Release of teichuronic acid from cell walls. Teichuronic acid released from purified cell walls by treatment with dilute acid was analyzed by electrophoresis on polyacrylamide slab gels. Treatment of the gels with alcian blue followed by silver staining revealed a ladderlike series of bands in which successive bands were separated by highly regular intervals (Fig. 1). Since polyacrylamide gel electrophoresis effects a separation based on charge and molecular size, and since teichuronic acid consists of two monosaccharide residues in a strictly alternating sequence, each band must differ from each of its nearest-neighbor bands by one disaccharide repeat unit. Results presented below support this conclusion.

Increased duration of acid treatment used to release teichuronic acid from walls gave banding patterns in which the distribution of bands was shifted toward those of shorter polymer length. The teichuronic acid liberated after a 20-min

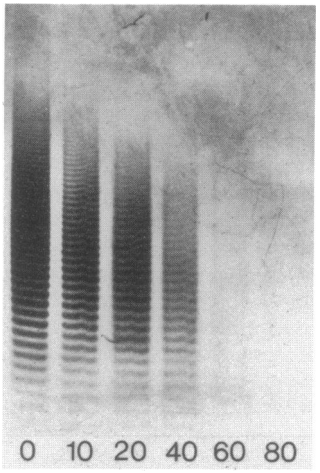

FIG. 2. Acid fragmentation of isolated teichuronic acid. Teichuronic acid recovered following release from cell walls by 10 min of acid treatment was additionally treated at a concentration of 12.5 mg/ml with 50 mM HCl at 90°C for the indicated time intervals (minutes). Aliquots were subjected to polyacrylamide gel electrophoresis (135-mm gel). The figure shows the portion of the gel which extended from 35 to 115 mm. Sample components were detected by fixation with alcian blue followed by silver staining.

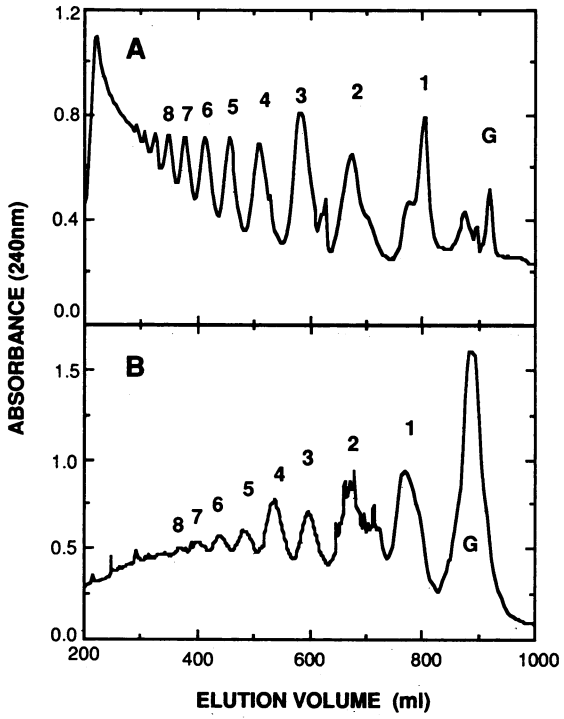

FIG. 3. Fractionation of acid-fragmented teichuronic acid. Teichuronic acid released from cell walls by 90 to 120 min of acid treatment (100 mg; 12.5 mg/ml) was treated with 50 mM HCl at 90°C for 60 min (A) or 90 min (B). Each digest was derivatized by reaction with aniline and fractionated on a Bio-Gel P-30 column (2.5 by 190 cm) equilibrated and developed with 0.25 M triethylammonium bicarbonate (pH 7.5).

treatment was distinctly shorter on average than that released in the initial 5 min (Fig. 1). After 40 min of treatment the amount of teichuronic acid detected was substantially diminished and consisted of significantly shorter chain lengths. These results suggest that the acid treatment used to cleave the phosphodiester linkage by which teichuronic acid is attached to peptidoglycan also effects a significant cleavage of glycosidic bonds within the teichuronic acid.

