The Isolation of Structural Components Present in the Cell Wall of *Bacillus licheniformis* N.C.T.C. 6346

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(Received 19 January 1965)

1. Four of the known components of wall preparations of vegetative cells of *Bacillus licheniformis* N.C.T.C. 6346 have been isolated free of each other after successive treatments of the walls with trichloroacetic acid and lysozyme: (a) a mucopeptide consisting of glucosamine, muramic acid, $\alpha\epsilon$ -diaminopimelic acid, glutamic acid and alanine in the molar proportions 1.0:0.8:1.0:1.2:1.7; (b) an insoluble protein; (c) teichoic acid containing phosphorus and glucose in equimolar amounts; (d) teichuronic acid containing equimolar amounts of *N*-acetyl-galactosamine and glucuronic acid, as found by Janczura, Perkins & Rogers (1961). 2. Evidence has been obtained for the presence in the soluble fraction obtained by lysozyme treatment of whole walls of a stable covalent complex of the teichoic acid and the mucopeptide components. 3. The molar ratio of phosphorus to glucose in the teichoic acid present in intact walls or the soluble fractions obtained by extraction of the walls with lysozyme or trichloroacetic acid is 1.0:0.25, in contrast with values of about unity obtained for the purified teichoic acid. 4. Intact walls have been shown to contain polyribitol phosphate chains bearing different amounts of glucose substituents. 5. Trichloroacetic acid extracts of walls also contain polyribitol phosphate compounds of different chain lengths. Dialysis of trichloroacetic acid extracts removes the short chains of polyribitol phosphate that have been found to carry only very low amounts of glucose side chains. By contrast, the longer chains present in the non-diffusible fraction contain phosphorus and glucose in almost equimolar amounts.

The wall of vegetative cells of *Bacillus licheniformis* N.C.T.C. 6346 has been shown to contain at least four components (Janczura, Perkins & Rogers, 1961). [Since the work of Janczura *et al.* (1961) it has been found that *Bacillus subtilis* N.C.T.C. 6346 is properly called *Bacillus licheniformis*. The organism used by Armstrong, Baddiley & Buchanan (1960, 1961) has been confirmed to be *B. subtilis* by Sharon, Shif & Zehavi (1964). The organism used by other workers (Salton, 1953; Salton & Marshall, 1959; Yoshida *et al.* 1957; Young, Spizizen & Crawford, 1963) is stated to be *B. subtilis*.] Extraction of the wall with trichloroacetic acid dissolves a teichoic acid, which was assumed to be structurally related to the teichoic acid isolated in a similar way from wall preparations of a strain of *B. subtilis* (Armstrong *et al.* 1960, 1961). In this organism the teichoic acid is a polymer of about nine ribitol phosphate groups with glucose and alanine attached, the latter in labile ester linkage. In addition to the teichoic acid, trichloroacetic acid extracts of *B. licheniformis* N.C.T.C. 6346 wall preparations contained an acidic

polysaccharide, teichuronic acid, which was purified by precipitation with cetylpyridinium bromide and by paper electrophoresis (Janczura *et al.* 1961). The pure material contained glucuronic acid and *N*-acetylglactosamine in equimolar amounts.

It was found that the teichoic acid and teichuronic acid were released into solution by the action of lysozyme on the wall preparation (Janczura *et al.* 1961) together with a component consisting of $\alpha\epsilon$ -diaminopimelic acid, glutamic acid, alanine, *N*-acetylglucosamine and *N*-acetylmuramic acid. This latter component is similar in composition to the mucopeptide polymers (Perkins & Rogers, 1959) that have been isolated from all bacterial cell walls so far examined (Salton, 1964). As found in previous work on *B. subtilis* cell walls (Salton, 1953; Salton & Marshall, 1959; Yoshida *et al.* 1957) a small insoluble residue remained after exhaustive enzymic treatment, and this was made up of an almost complete range of amino acids.

The present paper describes the separation of the four known components of the wall of vegetative cells of *B. licheniformis* N.C.T.C. 6346 by successive

treatments of the walls with trichloroacetic acid and lysozyme followed by ion-exchange chromatography. The fractionation of the intact wall was also carried out under milder conditions and evidence was obtained for the stable linkage of a part of the mucopeptide component to at least one of the other polymers present in the wall preparation.

MATERIALS AND METHODS

Growth of the organism and preparation of the cell walls. The strain of *B. licheniformis* used was N.C.T.C. 6346. The organism was grown as described by Janczura *et al.* (1961). The cell walls were isolated essentially by the method of Cummins & Harris (1956) as modified by Janczura *et al.* (1961) to eliminate autodigestion of the wall during isolation. Some experiments, as indicated, were also performed with walls prepared as described by Salton & Horne (1951).

