Autolysis of *Bacillus cereus* Cell Walls and Isolation of Structural Components

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Autolysis of *Bacillus cereus* N.R.R.L. 569 cell walls was accompanied by hydrolysis of the majority of the 4-*O*- β -*N*-acetylglucosaminyl-*N*-acetylmuramic acid linkages in mucopeptide, presumably by an endo- β -*N*-acetylglucosaminidase. Hydrolysis of the *N*-acetylmuramyl-L-alanine linkages by an amidase also occurred. Free D-alanine residues were detected in isolated cell walls and the proportion of these residues increased during autolysis, presumably due to D-alanine carboxypeptidase action. Fractionation and analysis of the products of autolysis confirmed these results. Among the products originating from mucopeptide were a disaccharide, *N*-acetylmuramyl-*N*-acetylglucosamine, and a tetrapeptide of sequence L-Ala-D-Glu-meso-Dap-D-Ala (Dap=diaminopimelate). A dimer fraction containing a D-Ala-meso-Dap cross-link was also isolated. Two polysaccharides were obtained from the products of autolysed cell walls and from walls made soluble by *Chalaropsis* B glycosidase. A neutral polysaccharide accounted for about 40% of the wall and contained *N*-acetylglucosamine, *N*-acetylgalactosamine and glucose. The neutral polysaccharide isolated from wall autolysates was attached to a part of the glycan moiety of mucopeptide. The molecular weight of the complex was approx. 28 000. Stoichiometric amounts of phosphorus were present, possibly in linkages between the polysaccharide and mucopeptide moieties. The second polysaccharide accounted for 12% of the wall and was very acidic. After acidic hydrolysis of the polysaccharide, glucosamine, galactosamine and unidentified acidic substances were detected. The acid polysaccharide isolated from wall autolysates contained only traces of mucopeptide constituents and no phosphorus.

The mucopeptide component present in cell walls of the Gram-positive Bacillaceae typically contains meso-diaminopimelic acid, D-glutamic acid, D- and L-alanine, glucosamine and muramic acid (Ghuysen, 1968) and is therefore identical in composition with the mucopeptide of many Gram-negative bacteria (Weidel & Pelzer, 1964). The glycan portion of mucopeptide consists of alternating β -(1 $\rightarrow$ 4)-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid and the D-lactate moiety of muramic acid is substituted by a short peptide side chain. Usually the peptide side chains are cross-linked by direct peptide bonds between a C-terminal D-alanine residue of one side chain and the free amino group of diaminopimelic acid in a second side chain. The degree of cross-linking is not extensive (Hughes, Pavlik, Rogers & Tanner, 1968; Hughes, 1970b) and resembles that occurring in the mucopeptide structure of Gram-negative bacteria.

Historically, *Bacillus cereus* is a noteworthy member of the group because of the serological similarity of certain strains to *Bacillus anthracis* (Ivanovics & Foldes, 1958). The cross-reaction is

due to a polysaccharide containing *N*-acetylhexosamines and galactose that is present in these organisms, probably as part of the cell wall (Mester, 1960; Mester, Moczar & Ivanovics, 1962). The present paper describes some chemical properties of *B. cereus* N.R.R.L. 569 cell walls, a strain that has been extensively used by other workers (Kogut, Pollock & Tridgell, 1956; Ozer, Lowery & Saz, 1970) for penicillinase induction. The cell wall is shown to contain at least three components; the mucopeptide, a neutral polysaccharide related to but not identical with the one mentioned previously, and an acidic polysaccharide. The starting material for isolation of wall components was the soluble fraction produced by autolysis of isolated walls, and the mode of action of the autolytic enzymes is described.

MATERIALS AND METHODS

Crystalline glucosaminol was a gift from Dr C. D. Warren and Dr R. H. Gigg. Sodium boro[^3H]hydride ($94\mu\text{Ci}/\mu\text{mol}$; The Radiochemical Centre, Amersham,

Bucks., U.K.) was diluted with unlabelled NaBH_4 to approx. $2\mu\text{Ci}/\mu\text{mol}$ before use. Pig epididymal exo- β -N-acetylglucosaminidase was prepared as described by Findlay & Levvy (1960). Lysostaphin was a product of Mead Johnson and Co., Evansville, Ind., U.S.A. The *Chalaropsis* B glycosidase preparation was from the American Cyanamid Co., Pearl River, N.Y., U.S.A.

Radioactivity counting of aqueous samples (0.5 ml) was performed in 10 ml of dioxan containing naphthalene (18%), 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenoxazol-2-yl)benzene (0.01%) in a Packard Tri-Carb liquid-scintillation spectrometer. The radioactivities of paper strips were counted in 2 ml of toluene containing 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%).

Preparation of cell walls. *B. cereus* N.R.R.L. 569 was obtained from the Torrey Institute, Aberdeen, U.K. (Cat. no. N.C.I.B., 7464), and grown at 35°C into late exponential phase in a casein hydrolysate medium described by Janczura, Perkins & Rogers (1961) and supplemented with 1% of glucose. Cells were harvested at 2°C and broken in a Braun homogenizer by shaking with glass beads. The temperature did not exceed 2°C . Walls were prepared (Janczura *et al.* 1961) by differential centrifugation at 2°C . When required, the wall autolytic enzymes were inactivated by heating a suspension of the walls (10 mg/ml) at 100°C for 30 min (Janczura *et al.* 1961). In one experiment the culture medium contained [^{32}P]-phosphate (The Radiochemical Centre) at $0.1\mu\text{Ci}/\text{ml}$ final concentration. The cell walls were washed with cold 0.1 M-potassium phosphate buffer, pH 7.0, at an early stage during the isolation procedure.

Analytical methods. Many of the procedures have been described by Hughes (1970a, b). Analysis for total amino acids and hexosamines were carried out after hydrolysis in 4 M-HCl at 105°C for 16 h, by using an amino acid analyser. Reducing power was determined by the method of Park & Johnson (1949).

