Components of the Cell Wall of *Clostridium welchii* (Type A)

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1. The cell wall of *Clostridium welchii* (type A) contains alanine, 2,6-diaminopimelic acid, glutamic acid, glycine, glucosamine, muramic acid, galactosamine, mannosamine, ethanolamine, rhamnose, galactose and phosphorus. 2. Heating with formamide at 150° resolved the wall into a formamide-soluble polysaccharide fraction and a formamide-insoluble mucopeptide fraction. 3. The formamide-soluble fraction contained two components: an electrophoretically neutral polysaccharide made up of galactose, rhamnose, galactosamine and phosphorus and an electrophoretically acidic polymer containing mannosamine, ethanolamine and phosphorus. 4. The formamide-insoluble residue has been digested by lysozyme to give soluble fragments of high molecular weight. 5. All fractions contain an unknown ethyl acetate-extractable substance that can be oxidized by sodium metaperiodate. 6. The amino acid compositions of the fragments produced by lysozyme are compatible with a mucopeptide structure which has cross bridges containing all of the constituent amino acids.

Current ideas on the structure of the cell walls of Gram-positive bacteria are derived largely from two organisms: *Micrococcus lysodeikticus* and *Staphylococcus aureus* (see reviews: Salton, 1960, 1964; Rogers, 1962, 1965; Perkins, 1963a). The major structural component of these cell walls has been found (Ghuysen & Salton, 1960; Perkins, 1960) to consist of a 'backbone' of alternating, 1→4-linked (Jeanloz, Sharon & Flowers, 1963; Young, Tipper & Strominger, 1964; Tipper, Ghuysen & Strominger, 1965) *N*-acetylglucosamine and *N*-acetylmuramic acid residues to which is linked, through the carboxyl group of muramic acid, a peptide composed of D- and L-alanine, D-glutamic acid, L-lysine and glycine. This polymer has been called mucopeptide (Perkins & Rogers, 1959), and such a component, which appears to be responsible for the shape of the organism, is present to a greater or lesser extent in the cell wall of all bacteria. Frequently, L-lysine is substituted by LL-, DD- or *meso*-2,6-diaminopimelic acid, and occasionally by 2,4-diaminobutyric acid (Perkins & Cummins, 1964) or by either D- or L-ornithine (Work, 1964).

No cell walls yet investigated consist entirely of mucopeptide, and this is especially true for Gram-negative organisms which have several, complex layers in their cell walls. In the cell wall of Gram-positive organisms, however, mucopeptide is com-

monly the major component, accompanied by one or more polysaccharides. These additional polymers appear to be the principal antigens of the cell wall. For example, the type-specific antigens of haemolytic streptococci are polysaccharides associated with the mucopeptide in the cell wall (Krause, 1963). A series of teichoic acids, which are polyribitol or polyglycerol phosphates, has been isolated from the cell walls of several organisms and shown to react with antibodies prepared against whole cells (McCarty, 1959; Baddiley, 1962; Elliott, 1963).

The cell-wall polysaccharides are in close association with mucopeptide, and can be removed only by the rather vigorous treatment of extraction with trichloroacetic acid in the cold or hot, or even more drastic conditions of extraction with formamide at 150°. From digestion with lytic enzymes it has been shown that the mucopeptide and teichoic acid polymers in *Staphylococcus aureus* cell walls are covalently linked together (Ghuysen & Strominger, 1963; Ghuysen, Tipper & Strominger, 1965).

The present investigation was undertaken to see what types of polymers are present in the cell wall of the Gram-positive anaerobe *Clostridium welchii* (type A). Preliminary accounts of some of these results have been given already (Pickering, 1964, 1965).

MATERIAL AND METHODS

Growth of organism. *Clostridium welchii* (N.C.T.C. 8237) was revived in broth containing meat and then grown in

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litre bottles each containing 11. of Hedley-Wright broth (Wright, 1933). Immediately before inoculation 10 ml. of 40% glucose and 5 ml. of 0.1 M-thioglycolic acid were added to each bottle and mixed in. The inoculum was then introduced to the bottom of the bottle, which was incubated for 9 hr. at 35°, followed by 12 hr. at room temperature (about 16°). The organism was killed by the addition of 50 ml. of 40% (w/v) formaldehyde to each bottle, which was then left for 1 hr. at 2°. The cells were harvested by centrifugation and washed twice with water.

Preparation of cell wall. The cell wall was prepared by a combination of the methods of Salton & Horne (1951) and of Cummins & Harris (1956). The bacteria were suspended in water to give a final concentration of about 20 mg./ml., and 10 ml. portions were mixed with equal volumes of glass beads (Ballotini no. 12) and shaken on a Mickel disintegrator for 10 min. at 0–4°. The beads were removed by filtration through a grade 1 sintered-glass filter, and washed. In an attempt to remove unbroken cells, the combined filtrate and washings were centrifuged at 1100g for 10 min. However, the broken cells flocculated and cell wall was sedimented along with unbroken cells. This sediment was washed twice with water, brought to 70° during the second wash, and taken up in a minimum volume of 0.05 M-phosphate buffer, pH 7.6 (prepared from m-Na₂HPO₄ and m-KH₂PO₄). A trace of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was added and the suspension was shaken for 2½ hr. at 35°. The digest was centrifuged at 1100g for 10 min. The cell wall was washed at the centrifuge four times with m-NaCl and three times with water, and then taken up in 0.05 M-phosphate buffer, pH 7.6, containing trypsin (0.3 mg./ml.) and ribonuclease (0.3 mg./ml.); the cell-wall concentration was 20–30 mg./ml. After shaking for 2½ hr. at 35° the cell wall was sedimented at 10000g for 10 min., washed three times with water and finally taken up in a minimum volume of water and freeze-dried.