Mild acid treatment fragments teichuronic acid. To confirm that glycosidic bonds were being hydrolyzed by the mild conditions used to release teichuronic acid from cell walls, a preparation of teichuronic acid already released from cell walls by 10 min of acid treatment was subjected to the same conditions of acid treatment and aliquots were removed over time for analysis by polyacrylamide gel electrophoresis. The teichuronic acid was gradually converted to shorter chain lengths (Fig. 2). Whereas the initial sample contained about 40 bands, samples removed later showed additional bands of shorter polymer length and a striking diminution of the bands of longer polymer lengths. By 80 min, the majority of the material was of short polymer lengths and very long polymers were no longer detectable. The decrease in total observable material is the consequence of extensive teichuronic acid fragmentation which produces fragments too short to be detected by the alcian blue and silver staining procedure.

Preparative fractionation of teichuronic acid. Reducing end groups of the teichuronic acid from digests like those described above were derivatized with aniline to facilitate detection of the material during preparative fractionation on a column of Bio-Gel P-30. Figure 3 shows the elution profiles of teichuronic acid which was acid fragmented for 60 min (Fig. 3A) and for 90 min (Fig. 3B). The uniformity of the size and position of the clearly resolved elution peaks suggests that the successive major peaks (identified by numbers) contain the phenylamine derivatives of teichuronic acid fragments which differ by the number of disaccharide repeat units, i.e., $(ManNAcA\text{-}Glc)_n\text{-}NH\text{-}\phi$. This series of fragments is generated by the random cleavage of α-D-glucosidic bonds. For convenience this major series of fragments is designated the normal or N series (see Discussion for alternate series of fragments).

Samples from the major elution peaks of Fig. 3A were analyzed by polyacrylamide gel electrophoresis (Fig. 4). Only the teichuronic acid fragments longer than the minimum detectable length were observed by the alcian blue- and silver-staining procedure. Material from peak 5 was the shortest that could be detected. A different major band predominated in each of peaks 5 through 10 as detected by polyacrylamide gel electrophoresis. The ladderlike progression of predominant bands correlated with the sequence of peaks observed in the Bio-Gel P-30 elution profile. As the resolution between major elution peaks from the Bio-Gel P-30 column diminished, an increasing overlap of bands was observed on polyacrylamide gels. The material which eluted in the excluded volume from the Bio-Gel P-30 column contained the long teichuronic acid chains as shown by the extensive ladder of bands detected by polyacrylamide gel electrophoresis (data not shown).

In addition to the major bands observed on the polyacrylamide gels, several minor bands were clearly evident (Fig. 4) with higher and lower mobilities than the major bands. These minor bands result from alternate fragmentation of teichuronic acid by cleavage of *N*-acetyl-β-D-mannosaminiduronate bonds, the presence of the unique linkage oligosaccharide at the reducing end of some fragments, and/or segments of peptidoglycan which contaminate the preparation.

HPLC analysis of phenylamine derivatives of teichuronic acid fragments. Pooled and desalted fractions corresponding to the peaks observed in the Bio-Gel P-30 column elution

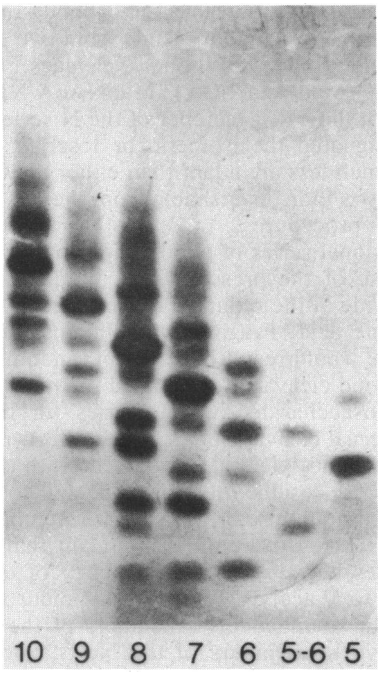

FIG. 4. Polyacrylamide gel electrophoresis of Bio-Gel P-30 column fractions. Bio-Gel P-30 column fractions from the experiment depicted in Fig. 3 were concentrated by lyophilization and redissolved in 0.1 volume of water. The polyacrylamide gel was 270 mm long. Electrophoresis was continued until the tracking dye had migrated for 210 mm. The figure shows only the portion of the gel which was 150 to 250 mm from the bottom of the sample wells. Sample components were detected by fixation with alcian blue followed by silver staining. Sample lanes are identified by the numbers of the corresponding peaks shown in Fig. 3B. The lane identified as 5–6 is a pool of the material in fractions which eluted between peaks 5 and 6.

