The Cell Wall of *Bacillus licheniformis* N.C.T.C. 6346

LINKAGE BETWEEN THE TEICHURONIC ACID AND MUCOPEPTIDE COMPONENTS

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1. After extraction of teichoic acid from cell walls of *Bacillus licheniformis* with dilute alkali, the insoluble residue contains the teichuronic acid and mucopeptide components and a small amount of residual phosphorus. 2. A complex of teichuronic acid and a part of the mucopeptide was isolated from the soluble fraction obtained by lysozyme treatment of alkali extracted walls. 3. Small-molecular-weight mucopeptide fragments, not containing teichuronic acid, are obtained from the soluble fraction in yields similar to those obtained after treatment of whole walls or acid-extracted walls with lysozyme. 4. The covalent linkages between teichuronic acid and mucopeptide are broken by treatment with dilute acid. The release of teichuronic acid chains is accompanied by the hydrolysis of *N*-acetylgalactosaminide linkages and the exposed *N*-acetylgalactosamine residues form chromogen under very mild conditions, indicating that they are substituted on C-3. 5. The initial rate of formation of reactive *N*-acetylgalactosamine residues during mild acid hydrolysis is parallel to the rate of extraction under the same conditions of teichuronic acid from alkali-treated insoluble walls, and to the rate of acid hydrolysis of glucose 1-phosphate. 6. The results suggest that the teichuronic acid chains are attached through reducing terminals of *N*-acetylgalactosamine residues to phosphate groups in the mucopeptide. 7. Muramic acid phosphate was isolated from the insoluble mucopeptide remaining after extraction of walls with dilute alkali followed by dilute acid.

Recent work has shown that covalent linkages exist between the teichoic acids and polysaccharides of several bacterial cell walls and the mucopeptide component (Knox & Hall, 1965; Hay, Archibald & Baddiley, 1965; Button, Archibald & Baddiley, 1966; Munoz, Ghuyssen & Heymann, 1967; Knox & Holmwood, 1968). In an earlier study (Hughes, 1965) of the cell wall of *Bacillus licheniformis* N.C.T.C. 6346 evidence for a firm attachment of the teichoic acid to the mucopeptide polymer was found. However, no direct evidence was obtained for the polysaccharide, teichuronic acid, that is also present in walls of this organism grown in batch culture (Janczura, Perkins & Rogers, 1961). Teichuronic acid is a polymer made up of glucuronic acid and *N*-acetylgalactosamine units, and accounts for approx. 18% of the dry weight of walls prepared from cells growing exponentially. Recently it was shown that teichuronic acid may account for about one-half of the dry weight of walls isolated from *Bacillus subtilis* var. *niger* and *Bacillus subtilis* W23 grown in continuous culture in phosphorus-limited medium. Under these growth conditions

the walls contained no teichoic acid (Ellwood & Tempest, 1969).

Extraction of *B. licheniformis* walls with dilute alkali selectively removes teichoic acid, and the teichuronic acid remains in the insoluble residue with the mucopeptide component (Hughes & Tanner, 1968). Using material prepared by this technique, I have found that teichuronic acid is indeed covalently bound to mucopeptide. A part of this work has appeared in a preliminary form (Hughes, Pavlik, Rogers & Tanner, 1968).

MATERIALS AND METHODS

Cell walls. *B. licheniformis* N.C.T.C. 6346 was grown into early stationary phase, as described by Janczura *et al.* (1961), except that the medium used to grow the cells also contained [32 P]phosphate (0.1 μ Ci/ml). The radioactive phosphate was carrier-free inorganic [32 P]phosphate dissolved in dil. HCl (The Radiochemical Centre, Amersham, Bucks., U.K.) and 1 mCi was diluted in 0.1 M-KH₂PO₄-NaOH, pH 7.0 (50 ml), before addition to the medium. Cell walls were prepared by differential centrifugation, after disruption of the cells by shaking with glass

beads in a Braun homogenizer fitted with a cooling device. The walls were heated at 100°C for 20 min at an early stage of preparation to inactivate autolytic enzymes. The walls were washed extensively with 0.1 M-KH₂PO₄-NaOH buffer, pH 7.0, followed by water, and freeze-dried.

Analytical methods. Glucuronic acid, phosphorus and total hexosamine were determined as previously described (Hughes, 1965). Glucose was measured either with the anthrone reagent or with glucose oxidase (Bergmeyer & Bernt, 1963). Mixtures of glucosamine and muramic acid were separated by adsorption of muramic acid on to charcoal in a batch modification (Hughes, 1968a) of the method of Perkins & Rogers (1959). When present in mixtures, galactosamine was eluted with glucosamine from charcoal by water. The relative proportions of the two amino sugars in the aqueous extract were determined by the method of Good & Bessman (1964). Amino acids were determined after acid hydrolysis by quantitative paper chromatography (Mandelstam & Rogers, 1959). Diaminopimelic acid was estimated by the Work (1957) method.