Lysozyme reaction mixtures. The procedure used was as described by Janczura *et al.* (1961).

Acid-phosphatase reaction mixtures. The substrate (4.0 ml.), containing about 0.5 mg. of total phosphorus, was incubated in 0.4M-ammonium acetate, pH 5.5 (0.8 ml.), with wheat-germ acid monophosphoesterase (0.2 ml., 5 mg./ml.; L. Light and Co. Ltd., Colnbrook, Bucks.) at 37°. At intervals samples were withdrawn and analysed for inorganic phosphorus and for total phosphorus after acid hydrolysis.

Dialysis. The dialysis tubing (Visking Co.) before use was placed in boiling distilled water for 10 min., then washed thoroughly with distilled water and stored in the cold.

Determination of hexosamine. Total hexosamine was estimated after hydrolysis in 4N-HCl for 4 hr. at 100° by the method of Elson & Morgan (1933) as described by Blix (1948). In this test the molar colour yields for glucosamine, galactosamine and muramic acid were found to be 100, 100 and 16% respectively. The muramic acid standard was a synthetic crystalline product kindly given by Dr R. H. Gigg. Glucosamine, galactosamine and muramic acid were separated and estimated in acid hydrolysates with the Beckman Spinco automatic amino acid analyser in conjunction with the amino acids. The colour yield of muramic acid given with the ninhydrin reagent was taken to be identical with the yields given by glucosamine and galactosamine. *N*-Acetylhexosamine or 'apparent' *N*-acetylhexosamine (Perkins, 1960) was determined by the Reissig, Strominger & Leloir (1955) modification of the method of Morgan & Elson (1934).

Determination of amino acids. After acid hydrolysis for 16 hr. at 105° in 6N-HCl, the amino acids were estimated by paper chromatography as described by Mandelstam & Rogers (1959). Mixtures containing the amino acids $\alpha\epsilon$ -diaminopimelic acid, glutamic acid, glycine and alanine were well resolved by one-dimensional chromatography on Whatman no. 3 paper in solvent *B* (see below). More complex materials were kindly analysed on the Beckman Spinco automatic amino acid analyser by Dr S. Jacobs at this Institute. The acid hydrolysates were carried out for a single time of 16 hr. at 105°, and each hydrolysate was analysed in duplicate for the amino acids and also for glucosamine, galactosamine and muramic acid. Allowance was made for the acid destruction during hydrolysis of muramic acid, galactosamine and glucosamine of 32, 13

and 9% respectively (Young *et al.* 1963). One sample was analysed for $\alpha\epsilon$ -diaminopimelic acid, glutamic acid, glycine and alanine by both methods and the values obtained agreed within 10%.

Determination of hexuronic acid. The method of Dische (1947) was used.

Determination of glucose. This was estimated with the anthrone reagent (Dreywood, 1946) either without prior acid hydrolysis of the samples or with fractions that had been previously hydrolysed in N-H₂SO₄ for 3 hr. at 100°. In the latter case, the acid hydrolysates were passed through columns of Amberlite IR-45 (acetate form) and Amberlite IRC-50 (H⁺ form) before analysis. Glucose was also estimated in the neutralized hydrolysates by the ferricyanide method of Park & Johnson (1949) and by quantitative paper chromatography with solvent *A* (see below) and the aniline hydrogen phthalate reagent of Baar (1954).

Determination of phosphorus. Phosphorus was estimated after hydrolysis for 16 hr. or 24 hr. at 105° in 6N-HCl. Suitable portions of the hydrolysate were analysed by the method of Allen (1940).

Paper electrophoresis. The apparatus of Kunkel (1954) was used with a potential difference of 1500V, usually applied for 3 hr. The following buffers were used: (i) *N*-acetic acid; (ii) 50mm-pyridine-acetic acid buffer, pH 5.1; (iii) 50mm-borate buffer, pH 8.4. When substances were to be eluted from the Whatman no. 3 paper after electrophoresis, the paper was washed before use with distilled water.