profiles were examined by HPLC on a TSK-DEAE-2SW column developed with a linear concentration gradient of sodium sulfate. This anion-exchange column fractionated teichuronic acid fragments according to total charge and therefore also according to polymer length. Table 1 shows a comparison of the HPLC retention times of fractions from the Bio-Gel P-30 column as well as selected authentic compounds. Material from peak G was not retained by the TSK-DEAE-2SW column and coeluted with authentic phenylamine derivative of glucose. Material from peaks 1 through 5 was retained on the TSK-DEAE-2SW column and was eluted with successively longer retention times (Table 1). Mixtures of comparable peaks from Fig. 3A and B gave identical retention times from the TSK-DEAE-2SW column. The shortest teichuronic acid fragments behaved analogously to the phenylamine derivatives of the di-, tetra-, and hexasaccharides obtained from a hyaluronidase digest of hyaluronic acid. Most of the teichuronic acid samples displayed a number of minor components on HPLC (each 5 to 20% of total sample absorbance), which undoubtedly are the minor bands observed on polyacrylamide gel electrophoresis gels. Comparison of the elution profiles of samples taken from the center of the Bio-Gel P-30 major peaks with samples taken from the valleys between major peaks permitted easy recognition of the components which were of the N series of fragments (retention times are given in Table 1). HPLC analysis was especially useful since it provided analytical resolution of the short-chain fragments which could not be detected on polyacrylamide gels. Some of the shortest fragments which could be detected on polyacrylamide gels were also resolved by the TSK-DEAE-2SW column, thus providing an overlap of the analytical range of the two procedures.

TABLE 1. HPLC retention times of teichuronic acid oligosaccharides on TSK-DEAE-2SW

Identifier	Compound	Retention time (min)
Reference	Glc-NH-ø	6.6
Reference	GlcA-GlcNAc-NH-ø[a]	7.9
Reference	$(GlcA\text{-}GlcNAc)_2$-NH-ø[a]	9.9
Reference	$(GlcA\text{-}GlcNAc)_3$-NH-ø[a]	12.6
Peak G	Glc-NH-ø	6.6
Peak 1	ManNAcA-Glc-NH-ø	9.6
Peak 2	$(ManNAcA\text{-}Glc)_2$-NH-ø	11.8
Peak 3	$(ManNAcA\text{-}Glc)_3$-NH-ø	15.7
Peak 4	$(ManNAcA\text{-}Glc)_4$-NH-ø	19.2
Peak 5	$(ManNAcA\text{-}Glc)_5$-NH-ø	22.1

[a] The phenylamine derivatives of the di-, tetra-, and hexasaccharides derived from hyaluronate by digestion with hyaluronidase (6). Because hyaluronidase is an eliminase, the nonreducing terminal residue of each hyaluronate oligosaccharide contains a 4,5-double bond.

Molecular weight verification of teichuronic acid fragments. Comparison of the Bio-Gel P-30 column elution profiles of teichuronic acid treated with mild acid for 60 min (Fig. 3A) with that treated for 90 min (Fig. 3B) showed the same series of major fragments, although the former sample was substantially enriched in the long-chain fragments (eluted at the column excluded volume), whereas the 90-min fragment contained about four times as much of the phenylamine derivative of glucose. Although the more extensive acid treatment released more glucose, it did not give rise to additional elution peaks of the N series, thereby indicating that the last major elution peak (peak 1) is the phenylamine derivative of the disaccharide. Furthermore, this component had an HPLC retention time on the TSK-DEAE-2SW column quite comparable to that of the phenylamine derivative of the disaccharide derived from hyaluronic acid.