Paper chromatography and electrophoresis. Descending paper chromatography on Whatman no. 3 or 3MM paper was done in the following solvent systems: solvent I, butan-1-ol-pyridine-water (6:4:3, by vol.); solvent II, butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase); solvent III, isobutyric acid-0.5 M-NH₃ (5:3, v/v). High-voltage electrophoresis was carried out on Whatman no. 3 or 3MM paper in the following solvent systems: solvent IV, 0.1 M-pyridine-acetate buffer, pH 3.6; solvent V, 0.1 M-pyridine-acetate buffer, pH 5.1. Electrophoresis was carried out at 2000 V for 3 h. After thorough drying at 40°C, the papers were developed with an Elson-Morgan reagent for hexosamines, alkaline AgNO₃ for sugars, ninhydrin for amino acids and amino sugars, or the starch-iodide reagent of Rydon & Smith (1952) for substituted amides. In each case a dip technique was used as recommended by Smith (1960).

Alkaline extraction of walls. The procedure of Hughes & Tanner (1968) was used. In a typical experiment, ³²P-labelled walls (508 mg, equivalent to 522 μmol of phosphorus, specific radioactivity 4570 c.p.m./μmol) were suspended in 0.1 M-sodium phosphate buffer, pH 8.0 (100 ml), containing sodium borohydride (20 mg) and the reduction of available reducing groups in the walls was carried out at 35°C for 5 h in an atmosphere of nitrogen. Then 0.2 M-NaOH (100 ml) containing sodium borohydride (20 mg) was added and the suspension was rapidly stirred at 35°C in an atmosphere of nitrogen. Samples (2.0 ml) of the suspension were removed at times and centrifuged. A portion (1.5 ml) of each supernatant solution was neutralized with HCl and diluted to 2.5 ml. The insoluble residues were washed with water by centrifugation and suspended in water (2.5 ml). Portions of the soluble and insoluble fractions were analysed for glucuronic acid and for radioactivity. Portions were also hydrolysed in 6 M-HCl and examined for amino acids by paper chromatography in solvents I and II. The amounts of diaminopimelic acid present in the acid hydrolysates were measured colorimetrically (Work, 1957). After a total extraction time of 40 h the suspension of walls was centrifuged. The insoluble residue was washed with water by centrifugation and finally suspended in water and freeze-dried. The yield was 195 mg. Allowing for material removed for analysis, this

represents approx. 43% of the weight of walls present originally.

Column chromatography. Chromatography on columns (2 cm × 140 cm) of Sephadex G-50 was carried out in 0.1 M-pyridine-acetate buffer, pH 5.1. Fractions (3.0 ml) were collected and portions were analysed for various constituents. Ion-exchange chromatography was performed on columns (2 cm × 40 cm) of DEAE-cellulose in pyridine-acetate buffer, pH 5.1. The washed resin (Whatman grade DE-11) was equilibrated with 0.01 M-pyridine-acetate buffer, pH 5.1, before preparation of the columns. The approximately linear concentration gradient used for elution was established by running 5.0 M-pyridine-acetate buffer, pH 5.1, into the mixing chamber containing 0.01 M-pyridine-acetate buffer, pH 5.1 (1 litre). Fractions (5.0 ml) were collected and analysed without prior removal of buffer salts. Peak fractions from the columns were combined and buffer salts were removed by freeze-drying.

Chromogen formation. A 3-O-substituted *N*-acetylhexosamine readily forms a Morgan-Elson chromogen on heating at pH 7.0 and 100°C for 45 min (Knox & Hall, 1965). This test was used to detect the 3-O-substituted *N*-acetylglactosamine residue at the free reducing terminals of teichuronic acid chains. The yield of chromogen from such a residue is 18 times that produced under the same conditions by *N*-acetylglactosamine and eight times that for free *N*-acetylglucosamine (Knox & Hall, 1965). Samples (0.1 ml) of teichuronic acid-mucopeptide complex or pure teichuronic acid containing approx. 1–2 μmol each of galactosamine or glucuronic acid equivalents were added to tubes containing 0.25 M-KH₂PO₄-NaOH buffer, pH 7.0 (0.1 ml), and water (0.3 ml). The tubes were heated at 100°C for various times, usually 45 min, and rapidly cooled in ice-water. Then acetic acid (3.5 ml) and the Morgan-Elson reagent (Gardell, 1958) (0.5 ml) were added. The tubes were kept at 37°C for exactly 20 min and the extinctions of the solutions were read at 550 nm. Control tubes containing *N*-acetylglactosamine (0.1 ml, 0.18 μmol), 0.25 M-KH₂PO₄-NaOH buffer, pH 7.0 (0.1 ml), and water (0.3 ml) were treated in the same way.

Radioactivity counting. The radioactivity of soluble samples dissolved in water (0.5 ml) was counted in 10 ml of dioxan containing naphthalene (18%, w/v), 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%) in a Packard Tri-Carb liquid-scintillation spectrometer.