Extraction with trichloroacetic acid. Cell wall was extracted either with 10% (w/v) trichloroacetic acid at 2° for 48 hr. as described by Baddiley, Buchanan, RajBhandary & Sanderson (1962), or with 5% (w/v) trichloroacetic acid at 90° for 15 min. In either case, trichloroacetic acid was removed from the extracts by extraction with ether.

Extraction with hot formamide. Cell walls were extracted with formamide for 20 min. at 150° by the method of Fuller (1938) as described by Perkins (1963b). After the first extraction, the insoluble residue was dried with alcohol and ether, and then re-extracted with formamide. Polysaccharide was recovered from the combined extracts by precipitation with 10 vol. of acetone.

Exclusion chromatography on Sephadex. Sephadex G-25 and G-75 (bead form) were packed into glass columns 150 cm. × 2.1 cm. as described by Flodin (1961). The columns were washed, and subsequently eluted with 0.1 N-acetic acid. Suitable portions of the eluate were partially hydrolysed with 2 N-NaOH and then caused to react with ninhydrin as described by Moore & Stein (1954).

Oxidation with periodate. A Smith degradation procedure (Smith & Montgomery, 1956) was used as follows: the polymer to be oxidized was dissolved in 0.2 M-sodium acetate (adjusted to pH 5.8 with acetic acid) and an equal volume of 0.04 M-sodium metaperiodate was added. The concentration of the polymer was such that the periodate in the final reaction mixture was present in fivefold excess.

The oxidation was allowed to proceed at 2° for 24 hr. in the dark, and periodate uptake was determined spectrophotometrically by the method of Dixon & Lipkin (1954).

The polyaldehyde produced during the periodate oxidation was freed from sodium acetate and from excess of periodate by dialysis, freeze-dried and dissolved in a small volume of water. Sodium borohydride was added to give approximately tenfold excess, and the reduction was allowed to proceed for 16 hr. at room temperature (about 16°). The reaction mixture was then acidified with acetic acid, freeze-dried and freed from borate by repeated addition and evaporation of methanol. The polyol was hydrolysed with 2 N-H₂SO₄ for 2 hr. at 105°.

Paper and thin-layer chromatography. Whatman no. 1 and no. 4 papers were used. For amino sugar separations, no. 1 paper was pre-soaked in 0.1 M-BaCl₂ as described by Heyworth, Perkins & Walker (1961). Silica thin layers were prepared with silica gel G (Merck) according to Stahl (1965) on plates 3½ in. × 4½ in. Solvents used were: A, butan-1-ol-acetic acid-water (63:10:27, by vol.); B, butan-1-ol-pyridine-water (6:4:3, by vol.); C, butan-2-one-acetic acid-sat. aq. boric acid (9:1:1, by vol.); D, propan-1-ol-aq. NH₃ soln. (sp.gr. 0.88)-water (6:3:1, by vol.).

The following reagents were used for the detection of components on plates and papers: organic substances on thin layers by spraying with 6 N-H₂SO₄ and subsequent charring, or by spraying with alkaline KMnO₄ (Hay, Lewis & Smith, 1963); amino compounds by dipping in 0.1% ninhydrin in acetone, and/or the papers or plates were exposed to chlorine gas for 5 min., excess of chlorine was removed from the paper or plates by dipping them in ammonia vapour, and they were then dipped in a 1:1 mixture of 0.1 M-KI and 2% acetic acid saturated with o-tolidine (Rydon & Smith, 1952; Reindel & Hoppe, 1954); acetamido and amino sugars by a modification (Perkins, 1963b) of the method of Partridge (1948); reducing sugars with alkaline AgNO₃ (Trevelyan, Procter & Harrison, 1950), or with aniline phthalate (Partridge, 1949); α-glycols with the periodate-Schiff reagent of Baddiley, Buchanan, Handschumacher & Prescott (1956) or with the periodate-benzidine test (Smith, 1960); phosphorus with the sulphosalicylic acid-FeCl₃ reagent (Wade & Morgan, 1953).

Paper electrophoresis. This was performed in an apparatus based on that of Kunkel (1954) with the following buffers: pH 2.4, 87 ml. of acetic acid, 29 ml. of 25% (w/v) formic acid and water to 31.; pH 6.5, 26.7 ml. of collidine, 9.2 ml. of acetic acid and water to 31.; pH 9.0, 22.95 g. of NaHCO₃ and 2.91 g. of Na₂CO₃ dissolved in 31. of water; pH 11.0, 0.01 M-Na₂CO₃.

Conversion of hexosamines into pentoses (Stoffyn & Jeanloz, 1954). The sample containing hexosamine was dissolved in water to give an amino sugar concentration of about 2 mg./ml. and 20 μl. of this was mixed with 20 μl. of 2% (w/v) ninhydrin in aq. 4% (v/v) pyridine. The mixture was then heated at 105° for 25 min. and the resultant pentoses were separated by chromatography on no. 4 paper in solvent C.

Conversion of hexosamines into N-acetyl derivatives. Small portions of hydrolysates were N-acetylated with acetic anhydride in aqueous trimethylamine solution as described by Perkins (1963b).

Digestion with lytic enzymes. Crystalline egg-white lysozyme was used at a final concentration of 50 μg./ml.

The substrate was suspended (4 mg./ml.) in 0.1 M-ammonium acetate (brought to pH 6.1 with acetic acid). Digestion was allowed to proceed at 35° for 16 hr.