Paper chromatography. Paper chromatography was carried out on Whatman no. 1, no. 3 or no. 4 paper with the following solvent systems: solvent *A*, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955); solvent *B*, butan-1-ol-pyridine-water (6:4:3, by vol.); solvent *C*, butan-1-ol-acetic acid-water (63:10:27, by vol.); solvent *D*, propan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); solvent *E*, butan-1-ol-ethanol-water-aq. NH₃ (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase) (Foster, Horton & Stacey, 1957). Solvent *A* separated galactosamine, glucosamine, muramic acid, glucose and ribitol after overnight development and was most useful in this work. Solvent *B* separated well $\alpha\epsilon$ -diaminopimelic acid, glutamic acid, alanine, galactosamine and the glucosamine-muramic acid pair, and was useful in the analysis of the simpler fractions obtained from the wall. Solvent *C* separated muramic acid from the glucosamine-galactosamine pair and was useful in conjunction with solvent *B* in detecting the presence of the hexosamines. Solvents *D* and *E* were used to detect products derived from the teichoic acid as described by Armstrong *et al.* (1961). The papers were subsequently dipped or sprayed with 0.1% (w/v) ninhydrin in butan-1-ol for amino acids, alkaline AgNO₃ (Trevelyan, Procter & Harrison, 1950), or aniline hydrogen phthalate (Baar, 1954) for sugars, the periodate-benzidine reagents for α -glycols (Smith, 1960), the *p*-dimethylaminobenzaldehyde reagent of Partridge (1948) for amino sugars, and modified molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949). The method of Rydon & Smith (1952) was used for substituted amides.

Chromatography on DEAE-cellulose. The washed resin (Whatman grade DE-50) was equilibrated with 10mm-pyridine-acetic acid buffer, pH 5.1, and packed under air pressure into a column (1.5 cm. $\times$ 15 cm.). The column was

eluted at room temperature and at 25 ml./hr. with a linear concentration gradient established by running 5.0M-pyridine-acetic acid buffer, pH 5.1 into the mixing chamber, containing 1 l. of 10M-pyridine-acetic acid buffer, pH 5.1.

RESULTS

Purification of teichoic acid and teichuronic acid. The wall preparation (about 0.11 g.) was extracted twice with 5 ml. of 5% (w/v) trichloroacetic acid at 35° overnight. The supernatants obtained after centrifugation were combined and the insoluble residue was washed with water several times. The soluble fraction contained 95% of the total recovered phosphorus and all of the uronic acid and glucose of the wall (Table 1), indicating that extraction of the teichoic acid and teichuronic acid was complete. The fraction was dialysed against water at 4° for 16 hr. The non-diffusible fraction weighed 35% of the initial whole wall preparation, and contained all of the uronic acid and about 35% of the phosphorus of the starting material (Table 1). The non-diffusible fraction was applied to a column of DEAE-cellulose that was developed as described in the Materials and Methods section, and fractions from the column were analysed for uronic acid and for anthrone-positive material. Peak T1 (Fig. 1) contained about equimolar amounts of glucose and phosphorus (Table 1), and paper chromatography of acid hydrolysates showed the presence of anhydrosorbitol, ribitol and alanine, as expected for the teichoic acid (Armstrong *et al.* 1961). Peak T2 contained glucuronic acid and hexosamine material (Table 1) that was shown to be galactosamine by paper chromatography. The two constituents were present in about equimolar amounts in fraction T2.

The yields of teichoic acid and teichuronic acid in the pooled fractions T1 and T2 respectively were 50–60% of the material placed on the column. On paper electrophoresis in N-acetic acid fractions T1 and T2 gave elongated spots moving 9–13 cm. and 5–7 cm. towards the anode, in agreement with the order of elution from the DEAE-cellulose column (Fig. 1). The chromatographic behaviour of the teichoic acid and teichuronic acid did not change when the pyridine-acetic acid buffers were replaced by sodium acetate buffers of the same ionic strength.

Action of lysozyme on the insoluble residue remaining after trichloroacetic acid extraction of walls. Extraction of walls with trichloroacetic acid leaves about 42% of the dry weight of the walls as an insoluble residue (Table 1). To determine whether the absence of teichoic acid and teichuronic acid from the mucopeptide altered the reaction of lysozyme, a portion (38 mg.) of the residue remaining after acid extraction of whole walls was incubated with lysozyme under the standard conditions. At the end of the reaction the amount of 'apparent' N-acetylhexosamine (Perkins, 1960) in the solution was estimated to be 370 μ moles/mg. of original whole walls. This value is comparable with that produced (340 μ moles/mg.) from original walls.

The insoluble residue remaining after centrifugation of the lysozyme reaction mixture was shown to yield amino acids but no hexosamines or α -diaminopimelic acid on acid hydrolysis, as expected

Table 1. *Recovery of components during fractionation of trichloroacetic acid extracts of B. licheniformis cell walls*

Experimental details are given in the text. Hexosamines were analysed after acid hydrolysis and are expressed as equivalents of glucosamine. Uronic acid was measured by the carbazole reaction, with glucuronic acid as a standard. Phosphorus was determined after acid hydrolysis as described in the Materials and Methods section. The values shown for glucose were obtained by analysis of unhydrolysed materials with the anthrone reagent. The dry weights are not corrected for ash content.