Lysozyme treatment of alkali-extracted walls. Insoluble walls (181 mg) were suspended in 0.1 M-ammonium acetate buffer, pH 6.5, at a final concentration of 5 mg/ml. Egg-white lysozyme (Armour Corp., Eastbourne, Sussex, U.K.) (5 mg) was added and the mixture was stirred overnight at 35°C. The insoluble residue remaining after lysozyme treatment was removed by centrifugation and washed extensively with water. Supernatants from the centrifugations were combined and freeze-dried repeatedly to remove the bulk of the ammonium acetate.

Preparation of teichuronic acid. Walls of *B. licheniformis* were extracted with 5% (w/v) trichloroacetic acid at 35°C for 3 days. Teichuronic acid was separated from teichoic acid by chromatography of the acid extracts on DEAE-cellulose. The product obtained was purified further by chromatography on a column (2 cm × 140 cm) of Sephadex

G-50 run in 0.1M-pyridine-acetate buffer, pH 5.1. The excluded peak containing teichuronic acid was passed through a column (2cm×40cm) of Dowex 50 (X4; H⁺ form; 200–400 mesh) to prepare the free acid.

Mild acid hydrolysis of the teichuronic acid-mucopeptide complex. The rate of hydrolysis of the linkages between teichuronic acid and mucopeptide in dilute acid was measured in the following way. Samples (0.1 ml) containing 1–2 μmol of galactosamine equivalents were pipetted into test tubes. Water (0.1 ml) and 0.3M-HCl (0.1 ml) were added and the stoppered tubes were kept in a water bath at 35°C. At known times the tubes were withdrawn, cooled rapidly in ice-water, frozen in a solid CO₂-acetone mixture and freeze-dried. Water (0.4 ml) and 0.25M-KH₂PO₄-NaOH buffer, pH 7.0 (0.1 ml), were added to each tube and the modified Morgan-Elson test was applied as described above.

Control tubes containing teichuronic acid (0.1 ml, respectively 1.04 μmol and 0.92 μmol of glucuronic acid and galactosamine equivalents) were treated with 0.1M-HCl at 35°C in exactly the same way, and then analysed for their content of reactive *N*-acetylgalactosamine.

Mild acid hydrolysis of glucose 1-phosphate. 0.8 mM-glucose 1-phosphate (0.2 ml) was heated with 0.2M-HCl (0.2 ml) at 35°C. At times varying from zero to 48 h the solutions were neutralized with 0.4M-NaOH. The concentration of free glucose was measured with glucose oxidase.

RESULTS

Preparation of the teichuronic acid-mucopeptide complex

The *B. licheniformis* walls used in the greater part of the present work were ³²P-labelled and contained 3.1% of phosphorus. Treatment with sodium borohydride removed no ³²P or glucuronic acid from the walls. Subsequent exposure of the walls to 0.1M sodium hydroxide solution rapidly extracted the ³²P-labelled teichoic acid. The insoluble residue contained 3.4 μmol of phosphorus/100 mg dry wt. of material, or approx. 1.4% of the total phosphorus originally present in the walls before treatment with alkali. During the alkali extraction no uronic acid, amino sugars or diaminopimelic acid were made soluble. The soluble extracts contained free alanine derived from the ester-linked alanine of the teichoic acids. The insoluble residue remaining after alkaline treatment of walls contained approx. 73 μmol of glucuronic acid/100 mg dry wt. of material.

Lysozyme treatment of alkali-extracted walls. Alkali-extracted walls (181 mg) were treated with lysozyme. The small residue (12.8 mg) that remained after treatment contained no detectable glucuronic acid or diaminopimelic acid, indicating that all of the teichuronic and mucopeptide components had been made soluble. The rate of dissolution of the alkali-treated walls by lysozyme was approximately the same as that found with intact walls of *B. licheniformis*.

Fig. 1. Chromatography on Sephadex G-50 of the soluble fraction obtained by alkaline extraction followed by lysozyme treatment of ³²P-labelled walls of *B. licheniformis*. The soluble fraction (148 mg) containing a small amount of residual ³²P was dissolved in water (4.0 ml) and eluted from the column (2.1 cm × 140 cm) in 0.1M-pyridine-acetate buffer, pH 5.1. Fractions (3.0 ml) were collected and analysed for glucuronic acid (●), for radioactivity (---) and, after acid hydrolysis, for total hexosamines (○).