To verify the identification of material in peak 1 as the phenylamine derivative of the teichuronic acid disaccharide repeat unit (ManNAcA-Glc-NH-ϕ), fast-atom-bombardment mass spectrometry was used to determine the molecular weight. Material from peak 1 in the acid form gave $m/z = 475$ for the $M + H^+$ ion. The methyl ester gave $m/z = 489$. Since derivatized materials are detected with substantially greater sensitivity, material from peak 1 was peracetylated, after which $m/z = 769$ ($M + H^+$) and 791 ($M + Na^+$) were major signals in the mass spectrum. The mass increase of 294 is indicative of the addition of seven acetyl groups corresponding to O-acetylation at C-2, C-3, C-4 and C-5 of glucose and C-3 and C-4 of ManNAcA, as well as *N*-acetylation of the secondary amine.

DISCUSSION

Teichuronic acid is a structural analog of hyaluronic acid, so it is expected that the electrophoretic system that separates the homologous series of hyaluronate oligomers and polymers into a ladderlike series of bands (19, 29) would have the same separation capability with a series of teichuronic acid oligomers and polymers. Similar electrophoretic separations resulting in ladders of bands were reported

recently for the acidic capsular polysaccharides of *Escherichia coli* K1 and K5, as well as several other bacteria (24). Hyaluronate oligomers must have a minimum of 10 repeat units to bind alcian blue as a prerequisite to detection on gels (29), but we have been able to detect teichuronic acid oligomers having as few as 5 repeat units.

The strict alteration of residues of glucose and *N*-acetylmannosaminuronic acid which was indicated by the simplicity of the natural abundance ^{13}C nuclear magnetic resonance spectrum of teichuronic acid (14) is confirmed by the uniformity of the progression of bands in the polyacrylamide gel electropherogram banding patterns. That each band in the ladder of bands represents material differing from its nearest-neighbor bands by one disaccharide repeat unit has been demonstrated by preparative fractionation of fragmented teichuronic acid on Bio-Gel P-30, resulting in clearly resolved elution peaks, each of which contains a unique band of the polyacrylamide gel electropherogram ladder. Furthermore, the lower members of the series behave analogously to the hyaluronate oligosaccharides on an HPLC anion-exchange column.

The samples released from cell walls by brief acid treatment contain many bands which indicates polydispersity in the teichuronic acid fraction recovered. Undoubtedly, native teichuronic acid has a range of polymer chain lengths, but some of the polydispersity observed is the consequence of fragmentation which occurs concomitantly with release of teichuronic acid from the cell wall. All conditions tested for cleavage of the phosphodiester (i.e., the bond by which the teichuronic acid is attached to peptidoglycan [7]) also show evidence of some simultaneous teichuronic acid fragmentation. Such fragmentation in mild acid is expected to involve cleavage of α-D-glucosidic bonds preferentially to *N*-acetyl-β-D-mannosaminiduronic acid bonds because of the preferential resistance of the glycosides of uronic acids (1, 2, 16).

The multiplicity of minor bands seen both in polyacrylamide gel electropherograms and in HPLC elution traces are a severe complication to the chain length analysis. The presence of minor components has been observed not only in the electrophoretic analysis of fractionated teichuronic acid fragments but also in unfractionated material separated on double-length gels, in which the extensive separation between bands of short fragments permitted obvious resolution of a few of the minor components. Minor bands have been observed both ahead and behind the major bands of the N series. Because long members of the N series are less well resolved, minor components are not apparent in this region of the electropherogram, although it is also possible that the relative abundances of long fragments of the minor series are quite low.

Some of the minor bands can be attributed to contaminating peptidoglycan. Other minor bands may arise from low-frequency random cleavage of *N*-acetyl-β-D-mannosaminiduronic acid bonds of teichuronic acid, giving rise to fragments with either an additional glucose residue at the nonreducing end or an additional ManNAcA residue at the reducing end of the fragment, resulting in two series of fragments. The former are represented by Glc-(ManNAcA-Glc)$_n$-NH-ϕ and can be referred to as the glucose or G series, whereas the latter are represented by (ManNAcA-Glc)$_n$-ManNAcA-NH-ϕ, which are known as the ManNAcA or M series.