The lytic enzyme from *Chalaropsis* B (Hash, 1963) was a gift from Dr J. H. Hash to Dr H. J. Rogers, and was used at a final concentration of 33 µg./ml. with substrate suspended in 0.025 M-sodium acetate (brought to pH 4.7 with acetic acid).

A preparation containing the muramylamidase from *Streptomyces albus* (Ghuysen, Leyh-Bouille & Dierickx, 1962) was obtained through the courtesy of Dr J. M. Ghuysen. Substrate (5 mg./ml.) was dissolved in 0.025 M-sodium acetate (brought to pH 5.3 with acetic acid), crude enzyme (200 µg./ml.) was added and the mixture was incubated at 35° for 16 hr.

Analytical methods

Amino acids. These were determined after hydrolysis at 105° for 16 hr. with 6N-HCl: (a) by the paper-chromatographic method described by Mandelstam & Rogers (1959), and (b) with a Beckman automatic amino acid analyser by the method of Spackman, Stein & Moore (1958) as modified by Jacobs (1964). The essential part of the modification is that Jacobs separates basic amino acids on a 50 cm. column instead of a 15 cm. column.

Amino sugars. These were determined by the method of Rondle & Morgan (1955) after the hexosamines and muramic acid had been separated by adsorption on charcoal-Celite as described by Perkins & Rogers (1959).

Neutral sugars. These were determined with either the ferricyanide method of Park & Johnson (1949) or with the anthrone method of Mokrasch (1954). Hydrolysates were prepared with 2N-H₂SO₄ at 105° for 2 hr. and amino sugars were removed from the hydrolysate by passage through Zeo-Karb-225 (Perkins & Rogers, 1959). 6-Deoxyhexoses were determined by the method of Dische & Shettles (1948).

Galactose. A value for the galactose content of mixtures of galactose and rhamnose was obtained by first determining the total reducing sugars, and then deducting the value for the amount of rhamnose found by the Dische & Shettles (1948) method. Standard mixtures of galactose and rhamnose were used for this purpose.

Sialic acid. This was sought for by the methods of Werner & Odin (1952) and Warren (1959).

Phosphorus. This was determined by the method of Chen, Toribara & Warner (1956).

Nitrogen. This was estimated by the ninhydrin method of Jacobs (1962).

Acyl groups. These were determined by the method of Ludowieg & Dorfman (1960).

RESULTS

The composition of unfractionated cell wall is given in Table 1, and the components listed account for 57% of the weight of the wall. The values for 'amino sugar' were obtained from the aqueous eluates from charcoal columns (Perkins & Rogers, 1959), and paper chromatography of these eluates showed that they contained galactosamine, glucosamine and an amino sugar which had a higher *R_F* than glucosamine when chromatographed under the conditions of Heyworth *et al.* (1961). When hydrolysis of the cell wall with 4N-hydrochloric acid was increased from 4 hr. to 24 hr. the value for 'amino sugar' went up, and is given in parentheses in the Table. There was no parallel increase in total amino sugar before adsorption on charcoal, and the observed apparent increase represented the breakdown of a component, which, in the 4 hr. hydrolysate, bound tightly to the carbon. This component could be partially eluted with 70% ethanol, contained phosphorus and, as will become apparent later, was probably hexosamine phosphate.

Preparation of soluble fractions of cell wall

The turbidity of cell-wall suspensions was unaffected by incubation for 48 hr. at 35° with either lysozyme or *Chalaropsis* B lytic enzyme.

Extraction of cell wall with trichloroacetic acid. It was thought that the resistance of the cell wall to the action of lytic enzymes might be due to the presence of a teichoic acid or similar polymer. However, the residue remaining after the walls had

Table 1. *Composition of the cell wall of Clostridium welchii: effect of hot formamide extraction*

Component	Component (µmoles/100 mg. of wall or wall fraction)		
	Cell wall	Formamide residue	Formamide extract
Muramic acid	25.6	34.4	
Amino sugar	54.4 (62.8)	35.7 (50.7)	89.5
Alanine	65.3	85.9	
Diaminopimelic acid	39.2	51.5	
Glutamic acid	40.5	56.3	
Glycine	40.1	53.5	
Galactose	20.8		81.6
Rhamnose	22.4		74
Acyl	156	173	181
Phosphorus	87.5	52.7	141

been extracted with either cold or hot trichloroacetic acid was not affected by either of the lytic enzymes. Hydrolysates of the trichloroacetic acid extracts were found to contain galactose, rhamnose, galactosamine and phosphorus, but extraction was not complete, and the residues also contained these components. Paper electrophoresis of the extracts at pH 6.5 showed two components which could be stained by the chlorine-potassium iodide technique: one was neutral and the other acidic.

Extraction with formamide. Hot formamide has proved useful for extracting polysaccharides from cell walls, notably from haemolytic streptococci (Fuller, 1938; Krause & McCarty, 1961) and from *Propionibacterium* (Allsop & Work, 1963). In the latter, the residue after formamide extraction was lysozyme-sensitive although the original cell wall was not.

Batches of cell wall (500–600 mg.) were extracted with hot formamide. The combined weights of formamide residue and acetone precipitate from the extract accounted for 80–85% of the cell wall and, of this, 70% was formamide residue and 30% extract. The analyses of residue and extract are given in Table 1: all of the galactose and rhamnose present in the cell wall was accounted for in the formamide-extractable polysaccharide mixture.