Chromatography of the lysozyme-solubilized fraction on Sephadex G-50. A sample (148 mg) of the fraction of alkali-extracted walls made soluble by lysozyme was placed on a column of Sephadex G-50. As shown in Fig. 1, glucuronic acid, as an index of the teichuronic acid component, was eluted from the column in the void volume together with material reacting in the Elson-Morgan test as hexosamine. In addition, hexosamine material was included in the gel and was eluted in a number of broad peaks (Fig. 1). The small amount of residual phosphorus present in alkali-extracted walls was eluted mainly in the peak containing teichuronic acid. No radioactive phosphorus was detected in the fractions S3 and S4 of lowest molecular size and eluted latest from the column. Fractions were pooled from the column as indicated and analysed (Table 1). Fraction S1 (74 mg) contained approximately equimolar amounts of glucuronic acid and galactosamine and accounted for 96% of the glucuronic acid recovered from the column. The mucopeptide constituents, diaminopimelic acid, glutamic acid, alanine, glucosamine and muramic acid, were also present in the approximate molar proportions 1:1:1.5:1:0.9. Fraction S1 accounted for 38% of the total diaminopimelic acid recovered from the

Table 1. Analysis of fractions obtained by chromatography of the lysozyme-solubilized portion of alkali-treated walls of *B. licheniformis*

Alkali-extracted walls (181 mg) were treated with lysozyme and the soluble fraction was separated by chromatography on Sephadex G-50. Fractions S1, S2, S3 and S4 were obtained from the column as described in Fig. 1, dissolved in water (5.0 ml) and analysed as described in the Materials and Methods section.

Substance	Composition ($\mu\text{mol}/\text{fraction}$)				Composition (molar proportions)			
	S1	S2	S3	S4	S1	S2	S3	S4
Diaminopimelic acid	34.8	22.5	23.6	11.7	1.00	1.00	1.00	1.00
Glutamic acid	34.0	23.2	24.2	12.5	0.98	1.03	1.03	1.06
Alanine	49.2	33.2	35.1	14.0	1.43	1.47	1.50	1.19
Glucosamine	38.4	24.3	21.7	12.7	1.09	1.07	0.92	1.06
Muramic acid	31.1	17.6	19.4	11.6	0.89	0.78	0.82	0.99
Galactosamine	82.0	2.9	0	0	2.37	0.13	—	—
Glucuronic acid	99.0	3.9	0	0	2.84	0.17	—	—
Phosphorus	4.2	1.1	0	0	0.12	0.05	—	—

column. Fraction S1 therefore is a mixture of the teichuronic acid component of *B. licheniformis* walls and a part of the mucopeptide.

In addition to the known constituents of the teichuronic acid and mucopeptide components, fraction S1 contained 78% of the total phosphorus recovered from the column (Table 1). The remaining phosphorus (22%) was recovered in fraction S2. Fraction S2 (24 mg) contained the mucopeptide constituents in molar proportions similar to those found for fraction S1 and accounting for 25% of the total diaminopimelic acid. Fractions S3 (23 mg) and S4 (14 mg) contained only mucopeptide and represented 24% and 13% respectively of the total diaminopimelic acid recovered from the column. The overall yield of material from the column was at least 90%.

DEAE-cellulose chromatography of Sephadex fraction S1. Fraction S1 (60 mg) was purified further by chromatography on DEAE-cellulose (Fig. 2). A single peak containing glucuronic acid and most of the hexosamine material was obtained. A small amount of hexosamine was eluted just in front of the main peak containing teichuronic acid. The peak fractions were pooled. The analysis of the main fraction from the DEAE-cellulose column is shown in Table 2. Examination of the minor peak (Fig. 2) after acid hydrolysis showed the presence of mucopeptide amino acids as well as glucosamine and muramic acid. No glucuronic acid or galactosamine was associated with the mucopeptide and the peak was not examined further. At least 85% of the radioactive phosphorus recovered from the column was associated with the main fraction containing teichuronic acid (Table 2). The overall yield of teichuronic acid in the fraction was 87%. Mucopeptide constituents were present in molar proportions very similar to those found for fraction S1 (Table 1). The fraction shown in Table 2 is referred to as the teichuronic acid-mucopeptide complex in

Fig. 2. Chromatography on DEAE-cellulose of fraction S1 from Fig. 1. Fraction S1 (60 mg) dissolved in water (3.25 ml) was applied to the column (2 cm $\times$ 45 cm) and eluted with pyridine-acetate buffers, pH 5.1, of increasing concentrations as described in the Materials and Methods section. Fractions (5.0 ml) were collected and portions were analysed for glucuronic acid ($\bullet$), for radioactivity (—) and for total hexosamine after acid hydrolysis ($\circ$). The fraction that was made by pooling tubes is indicated by the blocking along the abscissa.

the rest of the paper. The composition of this fraction is closely similar to that previously described for the complex isolated in the same way from another batch of cell walls (Hughes *et al.* 1968).

High-voltage electrophoresis of the complex. The complex moved to the anode as a single substance, as revealed by the starch-iodide reagent (Rydon & Smith, 1952), at pH 3.6 (Fig. 6) and pH 5.1. The mobilities relative to a teichuronic acid marker at pH 3.6 and pH 5.1 were respectively 0.89 and 0.93.

Table 2. Analysis of the teichuronic acid-mucopolysaccharide complex isolated from DEAE-cellulose

Fraction S1 (Fig. 1) was purified further by chromatography on DEAE-cellulose as shown in Fig. 2. Analyses were performed as described in the Materials and Methods section.