Fraction	Dry wt. (mg.)	Amount (μ moles)			
		Hexosamines	Uronic acid	Phosphorus	Glucose
1. Whole walls	106	98	45	147	37
2. Trichloroacetic acid extraction					
Insoluble	45	41	0	5	0
Soluble	—	48	45	115	37
3. Dialysis of soluble fraction					
Diffusible	—	0	0	52	12
Non-diffusible	37	48	45	38	29
4. DEAE-cellulose chromatography					
Fraction T1	—	4	3	17	19
Fraction T2	—	21	24	5	3

for the 'insoluble protein' component of the walls. Extensive dialysis of the soluble fraction removed material corresponding to no more than 5% of the dry weight and only 4% of the total hexosamines

(measured after acid hydrolysis) of the original unextracted wall preparation. These values are in close agreement with those found (respectively 4 and 3%) in the diffusible products of enzymic action on intact walls (Table 2). The diffusible fraction contained, after acid hydrolysis, glucosamine, muramic acid and the mucopeptide amino acids. The bulk of the mucopeptide constituents, however, remained in the non-diffusible material, which was further fractionated by chromatography on DEAE-cellulose under the standard conditions. A single hexosamine-containing peak was obtained, emerging at tubes 12–22 (Fig. 2), and was shown to contain, after acid hydrolysis, as well as glucosamine and muramic acid, $\alpha\epsilon$ -diaminopimelic acid, glutamic acid and alanine. On electrophoresis in *N*-acetic acid the mucopeptide component behaved as a neutral compound showing a slight movement to the cathode. At pH 5.1 it moved as an elongated spot anionically 4–11 cm.

Fractionation of the products of lysozyme action on whole walls. As shown by Janczura *et al.* (1961), analysis of the 'insoluble protein' obtained by treatment of several batches of walls with lysozyme showed the complete absence of $\alpha\epsilon$ -diaminopimelic acid, phosphorus, glucose, uronic acid and hexosamine (Table 2), indicating the total release by enzymic action of the other structural components of the wall. The soluble fraction contained $\alpha\epsilon$ -diaminopimelic acid, glutamic acid, alanine, glucosamine, muramic acid and galactosamine in the molar proportions 1.0:1.3:1.8:1.0:0.8:0.9. Very similar values were obtained on analysis of intact walls. After dialysis of the soluble fraction the diffusible products contained no phosphorus, glucose or glucuronic acid (Table 2), indicating the

Fig. 1. Chromatography on DEAE-cellulose of the non-diffusible products of trichloroacetic acid extracts of *B. licheniformis* cell-walls. The non-diffusible fraction obtained by dialysis of trichloroacetic acid extracts of intact walls was applied to a column (1.5 cm. $\times$ 15 cm.) of DEAE-cellulose. The column was developed with pyridine-acetic acid buffers, pH 5.1, of increasing concentration. Fractions (5 ml.) were analysed for uronic acid (.....) by the carbazole reaction and for glucose (●) with the anthrone reagent. —, Buffer concentration gradient.

Table 2. *Recovery of components during fractionation of lysozyme digests of B. licheniformis cell walls*

Experimental details are given in the text. The analytical values are expressed as in Table 1. The $\alpha\epsilon$ -diaminopimelic acid values were determined after acid hydrolysis by paper chromatography, with authentic $\alpha\epsilon$ -diaminopimelic acid as a standard. The dry weights are not corrected for ash content. N.D., Not determined.

Fraction	Dry wt. (mg.)	Amount (μ moles)				
		Hexosamines	Uronic acid	Phosphorus	Glucose	$\alpha\epsilon$ -Diaminopimelic acid
1. Whole walls	103	95	43	142	36	N.D.
2. Lysozyme action						
Insoluble	7	0	0	0	0	0
Soluble	—	100	43	149	38	45
3. Dialysis of soluble fraction						
Diffusible	4	3	0	0	0	N.D.
Non-diffusible	91	80	46	122	38	N.D.
4. DEAE-cellulose chromatography						
Fraction L1	30	19	0	0	0	8.0
Fraction L2	41	33	30	37	22	4.8
Fraction L3	14	7	0	53	9	1.7

Fig. 2. Chromatography on DEAE-cellulose of the non-diffusible products of lysozyme action on the trichloroacetic acid-insoluble fraction of *B. licheniformis* cell walls. The column (1.5 cm. $\times$ 15 cm.) was operated as described in Fig. 1 and fractions (5 ml.) were analysed after acid hydrolysis for hexosamine material (○) by the Elson-Morgan reaction.

absence of the teichoic acid and teichuronic acid components of the wall preparation. The unhydrolysed diffusible fraction contained about 6% of the 'apparent' *N*-acetylhexosamine (Perkins, 1960) produced on lysozyme treatment of the intact walls.

