Autolysis of Isolated Cell Walls of *Bacillus licheniformis* N.C.T.C. 6346 and *Bacillus subtilis* Marburg Strain 168

SEPARATION OF THE PRODUCTS AND CHARACTERIZATION OF THE MUCOPEPTIDE FRAGMENTS

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1. Cell walls were isolated from *Bacillus licheniformis* N.C.T.C. 6346 and *Bacillus subtilis* Marburg strain 168 *trp* grown on casein hydrolysate into exponential phase. Autolysis was carried out and the soluble products, separated by chromatography on DEAE-cellulose, from the two wall preparations are broadly similar in composition and are in agreement with autolysis proceeding with hydrolysis of amide bonds between L-alanine and N-acetylmuramic acid residues in the mucopeptide components. 2. Peptides originating from the mucopeptide components were isolated and shown to be a monomer peptide, L-alanyl-D-glutamyl-meso-diaminopimelic acid and a dimer peptide containing two monomer peptides linked through a residue of D-alanine. Approximately one amide group is present for each equivalent tripeptide unit and is probably substituted on diaminopimelic acid residues. 3. Oligosaccharides originating from the mucopeptide components were isolated and after hydrolysis contained almost equimolar amounts of glucosamine and muramic acid and only very small amounts of amino acids. The number-average chain length, estimated by the release of non-reducing end groups of N-acetylglucosamine with exo- β -N-acetylglucosaminidase, is approximately ten hexosamine residues for oligosaccharides isolated from either organism. The oligosaccharides are polydisperse. 4. N-Acetylglucosamine residues are the only reducing terminals detectable in the oligosaccharides isolated from *B. subtilis* or *B. licheniformis* cell-wall autolysates. The number-average chain lengths of the oligosaccharides were determined by estimation of the content of these residues and are higher than those found by enzymic assay. Possible reasons for the discrepancy are discussed.

The cell wall of *Bacillus subtilis* Marburg strain 168 contains an autolytic enzyme that hydrolyses the amide bond between the carboxyl function of N-acetylmuramic acid residues and L-alanine residues in mucopeptide (Young, Tipper & Strominger, 1964; Young, 1966). Autolysis of isolated walls suspended in moderately alkaline solutions appears to proceed exclusively by cleavage of most of these linkages. In this way the carbohydrate and peptide parts of mucopeptide that are linked together to form the insoluble polymer in the intact walls were separated. Fractions enriched in either one of the components were isolated by chromatography and partially characterized. The peptides contained diaminopimelic acid, glutamic acid and alanine, and the oligosaccharides contained about equimolar amounts of N-acetylglucosamine and N-acetylmuramic acid. Walls of *Bacillus licheniformis* N.C.T.C. 6346 are also solubilized during incubation at various pH

values. These walls contain an enzyme of specificity identical with that of *B. subtilis* Marburg strain 168 (Hughes, Pavlik, Rogers & Tanner, 1968; J. S. Thompson & H. J. Rogers, unpublished work). Previous work has shown that *B. licheniformis* N.C.T.C. 6346 mucopeptide (Fig. 1) contains meso-diaminopimelic acid, D-glutamic acid, L-alanine, D-alanine, N-acetylmuramic acid, N-acetylglucosamine and amide groups. The N-acetylmuramic acid residues are substituted by peptide side chains of L-Ala-D-Glu-meso-Dap* and some of the side chains are cross-linked by a D-alanine residue from a carboxyl group of diaminopimelic acid in one peptide side chain to a free amino group of diaminopimelic acid in a second tripeptide.

This paper describes a relatively simple fractionation of the main products of autolysis of *B. subtilis* Marburg strain 168 and *B. licheniformis*

* Abbreviation (in amino acid sequences): Dap, diaminopimelic acid.

Fig. 1. Peptide side chains in *Bacillus licheniformis* mucopeptide. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid. The figure shows a tripeptide side chain and a dimer formed from two tripeptides by a *D*-alanine residue cross-bridge.

N.C.T.C. 6346 cell walls and characterization of the fractions originating from mucopeptide. The results confirm and extend earlier work on these structures. A preliminary account of this work has appeared previously (Hughes *et al.* 1968).

MATERIALS

Pigepididymalexo- β -*N*-acetylglucosaminidase (Findlay & Levvy, 1960) was kindly provided by Dr H. R. Perkins. Some experiments were done with a preparation of enzyme provided by Dr D. H. Leaback. Disaccharide and tetrasaccharide from *Micrococcus lysodeikticus* were gifts from Dr N. Sharon (Sharon, Osawa, Flowers & Jeanloz, 1966). Crystalline glucosaminol (2-amino-2-deoxy-D-glucitol) was obtained from Dr C. D. Warren and Dr R. H. Gigg. Carrier-free [32 P]phosphate (1 mCi; The Radiochemical Centre, Amersham, Bucks., U.K.) was diluted in 0.1 M-potassium phosphate buffer, pH 7.0 (50 ml), before use. NaB^3H_4 (94 $\mu\text{Ci}/\mu\text{mol}$; The Radiochemical Centre) was diluted with unlabelled NaBH_4 to approx. 2 $\mu\text{Ci}/\mu\text{mol}$ before use. γ -Glutamylhydrazide was given by Dr H. R. Perkins.

METHODS

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

The radioactivity of paper strips was 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%).