	Composition	
	($\mu\text{mol}/\text{fraction}$)	(molar proportions)
Diaminopimelic acid	17.6	1.00
Glutamic acid	18.7	1.06
Alanine	25.2	1.44
Glucosamine	16.1	0.92
Muramic acid	13.1	0.75
Galactosamine	64.0	3.64
Glucuronic acid	69.0	3.93
Phosphorus	2.8	0.16

Properties of the teichuronic acid-mucopolysaccharide complex

Stability of the linkage between teichuronic acid and mucopolysaccharide. In a study of the rate of formation of chromogen by heating substances at 100°C and pH 7.0, Knox & Hall (1965) showed that the yield of chromogen from *O*- β -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetylgalactosamine was many times greater than that formed by *N*-acetylgalactosamine under the same conditions. Further, *O*- β -D-galactosyl-(1 $\rightarrow$ 6)-*N*-acetylglucosamine yielded the same amount of colour as *N*-acetylglucosamine. The extreme lability of substituents on C-3 of *N*-acetylhexosamines to alkali is well known (Kuhn & Krüger, 1956). The ready β -elimination of these substituents under even mildly alkaline conditions gives rise to enhanced colour yield in the Morgan-Elson test.

The results obtained with teichuronic acid by using the conditions of Knox & Hall (1965) suggested that the teichuronic acid molecules were terminated at the reducing ends with C-3 substituted *N*-acetylgalactosamine residues. A typical experiment is shown in Fig. 3. Samples of pure teichuronic acid, prepared from acid extracts of *B. licheniformis* walls as described in the Materials and Methods section, were heated at 100°C in 0.05 M-potassium phosphate buffer, pH 7.0, for various times. Chromogen was formed that reacted with the Morgan-Elson reagent. The formation of chromogen reached a maximum after 45–60 min of heating (Fig. 3). Free *N*-acetylgalactosamine formed little chromogen under the same conditions.

No chromogen was formed on heating the complex (Table 2) at pH 7.0 and 100°C for periods up to 120 min (Fig. 3). This result showed that the *N*-acetylgalactosamine residues present at the reducing terminals of the teichuronic acid chains were not free in the complex. It is concluded that

Fig. 3. Rates of chromogen formation at pH 7.0 and 100°C. Samples of teichuronic acid (1.04 μmol of equivalent glucuronic acid), teichuronic acid-mucopolysaccharide complex (1.72 μmol of glucuronic acid equivalents) and *N*-acetylgalactosamine (0.18 μmol) were treated at 100°C and pH 7.0 for the times indicated and the chromogen formed was estimated with the Morgan-Elson reagent as described in the Materials and Methods section. $\circ$, Teichuronic acid; $\bullet$, complex; ----, *N*-acetylgalactosamine.

these residues were combined with the mucopolysaccharide.

The stability of the linkages of the teichuronic acid chains with mucopolysaccharide was next studied. A series of tubes containing the teichuronic acid-mucopolysaccharide complex in 0.1 M-hydrochloric acid was heated at 35°C for periods up to 48 h and the amount of reducing terminal *N*-acetylgalactosamine residues exposed by acid hydrolysis was measured (Fig. 4). There was a relatively rapid increase in the number of such groups within the first 10 h of acid treatment. Thereafter increasing amounts of *N*-acetylhexosamine that was reactive in the modified Morgan-Elson test, appeared at a decreased rate up to at least 72 h. It seemed likely that the second, slower, phase of the curve was due to a breakdown of the internal glycosidic linkages of teichuronic acid by dilute acid. A control experiment, shown in Fig. 4, confirmed that teichuronic acid was degraded by 0.1 M-hydrochloric acid at 35°C. A slow rise in the amount of reactive *N*-acetylhexosamine was observed. The rate of increase in the reactive *N*-acetylhexosamine during the acid degradation of teichuronic acid (Fig. 4) was

Fig. 4. Rate of release of teichuronic acid chains from mucopeptide. Samples of the complex (Table 2, containing $1.7\mu\text{mol}$ of glucuronic acid equivalents) were heated in 0.1M-HCl at 35°C and the release of reactive *N*-acetylgalactosamine residues that were capable of forming chromogen on heating at 100°C and $\text{pH } 7.0$ was measured ($\circ$). The results obtained in two separate experiments are combined. The rate of appearance of new reactive *N*-acetylgalactosamine residues during treatment of a teichuronic acid standard (equivalent to $1.04\mu\text{mol}$ of glucuronic acid) in 0.1M-HCl at 35°C is also shown ($\bullet$).

almost parallel to the second phase of the curve obtained from the acid-treated complex and is also shown in Fig. 4.