It is probable therefore that the soluble diffusible fractions obtained after lysozyme action on both intact walls and the insoluble residue remaining after acid extraction contain small fragments of the mucopeptide component of the wall, as is the case with other bacterial cell walls (Salton, 1964). The mucopeptide content of the intact wall is about 24%, as indicated by the contents of residues of *N*-acetylglucosamine, *N*-acetylmuramic acid, $\alpha\epsilon$ -diaminopimelic acid, glutamic acid and alanine. The proportion is lower than the weight (36%) of the insoluble fraction remaining after extraction with trichloroacetic acid after allowance is made for the presence in this fraction for the 'insoluble protein' of the wall. About 20% of the weight of the mucopeptide component is made diffusible after lysozyme treatment.

The soluble and non-diffusible portion of the

Table 3. Composition of fractions obtained by DEAE-cellulose chromatography of the non-diffusible products of lysozyme action on *B. licheniformis* cell walls

Amino acids were determined after acid hydrolysis by quantitative paper chromatography. Phosphorus, glucose and glucuronic acid were determined as indicated in Table 1.

	Amounts (molar proportions)		
	Fraction L1	Fraction L2	Fraction L3
$\alpha\epsilon$ -Diaminopimelic acid	1.0	1.0	1.0
Glutamic acid	1.2	1.0	1.2
Alanine	1.7	1.5	2.3
Phosphorus	0	7.9	31
Glucose	0	4.7	5.2
Glucuronic acid	0	6.4	0

lysozyme reaction mixture (Table 2) was then subjected to column chromatography on DEAE-cellulose. The elution profile obtained is shown in Fig. 3 and was reproducible for fractions isolated from several different batches of walls prepared by the methods either of Janczura *et al.* (1961) or of Salton & Horne (1951). Three main fractions, named L1, L2 and L3, were obtained. Fraction L1 was found to contain no phosphorus, glucose or glucuronic acid but contained about a third of the total hexosamines recovered from the column (Table 2). Paper chromatography of acid hydrolysates showed that glucosamine and muramic acid but not galactosamine were present. In addition, fraction L1 contained $\alpha\epsilon$ -diaminopimelic acid, glutamic acid and alanine in the molar proportions 1.0:1.2:1.7 (Table 3). The yield of $\alpha\epsilon$ -diaminopimelic acid was 54% of the total recovered from the column (Table 2). This fraction represents the fragmented mucopeptide component of the wall. The molar proportions of the mucopeptide amino acids found in fraction L1 are essentially the same as those found in intact cell wall. The contribution to the alanine content of whole walls prepared by the method of Janczura *et al.* (1961) of the *D*-alanine residues of the teichoic acid polymer is therefore not great.

Fraction L2 on analysis showed the presence of glucuronic acid, phosphorus, glucose and hexosamine (Table 2). This fraction contains both the teichoic acid and teichuronic acid polymers of the wall. In addition to galactosamine, the mucopeptide hexosamines, glucosamine and muramic acid were detected on paper chromatography of acid hydrolysates. Similarly, the amino acids $\alpha\epsilon$ -diaminopimelic acid, glutamic acid and alanine were present in the molar proportions 1.0:1.0:1.5

Fig. 3. Chromatography on DEAE-cellulose of the non-diffusible products of lysozyme action on *B. licheniformis* cell-walls. The soluble non-diffusible fraction obtained after digestion of intact cell walls with lysozyme was fractionated on a column (1.5 cm. $\times$ 15 cm.) of DEAE-cellulose as described in Fig. 1. Fractions (5 ml.) were analysed for uronic acid (.....), glucose (●) and (after acid hydrolysis) for hexosamine (○). The contents of the tubes under the brackets shown were pooled, giving fractions L1, L2 and L3.

Fig. 4. Rechromatography on columns (1.5 cm. $\times$ 15 cm.) of DEAE-cellulose of the fractions L1 (a), L2 (b) and L3 (c), obtained by chromatography of the soluble non-diffusible products of lysozyme action on whole *B. licheniformis* cell-walls. The conditions of the chromatography were as given in Fig. 1., Uronic acid; ●, glucose; ○, hexosamine after acid hydrolysis.