Chromatography. Ion-exchange chromatography was carried out on columns (2 cm $\times$ 40 cm) of DEAE-cellulose (DE11, Whatman). The resin was equilibrated and run initially in 0.01 M-pyridine acetate buffer, pH 5.1, and 40 fractions (each 5.0 ml) were collected. Final elution of the columns employed a salt gradient established by running 5.0 M-pyridine acetate buffer, pH 5.1, into a mixing flask containing 0.01 M-pyridine acetate buffer, pH 5.1 (1.21). Columns of Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) G-50, G-25 and G-15 were operated at room temperature in 0.1 M-pyridine acetate buffer, pH 5.1. Material to be fractionated was applied to the columns (2 cm $\times$ 140 cm, 2 cm $\times$ 140 cm and 2 cm $\times$ 40 cm respectively) and fractions (2.5, 2.5 and 2.0 ml respectively) were collected. Pooled fractions were freeze-dried to remove buffer salts.

Paper chromatography on Whatman no. 3 MM or no. 1 paper employed the following solvent systems: A, isobutyric acid-0.5 M- NH_3 (5:3, v/v); B, butan-1-ol-pyridine-water (6:4:3, by vol.); C, butan-1-ol-acetic acid-water (4:1:5, by vol.); D, propan-1-ol-0.2% NH_3 (17:3, v/v); E, 0.75 M-sodium phosphate buffer, pH 6.1; F, butan-1-ol-acetic acid-water (25:6:25, by vol.).

Electrophoresis. Paper electrophoresis on Whatman no. 3 MM paper was carried out at 60 V/cm for various

times in the buffers: G, pyridine-acetic acid-water mixtures, pH 3.6 (6:46:323, by vol.), pH 4.7 (1:1:98, by vol.) or pH 6.5 (26:1:224, by vol.); H, 0.012 M-sodium molybdate, pH 5.0, as described by Mayer & Westphal (1968) except that the solvent was of lower molarity; I, 2 M-acetic acid.

Identification of hexosamine reducing groups with sodium borol[^3H]hydride. The methods were described in detail by Hughes (1970b). Briefly, a sample of soluble wall fragments (containing up to $4.0\mu\text{mol}$ of glucosamine after acid hydrolysis) was treated with an excess of NaB^3H_4 (1%, w/v, 0.4 ml) in 50 mM-sodium borate buffer, pH 8.9, at 35°C overnight. Excess of reagent was destroyed with 12 M-HCl (0.2 ml) and the mixture was heated at 100°C for 4 h. After repeated drying from water and methanol the hydrolysate was dissolved in water (0.5 ml) and the reduced sugar alcohol was separated from unchanged hexosamines by chromatography on Dowex 50 for further identification. Details of the chromatographic method (Hughes, 1970b) are given in Fig. 3. The amount of the sugar alcohol isolated by chromatography was determined either by the extent of incorporation of radioactivity, with glucosamine as a standard, or by the formaldehyde released during periodate oxidation at a low pH value. In acid solution standard glucosaminol gives exactly 1 mol.prop. of formaldehyde from the primary alcohol group at C-6 in the rapid stage of periodate oxidation while oxidation of the vicinal-amino alcohol group is hindered by ionization of the amino group at the low pH used.

RESULTS

Composition of cell walls and conditions for solubilization. The analyses (Table 1) of one batch of walls isolated from late exponential-phase cells accounted for approx. 80% of the dry weight of the preparation. In addition small amounts of amino acids not present in the mucopeptide were found and represented in total a further 5–6% of the dry weight. The mucopeptide constituents D-glutamic acid, L-alanine, meso-diaminopimelic acid and muramic acid were present in approx. molar ratios 1.0:0.8:1.0:0.6. In addition, D-alanine (about 1 mol.prop.) was found and was presumably totally derived from the mucopeptide since although phosphorus was detected (Table 1) these walls lack teichoic acid, as described below. Glucosamine was found in large molar excess over the other mucopeptide constituents because of the presence in the walls of polysaccharides containing this amino sugar as well as galactosamine and anthrone-positive hexose material. Glucose was identified in acid hydrolysates of walls by chromatography in solvent B and by reaction with glucose oxidase. Glucose was expected to be present in a polysaccharide from *B. cereus* N.R.R.L. 569 cell walls (Mester, 1960; Mester *et al.* 1962).

B. cereus cell walls suspended in various buffers (50 mM) between pH 5.5 and 8.5 at 35°C autolysed rapidly and in each case at the end of the reaction

Table 1. *Composition of B. cereus cell walls*

Walls were prepared from exponentially growing cells and analysed as described in the Materials and Methods section. Values refer to dry wt. of material and are corrected for ash content.

Substance	Composition		Molar ratios
	($\mu\text{mol}/100\text{mg}$)	(% of dry wt.)	
<i>meso</i> -Diaminopimelic acid	45.3	8.7	1.07
D-Glutamic acid	43.5	6.4	1.00
Total alanine	91.4	7.9	2.10
L-Alanine	35.7	—	0.83
Muramic acid	26.7	—	—
(as <i>N</i> -acetylmuramic acid)	—	8.3	0.62
Glucosamine	100	—	—
(as <i>N</i> -acetylglucosamine)	—	22.1	2.30
Galactosamine	64.3	—	—
(as <i>N</i> -acetylgalactosamine)	—	14.4	1.48
Hexose	58.5	10.8	1.35
Phosphorus	17.7	0.54	0.41
Ammonia	106	1.8	2.44
Recovery (%)	—	81.3	—

the turbidity of the suspension had decreased to less than 10% of the initial value (see Fig. 1*d*). The optimum pH was 7.0–7.5. The effects of three enzymes known to be lytic for other bacterial cell walls were tested with *B. cereus* N.R.R.L. 569 walls (Fig. 1). The cell walls were not solubilized by high concentrations (100 $\mu\text{g}/\text{ml}$) of egg-white lysozyme. The walls remained completely resistant after treatment with 0.1M-sodium hydroxide at 35°C for several hours, conditions that would be expected to remove ester groups from mucopeptide. The presence of such groups has been found to protect other cell walls from lysis by lysozyme (Brumfitt, Wardlaw & Park, 1958). Lysostaphin (Browder, Zygmunt, Young & Tavormina, 1965), an endo- β -*N*-acetylglucosaminidase, slowly solubilized *B. cereus* N.R.R.L. 569 cell walls but complete solubilization was never approached. The endo- β -*N*-acetylmuramidase from *Chalaropsis* B (Hash, 1963; Tipper, Strominger & Ghuyssen, 1964) caused a rapid initial decrease in the turbidity of wall suspensions to about 30% of the original value. A further decrease in turbidity required extended times of incubation and eventually the wall suspension was almost completely solubilized (Fig. 1*c*).