The members of the G and M series are expected to be generated with a lower frequency than members of the N series, since both G and M series fragments result from cleavage of one *N*-acetyl-β-D-mannosaminiduronic acid bond as well as one α-D-glucosidic bond. In the rare instances in which a fragment results from two *N*-acetyl-β-D-mannosaminiduronic acid bond cleavages, the fragment would be Glc-(ManNAcA-Glc)$_n$-ManNAcA-NH-ϕ, which is isomeric with the $n + 1$ member of the N series. This series may be designated the isomeric or I series. The I series should be much less abundant than either the G or M series and probably is indistinguishable from the N series by most fractionation procedures.

Two additional series of minor fragments are expected to arise because of the presence of a unique linkage region oligosaccharide at the reducing end of each native molecule of teichuronic acid. Fragments containing the linkage oligosaccharide would result either from random internal α-D-glucosidic bond cleavage (designated the normal series with linkage oligosaccharide, or NL series) or from the less frequent internal cleavage of an *N*-acetyl-β-D-mannosaminiduronic acid bond, yielding a fragment with a nonreducing terminal glucose residue (designated the glucose series with linkage oligosaccharide, or GL series). Members of the NL series are expected to be more abundant than members of the GL series because of the frequency of α-D-glucosidic with respect to *N*-acetyl-β-D-mannosaminiduronic acid bond cleavage. The abundance of members of the NL and GL series relative to members of the N, G, and M series is expected to decrease throughout the acid treatment period as internal fragmentation yields additional members of the N, G, M, and I series. All series are expected to be populated by successively shorter members as fragmentation continues during prolonged acid treatment.

At present it is not known which minor bands correspond to which minor series of fragments, but the G, M, and NL series are the most likely to occur at detectable concentrations. Because of the slight mass increase without additional charge, the G series would be expected to display a slightly slower electrophoretic mobility than the corresponding members of the N series. Following the same reasoning, the increased charge of the M and NL series would give rise to a slightly higher mobility than that of the corresponding members of the N series.

Evidence for two different nonreducing terminal residues was obtained from in vitro biosynthesis experiments with wall membrane preparations, which showed that only 25% of endogenous acceptors had terminal *N*-acetylmannosaminuronosyl residues (28). Therefore, some GL-series components may be native teichuronic acid polymers which have a nonreducing terminal glucosyl residue, whereas others may arise by fragmentation through the cleavage of *N*-acetyl-β-D-mannosaminiduronic acid bonds. Some G-series components may arise from the GL series by fragmentation involving cleavage of α-D-glucosidic bonds.

The mild acid treatment required to release teichuronic acid from its phosphodiester linkage to peptidoglycan in cell walls allows the preparation of a soluble polymer fraction amenable to electrophoresis; the possibility of polymer fragmentation, however, increases with the duration of acid treatment. The conditions we used for selective release of teichuronic acid from cell walls are quite similar to those reported by Pavlik and Rogers (pH 3 for 20 min at 100°C) (23). Although they reported no internal fragmentation of the polymer, our methods of assessing fragmentation are far more sensitive and clearly establish that some fragmentation does occur. Therefore, it is difficult to determine with certainty the actual distribution of native polymer lengths. We have shown that the rate of fragmentation is substantially lower than the rate of release, so samples obtained

from short acid treatment should closely approximate native chain length distribution. It is apparent that teichuronic acid is polydisperse with chain lengths ranging from 20 to 55 disaccharide repeat units.

The determination of polymer chain length as described in this paper can be used to evaluate the extent to which teichuronic acid chain length varies as a function of culture growth conditions and nutritional status. For mutants which do not produce the usual amount of teichuronic acid (31) the determination of polymer chain length may aid in distinguishing between loss of polymer initiation enzymes or loss of polymer elongation activities. This methodological approach should also be applicable to the evaluation of chain length distribution of any polyanion composed of a strictly repeated monomeric unit. The accompanying paper makes use of this methodology to show that incubation of UDP-glucose, UDP-ManNAcA, and acceptor teichuronic acid with solubilized enzymes from cytoplasmic membrane fragments leads to a clearly demonstrable increase in the chain length of the teichuronic acid (11).

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