The colour produced by the complex in the first phase of the curve (Fig. 4) was approx. 68% of the colour produced by an equivalent quantity of pure teichuronic acid. If it is assumed that all of the teichuronic acid chains terminate with *N*-acetylgalactosamine residues and that these residues are substituted on C-3, the initial low yield of chromogenic end groups of the acid-treated complex would suggest that the native polysaccharide chains present in the walls are in fact longer than those in the acid-extracted preparation. The result was expected, since the standard teichuronic acid was undoubtedly degraded to a certain extent during preparation. The molecular weight of the standard teichuronic acid used in the experiment shown in Fig. 4 was determined by Dr P. Charlwood, using ultracentrifugal analysis, to be 10500–11000 (Hughes & Thurman, 1970). On the assumption that the polysaccharide consists of alternating residues of glucuronic acid and galactosamine, the molecular weight determination indicates that each chain of teichuronic acid is made up of 25 disaccharide repeating units. From the above molecular-weight data and the results shown in Fig. 4 it is calculated that the molecular weight of the native polysaccharide is 14000–15000. This represents a chain composed of 35–40 disaccharide units.

Electrophoresis at $\text{pH } 3.6$ of the complex after treatment with dilute acid at 35°C for 40 h (Fig. 6)

Fig. 5. Extraction of teichuronic acid from alkali-insoluble walls by 0.1M-HCl at 35°C (—) compared with the rate of release of teichuronic acid chains from mucopeptide in the soluble complex (----). The amounts of glucuronic acid and galactosamine, used as a measure of teichuronic acid content, that were released from insoluble walls are expressed as percentages of the total cell-wall contents. The values obtained for both components were used to derive the curve shown. The amount of reducing terminal *N*-acetylgalactosamine residues released from the soluble complex are expressed as percentages of the value obtained at 40 h. The amounts of free glucose liberated from glucose 1-phosphate under the same acid conditions at various times are also shown ($\circ$).

Fig. 6. Separation of teichuronic acid and mucopeptide components after mild acid hydrolysis of the soluble complex. (a) *N*-acetylgalactosamine standard; (b) soluble complex before hydrolysis; (c) soluble complex after treatment with 0.1M-HCl at 35°C for 40 h; (d) teichuronic acid standard; (e) soluble mucopeptide fragments derived from acid-extracted walls by lysozyme treatment were separated on Sephadex G-50 and the material of highest molecular weight was used for the electrophoresis. High-voltage electrophoresis was carried out in $0.1\text{M-pyridine-acetate}$ buffer, $\text{pH } 3.6$, at 2000 V for 3 h, and the spots were revealed with the Rydon-Smith reagent.

showed that the linkages between the teichuronic acid and mucopeptide components had been broken. During electrophoresis at $\text{pH } 3.6$ the

mucopeptide component showed a low mobility towards the anode. In contrast, the teichuronic acid carried a strongly negative net charge either alone or when combined covalently with mucopeptide in the complex.

Rate of extraction of teichuronic acid from insoluble walls

The rate of release of reducing terminal *N*-acetyl-galactosamine residues from the soluble teichuronic acid-mucopeptide complex (Fig. 4) was compared with the rate of dissolution of teichuronic acid from insoluble cell walls under the same conditions. Samples of alkali-extracted cell walls of *B. licheniformis* (20mg) were suspended in 0.1M-hydrochloric acid (5.0ml) at 35°C for various times. The samples were centrifuged and portions of the supernatant and insoluble residue fractions were analysed. As shown in Fig. 5 there was an almost complete removal of teichuronic acid from the insoluble walls after 48h. The rate of dissolution of the polysaccharide was very similar to the initial rate of appearance of free reducing terminals of *N*-acetyl-galactosamine obtained on heating the soluble teichuronic acid-mucopeptide complex under the same conditions.

Rate of hydrolysis of glucose 1-phosphate by 0.1M-hydrochloric acid at 35°C

The stability of glucose 1-phosphate in dilute acid at 35°C was measured by following the liberation of free glucose with glucose oxidase. As shown in Fig. 5, the rate at which free glucose appeared during the acid treatment was very similar to the rate of dissociation in dilute acid solution of the teichuronic acid chains from mucopeptide, either in the soluble complex or in insoluble walls.

Isolation of muramic acid phosphate from alkali-treated walls

A batch of cell walls, not labelled with ^{32}P , was extracted successively with alkali and acid. A sample (130mg, containing $5.6\mu\text{mol}$ of phosphorus) of the insoluble residue was hydrolysed in 2M-hydrochloric acid at 100°C for 3h. After hydrolysis 39% of the total phosphorus was inorganic phosphorus. The hydrolysate was passed through a column ($0.84\text{cm} \times 22\text{cm}$) of Dowex 50 (H^+ form). The material eluted from the column with water (40ml) was evaporated to dryness and passed through a column ($0.84\text{cm} \times 22\text{cm}$) of Dowex 50 equilibrated with 0.1M-pyridine-acetate buffer, pH 2.8. The column was eluted with the same buffer and fractions (2.0ml) were collected. Hexosamine material was eluted in tubes 5 and 6. A muramic acid standard was eluted from a column operated under identical conditions, at peak tube 29. The