Formamide-extractable polysaccharides

Paper electrophoretograms of the formamide-extracted polysaccharides were run in collidine acetate (pH 6.5) and stained with several developing reagents. Staining with chlorine-potassium iodide showed the same two bands seen in trichloroacetic acid extracts. One was neutral and migrated 1.5 cm. towards the cathode under the influence of electroendosmosis, and the other migrated 6 cm. towards the anode and was therefore acidic. Throughout the rest of this paper these will be referred to as the 'neutral' and 'acidic' polysaccharides respectively. Both of the bands gave positive reactions in the test for phosphorus, but neither was stained by ninhydrin. Only the 'neutral' polysaccharide could be shown to react with periodate in the periodate-benzidine test.

Since there was an obvious charge difference between the two polysaccharides, attempts were made to separate them by chromatography on two anion exchangers (DEAE-cellulose and Amberlite CG-4B). With both these exchangers it was possible to remove the 'acidic' polysaccharide from the mixture, but all attempts at subsequent elution failed, even when eluents of pH 3 and containing sodium chloride at a concentration of 5 M were used. However, it was found possible to separate them by exclusion chromatography on Sephadex. Fig. 1 shows the result obtained when

Fig. 1. Sephadex G-75 chromatography of the polysaccharide mixture extracted from cell wall with formamide. —, Ninhydrin colour (E_{570}) obtained after partial alkaline hydrolysis of 0.2 ml. portions of the fractions; ----, anthrone colour from 0.5 ml. portions of the fractions. The eluent was 0.1 N-acetic acid and the column size was 139 cm. $\times$ 2.1 cm.

150 mg. of formamide-soluble polysaccharide mixture was chromatographed on Sephadex G-75. Three major peaks were obtained when ninhydrin colour was determined in partial alkaline hydrolysates (Moore & Stein, 1954). The first peak (A) was very sharp, accounted for less than 5% of the weight applied to the column and showed all the usual mucopeptide constituents after hydrolysis. Eluates in peak B reacted with the anthrone reagent and electrophoretograms of material from this peak showed that it was largely 'neutral' polysaccharide with a small amount of the 'acidic' one. Conversely, peak C, which was anthrone negative, was found by electrophoresis to be largely 'acidic' polysaccharide.

'Neutral' polysaccharide. Material from the pooled eluates from G-75 peak B (Fig. 1) was re-chromatographed on Sephadex and further purified by passage through a column (10 cm. $\times$ 2 cm.) of Amberlite CG-4B in the acetate form. The 'neutral' polysaccharide obtained in this way was examined by paper electrophoresis at pH 2.5, pH 6.5, pH 9.0 and pH 11.0. These papers were stained by the chlorine-potassium iodide method, and in each case there was a single spot which moved the same distance as the urea used as a neutral marker.

The polysaccharide was hydrolysed with 2 N-sulphuric acid and the neutral sugar fraction prepared and examined by chromatography in solvents A and B, and by paper electrophoresis in 0.025 M-sodium tetraborate. In each case two reducing sugars with identical R_f values or mobilities to galactose and rhamnose were found. The

Table 2. Analysis of 'neutral' polysaccharide

Nitrogen content = 4.2% N, i.e. 301 $\mu\text{g. atom}/100\text{mg.}$

Component	Composition of polysaccharide	
	($\mu\text{moles}/100\text{mg.}$)	(%)
Galactose	115	18.7*
Rhamnose	120	19.7*
Galactosamine	112	17.9*
Phosphorus	139	11.1 (HPO_3)
Acyl	137	5.9 (CH_3CO)
Ammonia	31	0.5

* Calc. as anhydrosugar.

quantities of these sugars in the polysaccharide are given in Table 2.

Hydrolysates prepared with 4N-hydrochloric acid for 4 hr. were subjected to paper electrophoresis in collidine acetate (pH 6.5) and stained either specifically for amino sugars or with ninhydrin. In each case two spots were seen, one with the mobility of the hexosamines and one which was just acid. If the hydrolysis was performed with 6N-hydrochloric acid for 17 hr., this second spot was very much reduced. A sample of such a hydrolysate was oxidized with ninhydrin, and the reaction mixture showed a single spot with the R_f of lyxose in solvent C. This pentose can arise from the ninhydrin oxidation of either galactosamine or talosamine. However, when the original hydrolysate was run in solvent B on paper previously soaked in 0.1M-barium chloride, it showed one amino sugar spot with the R_f of galactosamine. Galactosamine and talosamine are readily separated in this chromatographic system (Heyworth *et al.* 1961; Pickering, 1965).

A sample of the 4N-hydrochloric acid hydrolysate was *N*-acetylated and the reaction product examined by paper electrophoresis in 0.025M-sodium tetraborate buffer, pH 9.1, for 5 hr. After staining for acetamido-sugars, the *N*-acetylated hydrolysate showed two spots which had moved 2.8 cm. and 15.2 cm. towards the anode, whereas *N*-acetylated galactosamine had moved 2.8 cm. towards the anode. The faster-moving spot also stained for phosphorus.

The 'neutral' polysaccharide was oxidized with sodium metaperiodate and found to consume 101 μmoles of periodate/100 mg. of polysaccharide. The product was reduced with sodium borohydride, hydrolysed with 2N-sulphuric acid and examined with paper- and thin-layer chromatography. The only neutral sugar present in the hydrolysate was galactose; rhamnose had completely disappeared. When the thin-layer plates were stained with the periodate-Schiff reagent or with potassium per-

manganate, a new polyol could be detected. It had R_f slightly higher than that of rhamnose but could be differentiated from propylene glycol in solvents A, B and D. All of the rhamnose remained intact when the polysaccharide was reduced with borohydride without prior oxidation with periodate.