Characterization of the autolytic activity. Cell walls from late-exponential-phase cells were suspended at 4mg/ml in 50mM-potassium phosphate buffer, pH 7.6, and shaken at 35°C. Samples (0.45 ml) were removed immediately and after overnight incubation for the determination of *N*-terminal amino acids. Samples (0.1 ml) were removed more frequently for measurement of reducing power and residual turbidity. Autolysis of the walls was complete within 5h, as measured by the decrease in the

Fig. 1. Effect of enzymes on *Bacillus cereus* walls. (a) Heated walls (5 mg) were suspended in 0.05M-ammonium acetate, pH 6.5, (5.0 ml) containing lysozyme (500 μg); (b) heated walls (5 mg), suspended in 0.05M-ammonium acetate, pH 7.5, (5 ml), were treated with lysostaphin (500 μg); (c) heated walls (5 mg) were treated with *Chalaropsis* B enzyme (100 μg) in 0.05M-ammonium acetate, pH 5.5; (d) unheated walls (5 mg) were incubated in 0.05M-ammonium acetate, pH 7.5, (5 ml) to measure autolysis. All incubations were at 35°C and turbidity was measured at 675 nm. Walls were heated where indicated in water at 100°C for 30 min to inactivate autolytic enzymes.

turbidity (see Fig. 1*d*). After 24h the soluble mucopeptide contained (Table 2) approx. 0.75 mol of *N*-terminal alanine/mol of glutamate, indicating an incomplete hydrolysis of *N*-acetylmuramyl-L-alanine linkages by an amidase activity. A limited

Table 2. Reducing groups and amino groups liberated from *B. cereus* cell walls during autolysis at pH 7.6 and 35°C

Values are expressed as $\mu\text{mol}/100\text{ mg}$ of cell walls. The content of free alanine in the walls was measured as the DNP-derivative extracted into ether from acid solution before total acid hydrolysis of 1-fluoro-2,4-dinitrobenzene-treated material. Total quantities of constituents in this batch of cell walls, expressed as $\mu\text{mol}/100\text{ mg}$, were: glutamic acid, 38.1; diaminopimelic acid, 39.5.

Time of autolysis (h)	Reducing sugar (as <i>N</i> -acetylglucosamine)	<i>N</i> -Terminal groups of mucopeptide		Free alanine
		Alanine	Diaminopimelic acid	
0	3.2	5.7	21.3	9.1
24	20.7	29.5	23.4	14.5

amount of *N*-terminal alanine residues of mucopeptide were detected in the zero-time sample and this presumably was due to amidase action taking place either during the preparation of walls or during normal growth of the cells. A substantial amount of free alanine was found in the walls, even in the sample taken immediately after suspension, and this amount increased by 60% during autolysis. Free D-alanine was later identified among the products of autolysis. Presumably these residues were produced from *C*-terminal residues of the peptide side chains of mucopeptide, by D-alanine carboxypeptidase. There was no detectable change in the proportion of free amino groups of diaminopimelic acid during autolysis.

Reducing groups were also released during autolysis and reached a maximum of 0.6–0.7 mol of equivalent *N*-acetylglucosamine/mol of glutamate. This value is an approximation to the actual proportion of glycosidic bonds hydrolysed, since the reducing power of the oligosaccharides produced by hydrolysis of the mucopeptide glycan chains were unlikely to have the same reducing power as *N*-acetylglucosamine.

These results indicated that the autolytic system of *B. cereus* N.R.R.L. 569 cell walls contained an amidase, a glycosidase and possibly D-alanine carboxypeptidase activity. The glycosidase activity was identified as an endo- β -*N*-acetylglucosaminidase as follows. Cell walls (12 mg), made soluble by autolysis overnight at 35°C in 0.05 M-ammonium acetate buffer, pH 7.5, were treated with sodium boro[^3H]hydride. After acidification and hydrolysis, the reaction mixture was fractionated by chromatography on Dowex 50 (Fig. 2a). Radioactivity was found (Fig. 2a) in glucosaminol and, to a much smaller extent, in muramicitol [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucitol] (approx. 9% of the counts found in glucosaminol). In addition radioactivity was eluted unretarded from the Dowex 50 column and was found in control mixtures containing only acidified sodium boro[^3H]hydride.

Fig. 2. Identification of free reducing groups of hexosamines in solubilized cell walls. (a) Walls (12 mg) were autolysed in 50 mM-ammonium acetate, pH 7.5, (3.0 ml) overnight at 35°C; (b) walls (12 mg) were heated at 100°C for 30 min and incubated in 50 mM-ammonium acetate, pH 5.5, (3.0 ml) with *Chalaropsis* B endo- β -*N*-acetylmuramidase (100 μg) overnight at 35°C. The solubilized walls were freeze-dried and treated with NaB^3H_4 as described in the Materials and Methods section. After hydrolysis in 4 M-HCl (0.6 ml) at 100°C for 4 h the mixtures were dried, dissolved in water (0.5 ml) and portions (0.4 ml) were applied to columns (0.84 cm $\times$ 22 cm) of Dowex 50 (X8; 200–400 mesh), equilibrated in 0.1 M-pyridine acetate buffer, pH 2.8. With the same buffer 35 fractions (2.0 ml) were eluted and then additional fractions (2.0 ml) were eluted with 0.133 M-pyridine acetate buffer, pH 3.85. Portions (0.2 ml) of the fractions were analysed for radioactivity (—) and for hexosamines (E_{530}) (----). 1, [^3H]muramicitol; 2, muramic acid; 3, [^3H]glucosaminol; 4, glucosamine.

Presumably this material was present owing to incomplete exchange of tritium from the reaction mixture or to a radioactive impurity in the reducing agent. The recovery of glucosaminol from Dowex 50 (Fig. 2a) was 0.73 mol/mol of diaminopimelic acid equivalents in the material taken for reduction. Allowing for losses during hydrolysis and chromato-

graphy, this value suggests that the majority of the β -*N*-acetylglucosaminide linkages of mucopeptide had been hydrolysed during autolysis. Results described below confirmed that at least 85% of the bonds were broken. A sample of cell walls (12 mg) solubilized by *Chalaropsis* B endo- β -*N*-acetylmuramidase was also treated with sodium boro- ^{3}H hydride. ^{3}H Muramicitol was clearly identified by chromatography of the hydrolysed reaction mixture on Dowex 50 (Fig. 2b). Measurement of the muramic acid remaining after treatment showed that approximately two-thirds of the total *N*-acetylmuramic acid residues had reacted with the reducing agent. The small amount of radioactivity found in glucosaminol (Fig. 2b) presumably reflects an activity of the autolytic endo- β -*N*-acetylglucosaminidase *in vivo*. From the ratio of the radioactivity in the ^{3}H glucosaminol peaks isolated from walls solubilized by autolysis (Fig. 2a) and by *Chalaropsis* B enzyme (Fig. 2b), the extent of activity *in vivo* could not be more than hydrolysis of about 10% of the total number of β -*N*-acetylglucosaminide linkages in mucopeptide.