hexosamine material was freeze-dried before analysis. In the Elson-Morgan test a sharp absorption maximum at 510nm was found that is characteristic of 3-*O*-substituted hexosamines. With muramic acid as a standard there was $1.36\mu\text{mol}$ of hexosamine present. Further analysis showed the presence of $1.42\mu\text{mol}$ of phosphorus after vigorous acid hydrolysis, and 81% of this phosphorus was released by wheat-germ acid phosphatase. High-voltage electrophoresis of the hexosamine fraction at pH 4.5 gave a single spot moving to the anode and revealed by the specific hexosamine reagent and by alkaline silver nitrate. After treatment of the hexosamine fraction with acid phosphatase, the negatively charged spot did not appear on electrophoresis and an amino sugar was present in the neutral region on the paper. Its identity with muramic acid was confirmed by chromatography in solvents I and II.

Examination of Sephadex fractions S3 and S4 (Fig. 1)

Fraction S4 (Fig. 1) contained diaminopimelic acid, glutamic acid, alanine, glucosamine and muramic acid in the molar proportions 1.00:1.06:1.19:1.06:0.99 (Table 1). The almost equimolar proportions of alanine to the other constituents suggested that fraction S4 contained the monomer fragments characterized previously in *B. licheniformis* soluble mucopeptide (Hughes, 1968b; Mirelman & Sharon, 1968). Chromatography of fraction S4 in solvent III showed the presence of two ninhydrin-positive compounds. The faster of these (R_{Dap} , i.e. R_f relative to that of diaminopimelic acid, 0.71) had a mobility identical with that found previously for C1, the monomer fragment *N*-acetylglucosaminyl- β (1 $\rightarrow$ 4)-*N*-acetylmuramyl-tripeptide, and containing a single amide group substituted in the peptide moiety. Fraction S4 also gave a spot (R_{Dap} 0.55) that moved similarly to the fragment C2 studied earlier and identified as the non-amidated form of fragment C1. In the soluble mucopeptide fragment C1 predominates (80%), but there was an approximately equal distribution of fragments C1 and C2 prepared from the alkali-treated walls. Some amide groups may have been removed from the mucopeptide during the alkaline extraction.

Fraction S3, prepared as shown in Fig. 1, contained approximately 1.5 molecular proportions of alanine relative to the other constituents. Chromatography in solvent III showed the presence of two major ninhydrin-positive spots with R_{Dap} values of 0.31 and 0.21 respectively. The compounds were chromatographically identical with fragments B1 and B2 isolated from soluble mucopeptide prepared from acid-extracted walls (Hughes, 1968b). Fragment B1 is a dimer, formed by the cross-linking of two monomer units by a D -alanine residue from the C-terminus of one tripeptide to an amino group of

the diaminopimelic acid residue of a second tripeptide. Fragment B2 is of unknown constitution and is present as a relatively minor component in the fragments isolated either from acid-extracted walls or alkali-extracted walls.

DISCUSSION

The rate of dissolution of alkali-extracted walls of *B. licheniformis* by the action of lysozyme was similar to that obtained with acid-extracted walls containing neither teichuronic acid nor teichoic acid. Therefore the affinity of the enzyme for the mucopeptide of this organism was not appreciably affected by the presence of the acidic polysaccharide. The walls of certain organisms are resistant to lysozyme owing to inhibition of the enzyme by ester groups present in the mucopeptide (Brumfitt, Wardlaw & Park, 1958). In these cases alkali treatment of walls under the conditions used in the present paper might be expected to increase the susceptibility of the mucopeptide to lysozyme.

When intact walls of *B. licheniformis* were treated with lysozyme and examined by chromatography on DEAE-cellulose, 55% of the mucopeptide material was isolated in pure form. A second fraction was eluted later from the column and contained the rest (45%) of the mucopeptide together with the teichuronic acid and teichoic acid (Hughes, 1965). This value is similar to the yield (38%) of the mucopeptide present in fraction S1 (Fig. 1), and largely associated with the teichuronic acid as shown by subsequent chromatography on DEAE-cellulose (Fig. 2). Further, the relative proportions of the low-molecular-weight mucopeptide fragments were very similar in the products of lysozyme action on intact walls, alkali-extracted walls or acid-extracted walls. Thus the yields of the monomer fragments S4 and dimer fragments S3 obtained from the products of alkali-extracted walls were respectively 13% and 25% compared with 17% and 18% respectively obtained from intact walls and 18% and 26% respectively from acid-extracted walls (Hughes, 1968b). In this series of wall preparations, lysozyme is presented with substrates containing widely different amounts of associated polymers. These results support the suggestion (Hughes, 1968b) that lysozyme attacks with ease certain regions of *B. licheniformis* mucopeptide, producing reproducible amounts of fragments of low molecular weight. In the rest of the mucopeptide the hydrolysis of glycosidic linkages in the mucopeptide proceeds less efficiently and mucopeptide of high molecular weight is produced. This material of high molecular weight appears to be associated with one or both of the acidic polymers, or with the small amount of residual phosphorus remaining in walls after extraction of the teichuronic

acid and teichoic acid with dilute acids. Since muramic acid phosphate residues are present, as discussed below, it is noteworthy that the recent crystallographic model of lysozyme requires the C-6 position of *N*-acetylmuramic acid units of mucopeptide to point into the cleft. It is clear that steric hindrance would prevent binding of C-6 substituted residues (Phillips, 1967).