Table 2 shows that only 50% of the total nitrogen in the polysaccharide can be accounted for as galactosamine, and a search was made for another nitrogenous component. No unidentifiable ninhydrin-reacting spots could be detected in hydrolysates chromatographed in several solvent systems. A hydrochloric acid hydrolysate of the polysaccharide was examined with the automatic amino acid analyser. The effluent from the 50 cm. column showed two peaks, one corresponding to galactosamine and the other a small peak with the retention time of ammonia. The effluent from the 150 cm. column also showed two peaks, an early one, which was retained very little, after which the base line remained steady until the emergence of galactosamine. The early peak was in the position described for galactosamine 6-phosphate by Liu & Gotschlich (1963).

When hydrolysates prepared with 6N-hydrochloric acid for 17 hr. were chromatographed on thin layers of silica in solvent A and either stained with potassium permanganate or charred with sulphuric acid, a spot was revealed with R_f 0.6. Because of its high R_f , attempts were made to extract the unknown substance from aqueous solution with ether. Nothing could be extracted in this way but ethyl acetate would extract it. The ethyl acetate-extracted material was then chromatographed on silica thin layers and stained with a variety of reagents. It could not be stained with aniline phthalate or ninhydrin, but gave a weakly positive reaction with chlorine-potassium iodide reagent and gave a purple spot with the periodate-Schiff technique. The ethyl acetate-extractable material when dissolved in 2N-ammonia solution could be partially extracted with ether.

'Acidic' polysaccharide. The eluates from peak C (Fig. 1) of the Sephadex G-75 chromatography of the formamide extract were pooled, dried and rechromatographed on Sephadex G-75. On paper electrophoresis and staining with the chlorine-potassium iodide reagent, the rechromatographed peak C material was found to be largely 'acidic' polysaccharide with a small contaminant, about 5-10%, of 'neutral' polysaccharide.

After hydrolysis with 2N-sulphuric acid for 2 hr. and preparation of a neutral sugar fraction, the 'acidic' polysaccharide was found to contain very little neutral sugar (3%). This was consistent with the 'neutral' polysaccharide contaminant and suggests that the 'acidic' polymer itself contains no neutral sugars.

Hydrolysates were prepared with hydrochloric acid and examined by paper electrophoresis and chromatography for ninhydrin- and amino sugar-reacting components. Electrophoresis at pH 6.5 showed two amino sugar spots, one with the mobility of the hexosamines and another which was just acid and contained phosphorus. There was also a spot which stained only with ninhydrin and which had a mobility greater than that of lysine.

The amino sugar present in the hydrolysate was found to move with the R_F of mannosamine in the system of Heyworth *et al.* (1961) and to give rise to arabinose on ninhydrin oxidation. *N*-Acetylated samples of the hydrolysate showed, on electrophoresis in borate, an acetamido sugar with the mobility of *N*-acetylmannosamine together with a phosphorus-containing acetamido sugar with high mobility.

Paper chromatograms of hydrolysates run in solvent A showed a ninhydrin spot which ran just faster than alanine. This substance was isolated by preparative paper chromatography and found to be the basic component seen in paper electrophoresis. It produced formaldehyde on periodate oxidation and had the same R_F as ethanolamine in solvents A and B. When a hydrolysate was chromatographed on the 50cm. column of the amino acid analyser, it showed a peak with the retention time of mannosamine (which is identical with that for galactosamine), a peak corresponding to ammonia and one which just preceded ammonia. A sample of ethanolamine was included with a mixed amino acid marker on the column and found to emerge just before ammonia.

The ethanolamine content of the 'acidic' polysaccharide was estimated from the size of the peak from the analyser as 40 μ moles/100mg. Hexosamine was found by the Randle & Morgan (1955) method to be 91 μ moles/100mg. and phosphorus was estimated as 124 μ moles/100mg. This leaves some 70% of the weight of the 'acidic' polysaccharide unaccounted for. The only other component which could be detected, but not isolated in weighable amounts, was an ethyl acetate-extractable substance similar to that found in hydrolysates of the 'neutral' polysaccharide.

The 'acidic' polymer was shown to be unaffected by periodate both by the observation that no periodate was consumed during a reaction with 4.1mg. of polymer for 24hr. and by the fact that all of its components could be detected unchanged in hydrolysates of the reaction mixture.

Formamide-insoluble residue

The residue remaining after formamide extraction of the cell wall contained the usual mucopolysaccharide constituents: alanine, 2,6-diaminopimelic

acid, glycine, glutamic acid, glucosamine and muramic acid. In addition, paper chromatography showed the presence of an amino sugar which moved faster than glucosamine in the system of Heyworth *et al.* (1961) and which was subsequently shown (Pickering, 1965) to be mannosamine. Paper electrophoresis of acid hydrolysates in collidine acetate showed the same highly basic spot which was seen in hydrolysates of the 'acidic' polysaccharide, and which had all the properties of ethanolamine.

When the residue was incubated with lysozyme for 16hr. at 35°, 95% went into solution. The remaining 5% was removed by centrifugation, and the supernatant was freeze-dried, redissolved in water and freeze-dried again to remove ammonium acetate. The digest was chromatographed on Sephadex G-25 and the result is shown in Fig. 2.

High-molecular-weight fraction. Most of the lysozyme digest (70%) was in the form of a high-molecular-weight fraction (peak A, Fig. 2) which was excluded from Sephadex G-25. Material from this peak was chromatographed on Sephadex G-75 and Fig. 3(a) shows that the fraction was very heterogeneous. To follow any change in composition across the molecular-weight spectrum, fractions were taken as shown in Fig. 3(a) and rechromatographed on Sephadex G-75 to give fractions A₁, B₂ and C as shown in Fig. 3(b), (c) and (d).