First fractionation of autolysed walls. Cell walls (224 mg) labelled with ^{32}P were shaken at 35°C in 50 mM-ammonium acetate buffer, pH 7.5, for 24 h. A small residue (7 mg) was removed by centrifugation and was found to contain amino acids typical of protein, but no diaminopimelic acid. The soluble material was freeze-dried, dissolved in water (5.0 ml) and fractionated on a column (2 cm $\times$ 40 cm) of DEAE-cellulose (Fig. 3). A large peak fraction, A1, containing peptides, hexosamine material and glucose was eluted with dilute buffer and two other fractions, A2 and A3, were eluted with higher salt concentrations. Fraction A2 (Table 3) contained all of the constituents of mucopeptide and accounted for 15% of the total diaminopimelic acid recovered from the column. Galactosamine and glucose were absent or present in trace amounts. Fraction A3 (Table 3) contained only traces of amino acids or

Fig. 3. DEAE-cellulose chromatography of wall material made soluble after autolysis at pH 7.5. Walls (224 mg) were autolysed overnight at 35°C in 0.05 M-ammonium acetate, pH 7.5 (40 ml). The soluble fraction was applied to a column (2 cm $\times$ 40 cm) of resin in 0.01 M-pyridine acetate buffer, pH 5.1. Elution was started with the same buffer (200 ml) and fractions (5 ml) were collected. A gradient was then made by running 5 M-pyridine acetate buffer, pH 5.1, into 0.01 M-pyridine acetate buffer, pH 5.1, (1.2 l) and elution was continued. Fractions were analysed for ninhydrin-positive material (E_{570}) (—), neutral hexoses (E_{625}) (---) and, after acid hydrolysis, for total hexosamines (E_{530}) (○). Fractions were pooled as indicated by the arrows. Hexosamine material was also present in large amount in fraction A1 (not shown).

Table 3. Analysis of fractions isolated by DEAE-cellulose chromatography

B. cereus cell walls (224 mg) from Table 1 were autolysed and the soluble products were separated into three fractions as shown in Fig. 3. Analyses were carried out as described in the Materials and Methods section. tr, Trace.

Substance	Content ($\mu\text{mol}/\text{fraction}$)			Molar proportions	
	A1	A2	A3	A1	A2
Diaminopimelic acid	66	15	1	1.1	0.7
Glutamic acid	60	18	1	1.0	1.0
Alanine	129	24	2	2.1	1.3
Muramic acid	29	16	tr	0.5	0.9
Glucosamine	137	19	31	2.3	1.1
Galactosamine	130	tr	29	2.2	—
Hexose	139	0	0	2.3	—
Phosphorus	5.4	0	35	0.1	—

muramic acid and appeared to be a polysaccharide containing glucosamine and galactosamine. No glucose was present. In addition this fraction accounted for 87% of the total phosphorus content of the walls. The remaining phosphorus was found in fraction A1 (Table 3).

It is noteworthy that fraction A3 was eluted from DEAE-cellulose in the position at which the glycan moiety of the mucopeptide component of *Bacillus licheniformis* and *Bacillus subtilis* cell walls was eluted under identical conditions (Hughes, 1970b). Fraction A3 isolated from autolysates of *B. cereus* cell walls contained only traces of glycan fragments. Presumably the much shorter fragments of glycan present in *B. cereus* wall autolysates were eluted earlier from the DEAE-cellulose column in fraction A1 than were the longer glycan chains present in autolysed walls of the other organisms. Hughes (1970b) has shown that the glycan moieties isolated from *B. licheniformis* and *B. subtilis* wall autolysates contained on average 10 hexosamine residues whereas autolysis of *B. cereus* walls was accompanied by extensive hydrolysis of the glycosidic linkages of the glycan chains of mucopeptide-producing disaccharide and tetrasaccharide fragments.

A sample (equivalent to 55 μ mol of diaminopimelic acid) of peak A1 (Table 3) containing the major part of the mucopeptide constituents was separated further on a column (2 cm $\times$ 140 cm) of Sephadex G-25 (Fig. 4). Four well-resolved peaks were obtained and the analyses are shown in Table 4. Fraction S4 contained free D-alanine that presumably was produced by D-alanine carboxypeptidase action on the free C-terminal D-alanine residues of mucopeptide. Approx. 30% of the total D-alanine residues of walls was found in fraction S4. Consequently the alanine/glutamic acid molar ratios in the peptides present in the purified fractions S2 and S3 (1.7 and 1.5 respectively) were lower than in the starting fraction A1 (2.1). Fractions S2 and S3 were clearly mixtures of low-molecular-weight fragments of the mucopeptide and as well as the mucopeptide amino acids, these fractions contained smaller proportions of glucosamine and muramic acid in approximately equimolar amounts. Galactosamine was present in fraction S2. Mucopeptide fragments of higher molecular weight were also present in the intervening tubes between peaks S2 and S1. This material has not been studied further.

The large excess of glucosamine present in fraction A1 (Table 3) was recovered from the Sephadex G-25 column in a fraction, S1 (Table 4), that was excluded from the gel. This fraction contained a polysaccharide presumably of relatively large molecular weight and made up of glucosamine, galactosamine and glucose. The fraction contained

no amino acids but a part of the glycan of the mucopeptide was present since a small amount of muramic acid was found. All of the phosphorus of fraction A1 was present in the high-molecular-weight material from Sephadex and clearly was covalently linked to this material.