(Table 3). About 33% of the total $\alpha\epsilon$ -diaminopimelic acid was obtained in fraction L2 (Table 2). The most acidic fraction, L3, also contained constituents known to be characteristic of the teichoic acid and mucopeptide components of the wall material (Table 2). Phosphorus, glucose, glucosamine, muramic acid, $\alpha\epsilon$ -diaminopimelic acid, glutamic acid and alanine were found. The molar proportions of the amino acids were 1.0:1.2:2.3 respectively, and the $\alpha\epsilon$ -diaminopimelic acid content of fraction L3 represented 13% of the total recovered from the column (Table 2). Neither galactosamine nor glucuronic acid was detected in the fraction L3 shown in Table 2, indicating the absence of teichuronic acid. In other experiments small amounts of this component were found as contaminants of fraction L3.

Stability of the fractions obtained by DEAE-cellulose chromatography. The three fractions L1, L2 and L3 shown in Fig. 3 were separately applied to fresh DEAE-cellulose columns that were eluted in a manner identical with that used above. The elution profiles obtained are shown in Fig. 4. Fraction L1 on rechromatography was eluted (Fig. 4a) by the same salt concentration as found above. No further mucopeptide component was

detected throughout the remainder of the chromatography. To exclude more rigorously the presence of other mucopeptide fragments in low amounts, the contents of tubes 40–90 were pooled, freeze-dried, hydrolysed and examined by paper chromatography for hexosamines and amino acids. Only very small traces of these constituents were detected with large amounts of material. It was therefore concluded that the mucopeptide constituents found in fractions L2 and L3 could not be due to non-specific binding of a component normally eluted at low salt concentration. Similarly, no mucopeptide component was eluted at low salt concentration on rechromatography of fraction L2. This fraction was eluted (Fig. 4b) at a somewhat higher salt concentration than that found above, but again showed the presence of two poorly resolved peaks of teichoic acid and teichuronic acid, each containing constituents characteristic of mucopeptide. The chromatography of fraction L3 (Fig. 4c) gave a main anthrone-positive peak at the

expected position together with a smaller anthrone-positive peak in the position associated with the mucopeptide fraction L1. Chromatographic examination of acid hydrolysates of the minor peak revealed the characteristic mucopeptide amino acids and hexosamines as well as glucose to be present. This result is taken to mean that some breakdown of the complex had taken place, producing mucopeptide fragments and also a teichoic acid with the chromatographic properties of the acid-extracted teichoic acid (Fig. 1). Analysis of the minor and major peaks of Fig. 4(c) gave molar ratios of phosphorus to glucose 1.0:1.0 and 1.0:0.17 respectively.

Each major peak in the curves shown in Fig. 4 on analysis gave results very similar to those found above (Tables 2 and 3). It was apparent that, whereas fraction L1 was pure fragmented mucopeptide with no contamination from the other cell-wall polymers, the other fractions were relatively stable complexes of two or three components. Attempts were made to remove the mucopeptide component from fractions L2 and L3 and to resolve better the teichoic acid and teichuronic acid peaks of fraction L2, but without success. Thus the mucopeptide remained associated with the other polymers after high-voltage electrophoresis in acetate buffer, pH 3.6 and 5.6, and in borate buffer, pH 8.4. It should be noted that in the latter buffer it is probable that extensive hydrolysis of the ester-linked alanine residues of the teichoic acid had occurred. The resolution of the teichoic acid and teichuronic acid peaks of fraction L2 was attempted by DEAE-cellulose chromatography with shallower salt concentration gradients and longer columns than used above, on Sephadex G-100 columns and on agar-gel electrophoresis in 50mm-pyridine-acetic acid buffer, pH 5.1. Although fractions were obtained that were somewhat enriched in teichoic acid or in teichuronic acid, no clear-cut separation was obtained either from each other or from mucopeptide constituents. The effect of dilute acid on complex L2 was examined. The complex was heated for 16 hr. at 37° in 0.25N-hydrochloric acid. The acid was removed at room temperature and the fraction was examined by chromatography on DEAE-cellulose. As shown in Fig. 5 complete breakdown of the complex had taken place, giving a teichoic acid with the chromatographic properties of the purified teichoic acid (Fig. 1). The anthrone-positive peak (Fig. 5) was shown also to contain the constituents of the mucopeptide component by paper chromatography of total acid hydrolysates. The molar ratio of phosphorus to glucose in this peak was unity. Only very low amounts of mucopeptide or teichoic acid constituents were detected in acid hydrolysates of the uronic acid-containing peak, which was almost pure teichuronic acid.