Fractions A₁, B₂ and C were analysed for amino acids, amino sugars and phosphorus, and the results are given in Table 3. In addition to the components listed, the recording obtained from the amino acid analyser with a 6N-hydrochloric acid hydrolysate

Fig. 2. Lysozyme digest of formamide residue chromatographed on a column (68cm. $\times$ 3.5cm.) of Sephadex G-25. The column was eluted with 0.1N-acetic acid and the curve shows the ninhydrin colour after partial alkaline hydrolysis of 0.5ml. portions of the fractions.

of fraction B₂ showed an early peak where a hexosamine phosphate might occur (Liu & Gotschlich, 1963). Formamide residue was hydrolysed with 4*N*-hydrochloric acid and the hydrolysate was put on a column of Dowex 50 (X8) and eluted with 0.3*N*-hydrochloric acid as suggested by Gardell (1953) for the separation of amino sugars. Portions of the eluate were analysed by the method of Rondle & Morgan (1955) and three amino sugar peaks were obtained (Fig. 4). Peak Y contained a mixture of glucosamine and mannosamine and peak Z was largely muramic acid. Peak X also contained phosphorus and when it was run on the

amino acid analyser it gave a peak in the early position occupied by hexosamine phosphates. When, however, a sample of this phosphorylated hexosamine was hydrolysed for 16 hr. in 6*N*-hydrochloric acid and the hydrolysate put back on the analyser, the early peak was reduced in size and a new peak appeared in the position of mannosamine.

Low-molecular-weight fraction. The low-molecular-weight material (Fig. 2, peak C), obtained from several experiments by chromatography of lysozyme digests on Sephadex G-25, was combined and rechromatographed on G-25. The fraction obtained in this way was then further fractionated by preparative paper electrophoresis on Whatman no. 3MM paper in collidine acetate buffer, pH 6.5, for 5½ hr. with a potential gradient of 12.5 v/cm. Guide strips stained with the chlorine-potassium iodide reagent showed the presence of four major bands (I, II, III and IV) which were respectively 0, 5.5, 8.5 and 12.5 cm. from the origin towards the anode. These bands were eluted with 0.1*N*-acetic acid and freeze-dried. The eluted peptides were dissolved in 1 ml. of water and two 300 µl. portions were taken from each. One portion of each pair

Fig. 3. Fractionation of the high-molecular-weight products from the lysozyme digest of formamide residue by chromatography on Sephadex G-75 (139 cm. $\times$ 2.1 cm.). (a) Chromatography of the excluded fraction from Sephadex G-25 (Fig. 2, peak A). (b), (c) and (d) Recchromatography of the fractions marked in (a). The analyses of the fractions (A₁, B₂ and C) contained within the horizontal bars in (b), (c) and (d) are given in Table 3.

Fig. 4. Chromatography on a column (39 cm. $\times$ 0.9 cm.) of Dowex 50 (X8; H⁺ form) of a 4*N*-HCl hydrolysate of formamide residue according to Gardell (1953). The curve shows the Rondle-Morgan colour obtained from 0.1 ml. portions of the fractions.

Table 3. Analysis of G-75 peaks from lysozyme digest (Fig. 3)

Peak.....	A ₁		B ₂		C	
	µmoles/ 100 mg.	Mole ratio	µmoles/ 100 mg.	Mole ratio	µmoles/ 100 mg.	Mole ratio
Alanine	77.1	2.2	69.4	1.8	97.4	2.5
Diaminopimelic acid	46.8	1.4	40.0	1.0	60.6	1.6
Glutamic acid	46.4	1.4	39.4	1.0	58.0	1.5
Glycine	38.9	1.1	30.7	0.8	44.5	1.2
Muramic acid	34.4	1.0	38.4	1.0	38.4	1.0
Glucosamine	33.3	1.0	33.0	0.9	40.8	1.1
Mannosamine	22.0	0.6	35.1	0.9	15.2	0.4
Ethanolamine	20.5	0.6	33.0	0.9	15.6	0.4
Phosphorus	48.4	1.4	69.6	1.8	30.6	0.8

was hydrolysed with 4*N*-hydrochloric acid for 4 hr. and analysed for hexosamine and muramic acid; the other portion was hydrolysed with 6*N*-hydrochloric acid for 16 hr. and analysed with the automatic amino acid analyser. None of the fractions contained mannosamine or the phosphorylated hexosamine, and the molar ratios of the amino acids, 2,6-diaminopimelic acid (DAP), glucosamine (GlcM) and muramic acid (Mur) were found to be:

- I, Ala_{5.5}, DAP_{1.0}, Glu_{4.0}, Gly_{1.0}, GlcM_{4.1}, Mur_{2.9};
 II, Ala_{3.6}, DAP_{1.7}, Glu_{2.6}, Gly_{1.0}, GlcM_{2.1}, Mur_{1.6};
 III, Ala_{1.1}, DAP_{0.2}, Glu_{1.0}, Gly_{0.2}, GlcM_{0.8}, Mur_{0.8};
 IV, Ala_{2.5}, DAP_{1.5}, Glu_{1.6}, Gly_{1.0}, GlcM_{1.2}, Mur_{0.9}.

Digestions with amidase. Several attempts were made to digest the various fractions of the lysozyme digest with a preparation containing the muramyl amidase from *Streptomyces albus*. The product of amidase digestion was treated with 1-fluoro-2,4-dinitrobenzene (Sanger, 1945), hydrolysed with 6*N*-hydrochloric acid and the resulting DNP-amino acids were separated according to Blackburn & Lowther (1951). In this way it was found that amidase digestion released *N*-terminal alanine, but only in very small quantities. For example, the product of amidase digestion of the high-molecular-weight fraction A₁ had an *N*-terminal alanine content of 7.7 μ moles/100 mg.; in other words, only 10% of the total alanine in this fraction became *N*-terminal as a result of digestion with the amidase preparation. No other *N*-terminal amino acids could be detected.