Characterization of mucopeptide fragments in fractions S2 and S3. Fractions S2 and S3 (Table 4) contained respectively dimer and monomer peptides. Thus, each fraction after acid hydrolysis contained approximately equimolar amounts of L-alanine, D-glutamic acid and diaminopimelic acid and less than 1 mol.prop. of D-alanine. After treatment of fractions S2 and S3 with 1-fluoro-2,4-dinitrobenzene 47% and 94% respectively of the total diaminopimelic acid contents were recovered as the mono-DNP-derivative. In addition free amino groups of alanine were found and the amount of these groups (approx. 0.9 mol.prop.) suggested that most of the peptides in fractions S2 and S3 were unsubstituted by hexosamine material. The hexosamine material found in fractions S2 and S3 was present in large part as separate oligosaccharides, presumably tetrasaccharides and disaccharides respectively. Partial overlap of peaks containing tetrasaccharides and disaccharides with dimer and monomer peptides respectively would be expected on Sephadex G-25 chromatography.

The presence in fraction S3 of disaccharides containing exclusively *N*-acetylglucosamine reducing groups was confirmed by treatment of a sample

Fig. 4. Sephadex G-25 chromatography of fraction A1 obtained from DEAE-cellulose. Fraction A1 (Table 3) dissolved in water (4.5 ml) was applied to the column (2 cm $\times$ 130 cm). The column was previously washed with 0.1 M-pyridine acetate buffer, pH 5.1, and elution was performed with the same buffer. Fractions (2.5 ml) were collected and analysed for ninhydrin-positive material (E_{570}) (—), for neutral hexose (E_{625}) (---), and, after acid hydrolysis, for total hexosamines (E_{530}) (○). The fractions indicated by the arrows were made by pooling the appropriate tubes.

Table 4. *Analysis of fractions obtained by Sephadex G-25 chromatography*

Fraction A1 (Table 3) was separated into four fractions by gel filtration as shown in Fig. 4. Analyses were performed as described in the text. tr, Trace.

Substance	Total content (μmol)				Molar ratios		
	S1	S2	S3	S4	S1	S2	S3
Diaminopimelic acid	tr	24	15	0	—	1.1	1.1
Glutamic acid	tr	22	13	0	—	1.0	1.0
Alanine	tr	37	20	22	—	1.7	1.5
Muramic acid	5	14	9	0	1.0	0.6	0.7
Glucosamine	105	15	8	0	22	0.7	0.6
Galactosamine	117	8	0	0	24	0.3	—
Hexose	97	0	0	0	20	—	—
Phosphorus	4.7	0	0	0	1.0	—	—

(containing $2.2\mu\text{mol}$ of glucosamine after acid hydrolysis) with sodium boro[^3H]hydride. After acid hydrolysis and chromatography on Dowex 50 as described in Fig. 2 muramic acid (1mol.prop.) and [^3H]glucosaminol (0.83mol.prop.) were isolated.

Oligosaccharides containing *N*-acetylmuramic acid and *N*-acetylglucosamine were purified from the products of the second autolysate of walls as described below. No evidence was obtained for the origin of the galactosamine residues in fraction S2 (Table 4) isolated from the first autolysate of walls and containing dimer mucopeptide fragments. Possibly the galactosamine was present in oligosaccharides derived from the non-mucopptide components of *B. cereus* cell walls. For example, these polysaccharides contain *N*-acetylgalactosamine and *N*-acetylglucosamine residues and a limited number of glycosidic linkages in the polysaccharides may have been hydrolysed during autolysis by the endo- β -*N*-acetylglucosaminidase. However, it is not known at present if β -1 $\rightarrow$ 4-*N*-acetylglucosaminide linkages exist in the polysaccharides.

Chromatography of fraction S3 in solvent *A* gave two ninhydrin-positive spots with R_{Dap} values 1.42 and 1.21 (R_{Dap} =mobility relative to diaminopimelic acid). A larger sample of fraction S3 (containing $3.7\mu\text{mol}$ of diaminopimelic acid equivalents) was separated by preparative chromatography in solvent *A* and the minor (R_{Dap} 1.21) and major (R_{Dap} 1.42) bands were eluted from paper with water and purified further by electrophoresis at pH 3.6. No hexosamine material was present. The purified major band (R_{Dap} 1.42) contained equal amounts of L-alanine, D-glutamic acid, diaminopimelic acid and D-alanine. It accounted for 67% of the total diaminopimelic acid content of fraction S3. At least 90% of the diaminopimelic acid residues and L-alanine residues were available to 1-fluoro-2,4-dinitrobenzene and the relevant mono-DNP-derivatives were identified

by paper chromatography after acid hydrolysis. Hydrazinolysis of the purified peptide (R_{Dap} 1.42) released 0.65mol.prop. of D-alanine. The yield was not corrected for destruction during treatment and gives a minimum value for the content of C-terminal D-alanine residues. The results indicated that the sequence L-Ala-D-Glu-meso-Dap*-D-Ala, previously proposed (see Ghuysen, 1968) for the peptide side chains of several other mucopeptides containing meso-diaminopimelic acid, was also present in *B. cereus* N.R.R.L. 569 mucopeptide. Since the peptide was neutral at pH 6.5 and positively charged at pH 3.6 one of the carboxyl groups must be amidated.

The minor band (R_{Dap} 1.21) purified from fraction S3 contained approximately equimolar amounts of alanine, glutamic acid and diaminopimelic acid. Presumably this tripeptide was produced from the tetrapeptide by D-alanine carboxypeptidase action.

Hydrazinolysis released approx. 21% of the total D-alanine residues of fraction S2 (Table 4) as the free amino acid. This result, together with the presence of less than 1mol.prop. of total D-alanine in the fraction (Table 4), indicated that the dimer fraction was a mixture of an octapeptide formed from two tetrapeptide units, and a heptapeptide lacking a C-terminal D-alanine residue.

Second fractionation of autolysed walls. Oligosaccharides containing *N*-acetylmuramic acid and *N*-acetylglucosamine and lacking peptide substituents were isolated from autolysates by a method that avoided the apparent preferential loss of these fragments during purification of the products of the first autolysis.

Cell walls (181mg) were autolysed as described for the first autolysate and the products of autolysis were fractionated directly on a column (2cm $\times$ 140 cm) of Sephadex G-50. Fractions (2.5ml) were collected and portions were analysed for total

* Abbreviation: Dap (in amino acid sequences), $\alpha\epsilon$ -diaminopimelic acid.