Fig. 5. Effect of acid treatment on the complex fraction L2 of *B. licheniformis* cell walls. The fraction was kept in 0.25N-HCl for 16 hr. at 37°. The acid was removed at room temperature *in vacuo*. The residue was dissolved in water and applied to a column (1.5 cm. × 15 cm.) of DEAE-cellulose, which was developed as described in Fig. 1., Uronic acid; ●, glucose.

Co-chromatography of fractions prepared after trichloroacetic acid treatment of whole walls. To eliminate the possibility of ionic interactions being responsible for the chromatographic pattern obtained after the enzymic digestion of walls and shown in Fig. 3, the teichoic acid and teichuronic acid isolated from the trichloroacetic acid extracts (see Fig. 1) were mixed with the soluble mucopeptide material (see Fig. 2). The mixture was fractionated by DEAE-cellulose chromatography and the result is shown in Fig. 6. Neither the shapes of the curves nor the points of elution of the purified teichoic acid or teichuronic acid were changed by the presence of the purified mucopeptide fragments. The teichoic acid was eluted at tubes 14–30 and the teichuronic acid at tubes 32–45, and were clearly resolved as found above (Fig. 1). No mucopeptide constituents were detected in acid hydrolysates of the uronic acid-containing peak.

Treatment of fractions with phosphatase and the phosphorus and glucose contents of the teichoic acid. The purified teichoic acid contained equimolar amounts of phosphorus and glucose (Table 1), as found by Armstrong *et al.* (1961). Treatment of this teichoic acid with wheat-germ acid phosphatase at

Fig. 6. Chromatography on DEAE-cellulose of a mixture of the purified teichoic acid and teichuronic acid, and the non-diffusible products of lysozyme action on the insoluble residue remaining after extraction of *B. licheniformis* cell walls with trichloroacetic acid. Teichoic acid and teichuronic acid were chromatographically purified as shown in Fig. 1. The mucopeptide fragments were purified as shown in Fig. 2. The purified materials were mixed at room temperature and applied to a column (1.5 cm. $\times$ 15 cm.) of DEAE-cellulose, which was eluted as described in Fig. 1. Fractions (5 ml.) were analysed for uronic acid (.....), glucose (●) and, after acid hydrolysis, for hexosamines (○).

pH 5.5 for 20 hr. released 9.5% of the total phosphorus as inorganic phosphate. This indicates the teichoic acid to have a chain length of about ten ribitol phosphate units, in close agreement with other work (Armstrong *et al.* 1961). The phosphorus content of the wall preparations was 4.3%, but preliminary experiments showed that the glucose content in the walls was about 7%, giving a molar ratio of phosphorus to glucose only 1.00:0.25 in the intact wall. This value, obtained by direct analysis of unhydrolysed wall, was checked by the anthrone and ferricyanide reagents with total acid hydrolysates of the wall after treatment with ion-exchange resins to remove charged sugars and amino acids, which might interfere in the glucose assays. Values of 6.6% of glucose were obtained by all methods. Samples of the hydrolysates after treatment with ion-exchange resins were also estimated for glucose by quantitative paper chromatography (Baar, 1954), which gave slightly lower values. As shown in Table 1 the extract

obtained by extensive treatment of the walls with trichloroacetic acid at 37° gave a molar ratio of phosphorus to glucose similar to that found in intact walls. Incubation of this fraction with acid phosphatase under conditions identical with those used for the isolated teichoic acid released 27% of the total phosphorus as inorganic phosphate. Dialysis of the soluble fractions obtained by the acid extraction (Table 1) appeared to decrease preferentially the content of phosphorus in comparison with that of glucose. Thus over half of the phosphorus was removed after dialysis for 16 hr. at 4°, but about 75% of the glucose remained in the dialysis bag. The non-diffusible and diffusible fractions were analysed without further treatment and gave molar ratios of phosphorus to glucose 0.76 and 0.23 respectively. Acid hydrolysis of the non-diffusible and diffusible fractions gave products that were tentatively identified by paper chromatography as ribitol, glucose, alanine, anhydrosorbitol, glucosylribitol and phosphorylated derivatives of ribitol. These products are those expected to result from acid hydrolysis of the teichoic acid (Armstrong *et al.* 1961). Similarly, the main products of alkaline hydrolysis of both fractions were tentatively identified as ribitol, alanine, glucosylribitol and slow-moving phosphorylated compounds. The different values observed for the molar ratios of phosphorus to glucose in the non-diffusible and diffusible fractions were consistently observed on short-term (16 hr.) dialysis of total acid extracts of whole walls prepared by the methods of Janczura *et al.* (1961) or Salton & Horne (1951).