The amidase digest of A₁ was chromatographed on paper for 24 hr. in solvent A and the paper was stained with the diphenylamine-trichloroacetic acid reagent (Hough, Jones & Wadman, 1950; Ghuyssen & Strominger, 1963). Three pink spots were obtained with $R_{N\text{-acetylglucosamine}}$ values of 0.31, 0.50 and 0.71. A sample of the disaccharide *N*-acetylglucosaminyl-*N*-acetylmuramic acid obtained from the cell wall of *M. lysodeikticus* and kindly given by Dr Nathan Sharon was run under identical conditions and had $R_{N\text{-acetylglucosamine}}$ 0.84.

DISCUSSION

The cell wall of *Clostridium welchii* is a complex structure consisting of a mucopeptide-type polymer (Perkins & Rogers, 1959) which is very closely associated, probably by covalent linkage, with two other polymers. These can be extracted from the wall only by the drastic conditions of heating with formamide at 150°, and have been designated 'neutral' and 'acidic' on the basis of their electrophoretic properties. Even after this treatment the residue contains some components in common with the 'acidic' polymer.

From its behaviour during Sephadex chromatography, the 'neutral' polysaccharide would appear

to have a molecular weight in the neighbourhood of 20000. No change could be detected in the composition of the polymer after reduction with sodium borohydride, so that the number of reducing terminal groups must be very low, a conclusion which is supported by the low reducing power of the polymer. The known components of the 'neutral' polysaccharide are about equimolar, being (Table 2) rhamnose_{1.0}, galactose_{1.0}, galactosamine_{0.9}, phosphorus_{1.2}, acetyl_{1.1}. Since the polymer does not react with ninhydrin, the amino function of galactosamine is likely to be blocked by an *N*-acetyl group. It would seem that the phosphorus is linked to galactosamine, and from consideration of the acid stability of the hexosamine phosphate it is probably present as the 6-phosphate. During periodate oxidation, only rhamnose is destroyed, so that the galactose must have a substituent in the 3 position. The polyol which arises from the rhamnose after periodate oxidation and borohydride reduction is not propylene glycol and, since the rhamnose does not have a free C-1, the periodate must have cleaved a 2,3- α -glycol and the rhamnose must have been (1 $\rightarrow$ 4)-linked in the polymer. The polyol produced was presumably a 1,2,3-trihydroxybutane.

It seems, therefore, that the 'neutral' polysaccharide is a polymer containing equimolar proportions of rhamnose, galactose and *N*-acetyl-galactosamine 6-phosphate, in which the rhamnose occurs in (1 $\rightarrow$ 4)-linkage and the galactose is (1 $\rightarrow$ 3)-linked, or is 3-*O*-substituted or provides a branching point in a highly branched polysaccharide. Such a polymer, however, would be acidic, whereas it is in fact neutral, even at pH 2.4 and pH 11.0. There is an unknown constituent(s) which accounts for 27% of the weight of the polymer and for 50% of its nitrogen content, but even if this is a base which could compensate for the acidity of the phosphate at neutral pH, the resulting polymer might then be expected to be, at least slightly, negatively charged at pH 11.0 and positively charged at pH 2.4. The polymer, however, showed no evidence of being an ampholyte.

The 'acidic' polysaccharide contains mannosamine, ethanolamine and phosphorus, and, since the molar content of phosphorus is equal to the sum of those for mannosamine and ethanolamine, it is probably a polymer of mannosamine phosphate and ethanolamine phosphate. The observations that the polymer is neither affected by periodate nor has free reducing groups suggests that the mannosamine residues are linked into the polymer and are not present as short substituent side-chains. The known components together account for only 30% of the polymer and it is possible that it may be a lipopolysaccharide, especially since ethanolamine phosphate is usually associated with lipid.

However, attempts to find glycerol and fatty acid constituents were unsuccessful. Nothing is extracted from acidic hydrolysates with ether, and any fatty acids present must therefore be very polar ones. The only unknown constituent that has been detected is the ethyl acetate-extractable substance, which, however, seems to be basic rather than acidic. It can be partially extracted from dilute ammonia solution with ether, and, in view of its susceptibility to periodate oxidation, has many of the properties of a long-chain base of the sphingosine type. This unknown substance is common to both polysaccharides and has also been found in the formamide-insoluble residue.

The products of lysozyme digestion of the mucopeptide fraction were largely of high molecular weight, a large proportion being excluded from the gel matrix of Sephadex G-75 and thus having a molecular weight of 40000 or greater. The mucopeptide of *Staph. aureus* (Copenhagen) has been shown (Mandelstam & Strominger, 1961; Ghuyssen *et al.* 1965) to consist of a backbone of *N*-acetylglucosaminyl-*N*-acetylmuramic acid units, to each of which is linked the tetrapeptide L-Ala-D-Glu-L-Lys-D-Ala as a 'tail' from the carboxyl group of each muramic acid residue. This structure is then knitted together by pentaglycine cross-bridges

from the D-alanine of one 'tail' to the ϵ -NH₂ group of the lysine of another; these bridges may be intra-chain (forming a link between primary peptides derived from the same polysaccharide backbone) or inter-chain (joining primary peptides that stem from different polysaccharide chains). Lysozyme cleaves the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the backbone, and thus the products of lysozyme digestion of a highly cross-bridged mucopeptide similar to that described for *Staph. aureus* would be largely of high molecular weight, the proportion of high-molecular-weight product being directly related to the degree of cross-bridging in the original mucopeptide.