Fig. 5. Chromatography on Sephadex G-50 of the soluble fraction of walls solubilized at pH 7.5. Walls (181 mg) were autolysed overnight at 35°C. The soluble products were separated on a column (2 cm × 140 cm), equilibrated and run in 0.1 M-pyridine acetate buffer, pH 5.1. Fractions (2.5 ml) were analysed for neutral hexoses (E_{625}) (○), free amino groups (E_{570}) (—) and free reducing groups (E_{700}) (----).

hexosamines, neutral hexoses, free amino groups and free reducing groups (Fig. 5). A large ninhydrin-positive peak containing free D-alanine was eluted last from the column. Material eluted from the column at the void volume contained after acid hydrolysis hexosamines detected with the Elson-Morgan reagent and glucose; only traces of amino acids were present. The fraction (92 mg) was applied to a column of DEAE-cellulose and chromatography was carried out exactly as described in Fig. 3. Two peaks were eluted from the columns in the positions at which fractions A1 and A3 were eluted (Fig. 3) during a similar fractionation of the first autolysate of walls. Analyses of the two peaks obtained by pooling the appropriate tubes confirmed that the materials referred to as fractions A1 and A3 in the products of the first autolysate (Table 3) were present in the material of the second autolysate that was excluded from Sephadex G-50 (Fig. 5).

The fraction of high molecular weight that was excluded from Sephadex G-50 contained muramic acid after acid hydrolysis accounting for approx. 13% of the total muramic acid present in the second autolysate. The rest of the muramic acid content of the second autolysate of walls was found in two fractions that were eluted later from the column of Sephadex G-50. The fractions of low molecular weight were detected by reducing-power measurements (Fig. 5). The appropriate tubes were pooled and freeze-dried before analysis. Both fractions contained after acid hydrolysis muramic acid, glucosamine, diaminopimelic acid, glutamic acid and alanine. The fractions clearly contained fragments of the mucopeptide component of walls, and are referred to in the present paper as dimer

fraction and monomer fraction respectively in their order of elution from Sephadex G-50.

Samples of the dimer and monomer fractions containing 5 μ mol of muramic acid equivalents were subjected to preparative paper electrophoresis at pH 3.6 for 3 h. Marker strips were developed with ninhydrin or by the method of Rydon & Smith (1952). Major spots were detected by both reagents moving 18 cm to the cathode and presumably these were the peptides carrying a positive charge at pH 3.6 and identified among the products of the first autolysis. Material eluted from this band contained diaminopimelic acid, glutamic acid and alanine. Marker strips developed with the Rydon & Smith (1952) reagent showed the presence of compounds moving towards the anode. Material was eluted with water from paper and the eluates were dried before analysis. The purified materials contained after acid hydrolysis equimolar amounts of muramic acid and glucosamine. During electrophoresis at pH 3.6 the purified oligosaccharides from the dimer and monomer fractions moved as single spots with mobilities (m_{MurNAc}) respectively 0.69 and 0.57 relative to a standard of *N*-acetylmuramic acid. Homogeneous spots were also obtained after chromatography in solvent *F*. The mobilities relative to *N*-acetylglucosamine (R_{GlcNAc}) were respectively 0.43 and 0.85. An authentic standard of the disaccharide 4-*O*- β -*N*-acetylglucosaminyl-*N*-acetylmuramic acid (Sharon, Osawa, Flowers & Jeanloz, 1966) moved in solvent *F* with R_{GlcNAc} 0.87. Treatment of samples (containing 0.25 μ mol of muramic acid equivalents) of the oligosaccharides purified from the dimer and monomer fractions with sodium borohydride destroyed 43% and 91% respectively of the glucosamine contents. The contents of muramic acid were unchanged. It was concluded that the oligosaccharide present in the monomer fraction (Fig. 5) was *N*-acetylmuramyl-*N*-acetylglucosamine. The dimer fraction from Sephadex G-50 contained a tetrasaccharide consisting of equal proportions of *N*-acetylmuramic acid and *N*-acetylglucosamine and carrying a reducing terminal residue of *N*-acetylglucosamine. Presumably the tetrasaccharide was formed from two molecules of the disaccharide present in the monomer fraction. The tetrasaccharide fraction contained approx. 25% of the total muramic acid equivalents recovered in the peptide-free oligosaccharides. This value suggested that approx. 87% of the β -*N*-acetylglucosaminide linkages of mucopeptide were hydrolysed during autolysis. A similar conclusion was obtained by direct analysis of cell wall autolysates with sodium boro[^3H]hydride as described above.

The disaccharide reacted negatively in the Morgan-Elson test even after heating for 35 min in borate buffer, pH 9.2. This result was consistent

with the proposed structure and was different from the result obtained with the disaccharide 4-*O*- β -*N*-acetylglucosaminyl-*N*-acetylmuramic acid. This disaccharide gave a high colour yield in the modified test due to the lability of the glycosidic linkage at alkaline pH (Tipper, Ghuyssen & Strominger, 1965).

Properties of the wall polysaccharides. Fraction A3 (Table 3) was passed through a column (2 cm $\times$ 40 cm) of Sephadex G-15. The collected effluent fractions (2.0 ml) were analysed for total hexosamine material after acid hydrolysis and for ^{32}P . Hexosamine material was eluted in a sharp peak in the void volume and all of the radioactivity was found in the fractions containing salts. Analysis showed that P_1 was present. The purified polysaccharide obtained from Sephadex G-15 contained *N*-acetylglucosamine and *N*-acetylgalactosamine (25 and 23% of the dry wt. of polysaccharide respectively). Very small amounts of the mucopeptide amino acids and muramic acid were found. The material was non-reducing in the Park-Johnson (1949) test and failed to incorporate radioactivity after treatment with sodium boro[^3H]hydride. This last method was sufficiently sensitive to make it almost certain that the polysaccharide carried a blocked reducing group rather than being a highly branched molecule of very high molecular weight. The material was homogeneous during electrophoresis at pH 3.6 moving towards the anode (m_{MurNAc} 0.67) in a position similar to that of the disaccharide 4-*O*- β -*N*-acetylglucosaminyl-*N*-acetylmuramic acid (Sharon *et al.* 1966). In solvent *I* at pH 2.4, the polymer still moved 4.6 cm as an anion (m_{MurNAc} 2.1). As a marker the teichuronic acid isolated from cell walls of *B. licheniformis* N.C.T.C. 6346 (Janczura *et al.* 1961; Hughes & Thurman, 1970) moved 10.1 cm. Therefore it seemed that the only ionizable groups in the polysaccharide from *B. cereus* cell walls were probably carboxyl groups and that the amino groups of the hexosamines were blocked. Tests for likely compounds that would account for these properties, e.g. glucuronic acid, were unsuccessful and, as mentioned above, phosphorus was absent. Samples of the polysaccharide (containing approx. 0.5 μmol of glucosamine) were hydrolysed in *m*-hydrochloric acid at 100°C for 1–4 h. The hydrolysates contained glucosamine, galactosamine and material, detected with ninhydrin, silver nitrate and periodate-benzidine reagents, that was neutral during electrophoresis at pH 6.5 and moved slowly in solvent *B*. The *N*-acetylated derivatives of glucosamine and galactosamine were also present in the early hydrolysates. A sample of the substance neutral during electrophoresis at pH 6.5 and moving slowly in solvent *B* was isolated. Electrophoresis in solvent *H* showed a single spot, detectable with silver nitrate or ninhydrin, which moved towards