The residual phosphorus remaining in different batches of *B. licheniformis* cell walls after extraction with dilute alkali was found to be 0.1–0.2% of the weight of insoluble material. This amount is similar to that present in these walls after exhaustive treatment with dilute acid (Hughes, 1968a). When alkali-treated walls were extracted with dilute acid, the insoluble residue remaining at the end of the reaction contained only 6% of the total glucuronic acid, but all of the phosphorus originally present. The teichuronic acid present in the acid extracts contained no detectable phosphorus. These results are best explained if the bonds between the polysaccharide and the insoluble mucopeptide involve phosphodiester linkages from the reducing ends of the teichuronic acid chains to the mucopeptide. The ready cleavage by acid, of a phosphate ester on C-1 of *N*-acetylgalactosamine residues at the reducing ends of the teichuronic acid chains, would be expected in view of the extreme acid lability of glucose 1-phosphate or *N*-acetylglucosamine 1-phosphate (Leloir & Cardini, 1956). Indeed the stability in dilute acid of the linkages between teichuronic acid and mucopeptide was found to be very similar to the stability of glucose 1-phosphate under the same conditions (Fig. 5). This hypothesis would also explain the relative resistance of the linkage to alkali, if it is assumed that the group in the mucopeptide that is substituted by the phosphodiester is suitably protected from cyclic ester formation, since it is well known that alkaline hydrolysis of phosphate esters proceeds by such a mechanism (Archibald & Baddiley, 1968).

As shown in Table 2, there were approx. 0.9 mol of phosphorus/mol of a teichuronic acid consisting of 25 repeating disaccharide units. However, as discussed above, the chains of undegraded teichuronic acid present in the native walls are almost certainly longer than this. The amount of phosphorus present in the complex is therefore more than enough to account for the attachment of the teichuronic acid chains to mucopeptide. Teichoic acids extracted from walls of several organisms by alkali have been shown to carry terminal phosphate groups that presumably originated from the phosphodiester linkages to mucopeptide (Hay *et al.* 1965; Archibald, Coapes & Stafford, 1969). In this situation any residual phosphorus found to be associated with the mucopeptide after a complete extraction of teichoic acid with alkali could arise

only from a source other than the teichoic acid. One possible source, as suggested originally by Knox & Hall (1965) and Knox & Holmwood (1968) and in this paper, is the linkage point of a cell-wall polysaccharide to mucopeptide.

The nature of the group to which the teichuronic acid chains are attached has not been established. However, the fact that muramic acid phosphate was isolated from the residue remaining after acid extraction of alkali-treated walls may be significant. This compound has been found in the walls of a large number of organisms (Ågren & de Verdier, 1958; Liu & Gottschlich, 1967; Heymann, Maniello & Barkulis, 1967; Montague & Moulds, 1967; Knox & Holmwood, 1968; Grant & Wicken, 1968). Recently the structure has been shown conclusively to be the attachment point of an atypical teichoic acid to mucopeptide in the walls of *Staphylococcus lactis* 13 (Button *et al.* 1966). A similar role is possible for muramic acid phosphate in the attachment of the chains of teichuronic acid to the mucopeptide in *B. licheniformis* cell walls.

Earlier studies (Hughes, 1966) have shown that the precursors of *B. licheniformis* teichuronic acid are UDP derivatives of *N*-acetylgalactosamine and glucuronic acid. Incorporation of the monosaccharide of either precursor into teichuronic acid material was catalysed by a particulate fraction from broken cells of *B. licheniformis*, and required the presence of the other precursor. Approximately equimolar amounts of glucuronic acid and *N*-acetylgalactosamine were incorporated. The phosphodiester structure that is believed, on the available evidence, to link the teichuronic acid chains to the mucopeptide of the walls, could conceivably be formed by transfer of a residue of *N*-acetylgalactosamine 1-phosphate from UDP-*N*-acetylgalactosamine to an acceptor site in mucopeptide or mucopeptide precursor with the simultaneous formation of UMP. Incorporation of *N*-acetylglucosamine 1-phosphate residues into the wall teichoic acid of *Staphylococcus lactis* 13 proceeds by a similar mechanism (Baddiley, Blumsom & Douglas, 1968). Later events in teichuronic acid biosynthesis would be transfer of the monosaccharide residues to non-reducing ends of the growing chains in a manner similar to that taking place in hyaluronic acid biosynthesis in streptococci (Stoolmiller & Dorfman, 1969), followed by chain termination.

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