In common with many other organisms, there is insufficient glycine in the cell wall of *Cl. welchii* for pentaglycine bridges to occur in the mucopeptide of this organism (see Tables 1 and 3). Thus the cross-links must be composed of the same amino acids as the peptide 'tails' and the simplest way of achieving this is to suggest that the cross-bridge is identical with the 'tail' as shown in Fig. 5. The mucopeptide would then be a polymer of a unit similar to that shown in the Figure, and although for convenience the cross-bridge is shown as intra-chain, it is possible that the bridges are both intra- and inter-chain. Moreover, the presence of the multifunctional amino acid diaminopimelic acid makes it very likely that there are also some inter-bridge linkages.

The evidence for the type of structure shown in Fig. 5 is largely circumstantial. The structure satisfies the observed amino acid compositions of fractions A₁ and C (Table 3) and of the low-molecular-weight fraction IV, i.e. Ala₅, DAP₃, Gly₂, Glu₃, GlcM₂, Mur₂. It is also analogous to the structure suggested by Ghuyssen *et al.* (1965) for the mucopeptide of *Staph. aureus* (Copenhagen). Moreover, the results of analysis, titration and end-group determinations in the cell wall of *M. lysodeikticus* (Salton, 1961; Czerkawski, Perkins & Rogers, 1963) allow a similar structure to be

Fig. 5. Suggestion of a possible structure for a unit of the mucopeptide polymer of *Clostridium welchii*. For details see the text.

Fig. 6. Suggestion of a possible structure for a unit of the mucopeptide of *Micrococcus lysodeikticus*. See also Table 4.

Table 4. *Cell wall of Micrococcus lysodeikticus: comparison of composition found by experiment with that expected from hypothetical structure given in Fig. 6*

Component	Moles/unit	Mol. prop. (Mur=1.00)	
		Expected	Found*
Muramic acid	5	1.00	1.00
Glucosamine	5	1.00	1.28
Alanine	14	2.80	2.54
Glutamic acid	7	1.40	1.33
Glycine	7	1.40	1.44
Lysine	7	1.40	1.35
-NH ₂ -termini	4	0.80	0.64, 0.90†
-CO ₂ H-termini { Gly	7	1.40	0.68‡
	Ala	1	0.10‡
-CO ₂ H (titratable)	9	1.80	2.18, 1.58§

* Czerkawski *et al.* (1963).

† Perkins (1963b).

‡ Salton (1961).

§ After allowance for aminomannuronic acid-glucose polymer (Perkins, 1963b).

postulated for the mucopeptide of this organism. A further complication with *M. lysodeikticus* is that 20% of the muramic acid in its cell wall is unsubstituted by peptide (Perkins & Rogers, 1959; Rogers, 1962) and this has been allowed for in the hypothetical structure shown in Fig. 6. Again, for convenience, only intra-chain bridges are shown, although both intra- and inter-chain links almost certainly occur. Glycine has been assigned to the free carboxyl group of glutamic acid as suggested by Tipper & Strominger (1965). Table 4 compares the analytical values obtained for *M. lysodeikticus* with those expected from the hypothetical structure, and it will be seen that there is good agreement.

It has been pointed out above that the hypothetical structure suggested for the mucopeptide of *Cl. welchii* can accommodate the high-molecular-weight fractions A₁ and C and also the low-molecular-weight fraction IV. Low-molecular-weight fractions I and II are almost certainly mixtures, and III probably represents a fragment of the unit namely: *N*-acetylGlcM-*N*-acetylMur-Ala-Glu. The amino acid composition of high-molecular-weight fraction B₂ (Ala₂, DAP₁, Glu₁, Gly₁, GlcM₁, Mur₁) is, however, just what would be expected for a mucopeptide without cross-bridges. Such a mucopeptide would, however, be digested by lysozyme to disaccharide-tetrapeptide which has a relatively low molecular weight. How then could fraction B₂ arise? Table 3 shows that B₂ has the highest concentration of mannosamine, ethanolamine and phosphorus; there is in fact 1 mole of each of the amino compounds and 2 moles of phosphorus for each mole of muramic acid, and it may be that the digested mucopeptide is held together by linkage to some kind of polymer of

mannosamine phosphate and ethanolamine phosphate. It can be seen from Table 3 that although the amount of mannosamine and ethanolamine relative to the other constituents varies from fraction to fraction, these two components are in each case present in equimolar proportions and, moreover, the sum of their molar proportions is equal to the molar proportion of phosphorus in each fraction. Attempts have been made to separate a mannosamine-ethanolamine-phosphorus polymer from mucopeptide by chromatography on DEAE-cellulose and by digestion with phosphodiesterases, but they have all been unsuccessful. The 'acidic' polysaccharide also contains mannosamine phosphate and ethanolamine phosphate, and it may be that the presence of these compounds in the formamide-insoluble residue reflects the linkage points of mucopeptide and 'acidic' polysaccharide in the intact cell wall. The possibility that mannosamine phosphate and ethanolamine phosphate are integral parts of the mucopeptide of *Cl. welchii* cannot, however, be excluded.

In conclusion, the cell wall of *Cl. welchii* (type A) would appear to consist of a mucopeptide which is highly cross-linked by peptide bridges and perhaps also by bridges containing mannosamine phosphate and ethanolamine phosphate. In the intact cell wall the mucopeptide is very closely associated with two polysaccharides, which can be extracted only by the very harsh treatment of heating with formamide at 150°.

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