the anode more slowly than standards of glucosaminuronic acid, galactosaminuronic acid and mannosaminuronic acid. Glucosamine was immobile in the system. The spot gave a normal purple colour with ninhydrin, unlike the known aminohexuronic acids (Perkins, 1963). Further, the spot was rather stable to acid hydrolysis and survived heating in *m*-hydrochloric acid at 100°C for 4 h, in contrast with the lability of known aminohexuronic acids (Perkins, 1963) and, for example, the pyruvic acid ketals of sugars (Sutherland, 1969). Further work is required to identify the acidic constituents of the polysaccharide. To avoid confusion in the description of the different polysaccharides present in cell walls of *B. cereus* N.R.R.L. 569 it is proposed to call the acidic fraction polysaccharide A.

Polysaccharide A accounted for approx. one-fifth of the total galactosamine content and approx. 12% of the dry weight of *B. cereus* N.R.R.L. 569 cell walls.

The polysaccharide present in fraction S1 (Table 4) from Sephadex G-25 is called polysaccharide S in the present paper and was familiar as the antigen studied in detail by Mester (1960) and Mester *et al.* (1962). My analyses showed the presence of glucose (21%), *N*-acetylglucosamine (39%) and *N*-acetylgalactosamine (32%) corresponding to a content of total hexosamines of 56%. Mester (1960) found 17% glucose, 47% total hexosamines (glucosamine and galactosamine) and 13.5% acetyl groups. The polysaccharide studied by Mester (1960) was prepared from cell lysates by fractional precipitation with barium acetate followed by graded ethanol solutions. The cells were lysed in dilute ammonium carbonate at pH 8.5. Methylation analysis and periodate oxidation (Mester *et al.* 1962) showed that all of the glucose residues and a part of the hexosamines were non-reducing end groups. These groups were substituted on to a branched core consisting of *N*-acetylglucosamine residues. Small amounts of the mucopeptide amino acids and muramic acid were also present. By contrast fraction S1 (Table 4), after purification from cell-wall autolysates by chromatography, contained only traces of amino acids. However, a significant amount of muramic acid was found and phosphorus was detected (Table 4).

A sample of fraction S1 containing 1 μmol of total phosphorus was hydrolysed in 2 *M*-hydrochloric acid at 100°C for 3 h. The hydrolysed material was passed through a column (0.84 cm $\times$ 22 cm) of Dowex 50 (H^+ form) and the substances eluted with water were dried and examined by chromatography in solvent *C* and by electrophoresis at pH 4.7. A spot was detected with silver nitrate that had moved towards the anode during electrophoresis and very slowly in solvent *C*. Glucose was also

present as expected. After treatment of the neutral fraction from Dowex 50 with wheat-germ acid phosphatase the negatively charged spot did not appear on electrophoresis at pH 4.7. The properties described were consistent with the presence of muramic acid phosphate in fraction S1.

Fraction S1 (Table 4) was neutral during electrophoresis at pH 3.6 and was immobile in solvents *B* and *C*, indicating a high molecular weight. The molecular weight of the material in fraction S1 (Table 4) was kindly determined by Dr P. A. Charlwood of this Institute. The fraction was dialysed against buffers of the following composition: (a) sodium phosphate buffer, pH 6.6 and *I* 0.05, containing 0.5M-sodium chloride, or (b) sodium phosphate buffer, pH 6.4 and *I* 0.05, containing 1.0M-sodium chloride. The solutions were ultracentrifuged in each buffer at concentrations of 0.3 and 0.6% at 14290 and 17980 rev./min. Yphantis (1960) short-column experiments were carried out in a Spinco Model E analytical ultracentrifuge at about 20°C. A value of 0.66 was taken for the partial specific volume (Smith & Zwartouw, 1956). The results at the different concentrations and speeds were very similar with a mean value of approx. 28000. The minimum molecular weight of fraction S1 was calculated from the analytical results in Table 4 to be approx. 13400 and strongly suggested that the complex was a dimer molecule containing 2 mol.prop. each of phosphate and muramic acid.

Reducing groups were detected in fraction S1 by a ferricyanide reagent (Park & Johnson, 1949) amounting to 0.4mol/mol of muramic acid. A sample (containing 0.5 μ mol of muramic acid residues) of fraction S1 was treated with sodium borotrihydride. After acid hydrolysis [³H]glucosaminol (0.4mol/mol of muramic acid) was isolated from a column of Dowex 50 eluted as described in Fig. 2. [³H]Glucosaminol was distinguished by electrophoresis in solvent *H* from galactosaminol, which is eluted with glucosaminol from the Dowex 50 column. Theoretically, the reducing *N*-acetylglucosamine residues could have been derived from the polysaccharide molecule. However, it seemed more likely that a short fragment of mucopeptide glycan, probably a tetrasaccharide containing a reducing *N*-acetylglucosamine unit, was present in fraction S1. The tetrasaccharide was possibly substituted with the two phosphate groups present in the complex molecule of mol.wt. 28000 but direct evidence was not obtained. It is equally possible that one of the phosphate residues was present internally in the chains of polysaccharide S. A phosphorylated derivative other than muramic acid phosphate was not detected, however, in acid hydrolysates of fraction S1.