DISCUSSION

The chemical structure of the teichoic acid and teichuronic acid components isolated in the present work after acid treatment of the walls have not been extensively studied, but there is no reason to believe that the purified materials obtained in the present work differ greatly from those isolated previously by different methods from walls of *B. licheniformis* or of *B. subtilis*. Examination of the soluble fraction obtained by lysozyme action on intact wall preparations showed that, in the absence of acid treatment, about half of the mucopeptide fragments of the reaction mixture were associated on chromatography with the teichoic acid and teichuronic acid components. The points of elution of teichuronic acid in the chromatography of the products both of acid and enzyme action were identical, as shown in Figs. 1 and 3. This is interpreted as being circumstantial evidence that the teichuronic acid is not involved in binding the mucopeptide or teichoic acid components. This view is supported by the finding that a strong complex is present in fraction L3, which contains

little or no teichuronic acid (Table 3). The difference in the chromatographic behaviour of purified teichoic acid and the teichoic acid present in fractions L2 and L3 is pronounced. The component that contributes the greater negative charge in these last two fractions has not yet been identified, but clearly cannot be either the mucopeptide or teichuronic acid components. The evidence presented above demonstrates that the linkages between the teichoic acid and mucopeptide fragments resist further fractionation but are easily broken by treatment with dilute acid. Ghuysen & Strominger (1963) reported that the teichoic acid present in the cell walls of *Staphylococcus aureus* (Copenhagen) is joined in stable linkage to a part of the mucopeptide component of the walls. In this case also the complex was broken on treatment of the walls with trichloroacetic acid. The chemical analysis of the complexes L2 and L3 (Table 3) shows that the molar ratios of $\alpha\epsilon$ -diaminopimelic acid, used as an index of mucopeptide content, to glucose are relatively constant for fractions L2 and L3. By contrast, the molar ratios of $\alpha\epsilon$ -diaminopimelic acid to phosphorus are different, suggesting that the extent of formation of the complex is independent of the total phosphorus content of the fractions.

The purified teichoic acid of the strain of *B. subtilis* used by Armstrong *et al.* (1960) contained phosphorus and glucose in the molar ratio 1.0:0.93 and accounted for at least 30% of the weight of the wall. In the present work it was found that the molar ratio of phosphorus to glucose was 1.0:0.25 in intact wall preparations, although purified teichoic acid isolated here contained these constituents in almost equimolar amounts (Table 1). Similar results have been reported (Young *et al.* 1963) for several strains of *B. subtilis*. By using an enzyme test for the glucose content of whole cell-wall preparations of four strains of the organism, these authors found molar ratios of phosphorus to glucose ranging from 0.4 to 0.6.

The analyses of whole walls of *B. licheniformis* and of the complex fractions L2 and L3 (Fig. 3) clearly show that the teichoic acid in intact walls is heterogeneous with respect to side-chain substitution by glucose. The teichoic acid present in fraction L2 contains polyribitol phosphate chains with higher amounts of glucose than the teichoic acid of fraction L3. It is not known to what extent this heterogeneity is controlled by the time or conditions of growth of the organism or of the method of isolation of the cell walls.

The products obtained by acid extraction of walls were also shown to be heterogeneous in the length of the polyribitol phosphate chains by end-group assay with acid phosphatase. The average chain length of the ribitol phosphate

polymers of total trichloroacetic acid extracts of walls was considerably smaller than the value obtained by the same method for the purified teichoic acid. The acid extraction in the present work was carried out at 35°, and it is likely that fragmentation of the polyribitol phosphate chains had occurred (Strominger & Ghuysen, 1963). However, it is not ruled out that the short chains found in the diffusible fraction of acid extracts of walls may exist as such in the intact walls together with longer chains containing higher amounts of glucose units. No phosphorus is removed on dialysis of the soluble fraction obtained by lysozyme treatment of intact walls and containing 95% of the total phosphorus of the wall. If the short diffusible chains of polyribitol phosphate present in acid extracts exist as such in intact walls they are rendered non-diffusible by attachment to a component of high molecular weight.

I am grateful to Dr H. J. Rogers for advice and encouragement throughout this work and to Dr J. Walker for making available laboratory space. I thank Mr M. Stevens for technical assistance, and Mr I. Mathison for preparing the walls. This work was done during the tenure of a Helen Hay Whitney Foundation Fellowship.

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