Isolation of the cell-wall polysaccharides by other methods. Fractions containing polysaccharides A

and S were isolated from *B. cereus* N.R.R.L. 569 cell walls solubilized with *Chalaropsis* B endo- β -*N*-acetylmuramidase. Heated cell walls (130mg) labelled with ³²P were treated with the enzyme for 24h (see Fig. 1c) and the soluble material was dialysed. Fractionation of the non-diffusible material on a column (2cm $\times$ 140cm) of Sephadex G-50 gave an excluded peak containing glucose in excellent yield (87%), hexosamine material and about 18% of the total ³²P. This fraction was applied to a column (2cm $\times$ 140cm) of DEAE-cellulose run exactly as described in Fig. 3, and the column fractions (5.0ml) were examined for glucose and after hydrolysis for total hexosamines. Two main fractions were obtained. One fraction was eluted unretarded from the column and contained all of the phosphorus and glucose recovered as well as glucosamine and galactosamine. The components were present in this fraction in relative amounts that were very similar to those in fraction S1 (Table 4). It differed from fraction S1 in that diaminopimelic acid, glutamic acid, alanine and muramic acid were present in higher amounts, respectively 4, 4, 7 and 3mol/mol of phosphorus. This fraction was polysaccharide S attached to a part (16%) of the wall mucopeptide.

The second peak was eluted from the column in the region expected for fraction A3 (Fig. 3) and contained equimolar amounts of glucosamine and galactosamine. Glucose was absent and muramic acid and the amino acids characteristic of the mucopeptide were found in amounts similar to those shown for fraction A3 in Table 3.

Polysaccharide material was extracted from insoluble cell walls (4mg/ml) by 0.1M-hydrochloric acid at 35°C. After 2 days approx. 28 and 33% respectively of the total glucose and hexosamine material of the walls had been removed. After acid hydrolysis glucosamine and galactosamine were identified by paper chromatography. The soluble material was examined by electrophoresis in solvent *G* at pH 3.6. A large neutral spot was detected by chlorination, as expected for polysaccharide S. No negatively charged polysaccharide was detected, indicating that polysaccharide A was not extracted by dilute acid. This result was consistent with the view that the two polysaccharides were attached to the mucopeptide by different types of covalent bonds. Dilute alkali at 35°C (Hughes & Tanner, 1968) failed to extract significant amounts of either polysaccharide.

DISCUSSION

Autolysis of isolated cell walls of *B. cereus* N.R.R.L. 569 as described in the present paper is due to the action of an endo- β -*N*-acetylglucosaminidase and an amidase. Other activities are present including

the presumptive D-alanine carboxypeptidase that removes C-terminal D-alanine residues from both monomer peptides and cross-linked dimer peptides of mucopeptide. Similar activities are present in the cell walls of *B. licheniformis* N.C.T.C. 6346 (Hughes *et al.* 1968; Hughes, 1968). In this organism, however, the removal of C-terminal D-alanine residues from mucopeptide occurs as a normal part of cell-wall growth and no free D-alanine carboxyl groups remain in the completed mucopeptide. A lytic enzyme induced in *B. cereus* N.R.R.L. 569 cells by u.v. irradiation (Csuzi, 1968, 1969) has since been purified 15-fold from the cell sap by ion-exchange chromatography. The purified enzyme lysed heated walls of *B. cereus* N.R.R.L. 569 by hydrolysis of a proportion of the N-acetylmuramyl-L-alanine bonds of mucopeptide. There was no parallel induction by u.v. irradiation of a glycosidase (S. Csuzi, personal communication). The relationship between the amidase present in the wall fraction prepared from uninduced cells as described in the present paper and the soluble amidase induced by u.v. irradiation of cells (Csuzi, 1968, 1969) is not known at present. Presumably the amidase exists in uninduced form in the cell at a low activity and together with the other wall-associated enzymes may be involved in expansion of the wall during growth.

The main peptide side chains of *B. cereus* N.R.R.L. 569 mucopeptide are tetrapeptides (L-Ala-D-Glu-meso-Dap-D-Ala), although tripeptides lacking the C-terminal D-alanine residue were found among the products of autolysis. Some of the carboxyl groups are amidated as shown by the electrophoretic behaviour of the peptides, although the extent of amide substitution in the autolysis products is less than expected from the apparent amide content of whole walls (Table 1). Two explanations are possible for this discrepancy. The ammonia released from walls by acid hydrolysis may be derived in part from breakdown of unstable compounds. Alternatively, amide groups may have been removed from mucopeptide carboxyl groups during growth or in the autolysis of isolated walls. Evidence for a diaminopimelic acid deamidase in *Corynebacterium diphtheriae* has been reported (Kato, Strominger & Kotani, 1968).

Cross-linking between peptide side chains in *B. cereus* N.R.R.L. 569 is probably through D-Ala-meso-Dap bridges. A low degree of cross-linking in the mucopeptide was indicated by the extent of reaction of whole walls and autolysed walls with 1-fluoro-2,4-dinitrobenzene. The analysis of ϵ -amino-terminal groups of diaminopimelic acid in walls (Table 2) showed that approx. 54% of such amino groups were free. A value of 66% would be expected for equal amounts of monomers and dimers.

The isolation of polysaccharide A from *B. cereus* N.R.R.L. 569 provides another instance of a cell wall containing a strongly anionic polymer. The physiological importance to the cell of highly acidic components present at the cell surface has been emphasized (Heptinstall, Archibald & Baddiley, 1970). The attachment of the wall polysaccharides to mucopeptide by covalent linkages is inferred by analogy with many other bacterial cell walls. The presence of mucopeptide constituents in the polysaccharide fractions isolated from solubilized *B. cereus* N.R.R.L. 569 walls is consistent with this proposal. A direct covalent linkage between polysaccharides A and S is improbable because of the known (Tipper *et al.* 1964) specificity of *Chalaropsis* B glycosidase and the separate release of the two polysaccharides into a soluble form by this enzyme. The presence of phosphorus and muramic acid in the complex containing polysaccharide S and the isolation of a compound with the properties of muramic acid phosphate after acid hydrolysis suggests that a phosphodiester linkage exists between the polysaccharide chains and glycan. This is consistent with the release of polysaccharide S from insoluble walls by dilute acid due to the known lability of sugar 1-phosphate linkages (Leloir & Cardini, 1956). Similar linkages for polysaccharides present in other cell walls have been proposed (Knox & Hall, 1965; Hughes, 1970a). Clearly the bonds linking polysaccharides A and S respectively to mucopeptide are chemically different, since polysaccharide A was not made soluble by dilute acid and contained no phosphorus. Munoz, Ghuyssen & Heymann (1967) proposed a glycosidic attachment to mucopeptide for the C-polysaccharide of *Streptococcus pyogenes* cell walls. A similar glycosidic attachment site could exist between polysaccharide A and the glycan moiety of mucopeptide, although clearly other alternatives are possible.

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