Growth of bacteria and preparation of cell walls. *B. licheniformis* N.C.T.C. 6346 was grown into mid-exponential phase in a casein-hydrolysate medium supplemented with inorganic salts (Janczura, Perkins & Rogers, 1961). In some experiments the medium also contained [32 P]-phosphate (0.1 $\mu\text{Ci}/\text{ml}$). *B. subtilis* Marburg strain 168 *trp* was grown into mid-exponential phase in a medium containing casein hydrolysate (0.5%), yeast extract (0.5%), MgSO_4 (1 mM), β -glycerophosphate (60 mM) and glucose (1%). This medium was supplemented with a salt solution (0.02 ml/l) containing MnCl_2 (0.2%), CuSO_4 (0.5%), ZnSO_4 (6.5%), FeSO_4 (0.5%) and HCl (10%). Cell walls containing active autolytic enzymes were prepared by differential centrifugation after breakage of the cells by shaking with glass beads in a Braun homogenizer fitted with a cooling device. The walls were washed extensively with water at 4°C and freeze-dried. The yield of walls was approx. 30 mg from 1 litre of culture.

Analytical methods. Analyses for glucose, glucuronic acid, total phosphorus, L-alanine, D-alanine, *N*- and *C*-terminal amino acids were performed as previously described (Hughes, 1968a). Amino acids and hexosamines were estimated by Dr S. Jacobs with a Beckman-Spinco automatic analyser after overnight hydrolysis of samples in 4 M-HCl at 105°C. Acetyl-amino sugars were determined by the Morgan-Elson reaction (Reissig, Strominger & Leloir, 1955), with a heating time of 3 min in potassium borate buffer, pH 9.2.

Paper chromatography and electrophoresis. Descending chromatography on Whatman no. 3 or no. 3MM paper employed the following solvent systems: *A*, isobutyric acid-0.5 M- NH_3 (5:3, v/v); *B*, butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase); *C*, butan-1-ol-acetic acid-water (25:6:25, by vol., upper phase) (Chipman, Pollock & Sharon, 1968); *D*, butan-1-ol-pyridine-water (6:4:3, by vol.); *E*, propan-1-ol-aq. 0.2% NH_3 (17:3, v/v); *F*, 0.75 M-sodium phosphate buffer, pH 6.1. Electrophoresis on Whatman no. 3MM paper was carried out in the buffer systems: *G*, pyridine-acetic acid-water, pH 3.6 (6:46:323, by vol.) or pH 6.5 (25:1:224, by vol.) run at 2000 V for 50 min or 3 h as indicated; *H*, 0.1 M-sodium molybdate, pH 5.0 (Mayer & Westphal, 1968), run at 2000 V for 50 min.

Column chromatography. Sephadex G-15, G-25 or G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) was suspended in 0.1 M-pyridine-acetate buffer, pH 5.1, and fines were decanted. A column (2 cm $\times$ 140 cm) containing Sephadex G-25 was washed extensively with the same buffer. Samples (2-4 ml) were applied to the top of the column and eluted at room temperature at a flow rate of 30 ml/h. Fractions (2.5 ml) were collected and suitable portions analysed. Smaller columns (2 cm $\times$ 40 cm) containing Sephadex G-50 or G-15 were used in some experiments, when 2 ml fractions were collected. Ion-exchange chromatography was carried out on columns (2 cm $\times$ 40 cm) of DEAE-cellulose in pyridine-acetate buffers at pH 5.1. The resin was equilibrated with 0.01 M-pyridine-acetate buffer, pH 5.1. After application of the sample the column was eluted with the same buffer (100-150 ml) and fractions (5.0 ml) were collected. Then an approximately linear concentration gradient was established by running

5.0M-pyridine-acetate buffer, pH 5.1, into a mixing chamber containing 0.01M-pyridine-acetate buffer, pH 5.1 (1 litre). Buffer salts were removed from peak fractions by freeze-drying.

Autolysis of isolated cell walls. *B. subtilis* cell walls (320mg) were suspended in 20mM-(NH₄)₂CO₃ (50ml) at pH 8.9 and kept overnight at 35°C. The turbidity as measured by the extinction at 675nm decreased to 4% of the starting value. A residue (10mg) was removed from the autolysate by centrifugation and washed with water several times. Supernatants were combined, freeze-dried and dissolved in water (5.0ml).

Autolysis of *B. licheniformis* cell walls was carried out with several batches of walls. In a typical experiment walls (226mg) were suspended in 50mM-ammonium acetate, pH 7.5. After incubation at 35°C overnight the turbidity of the suspension decreased to 3% of the initial value. The soluble material was obtained by centrifugation, freeze-dried and dissolved in water (5.0ml).

Treatment of oligosaccharides with exo-β-N-acetylglucosaminidase. Samples of oligosaccharides (0.1ml containing up to 0.5μmol of total glucosamine) were mixed with 0.1M-sodium citrate buffer, pH 4.4 (0.05ml), and enzyme (0.05ml). After incubation of the mixtures at 35°C for various times, water (0.3ml) was added and the amount of *N*-acetylglucosamine liberated was measured with the Morgan-Elson reaction. Control tubes contained enzyme or *N*-acetylglucosamine in sodium citrate buffer, pH 4.4. The determination of *N*-acetylglucosamine was not affected by the presence of the buffer salts. None of the oligosaccharides examined reacted positively in the Morgan-Elson reaction.

Reduction of oligosaccharides with sodium boro[³H]-hydride. Reducing groups of oligosaccharides were detected by reduction with NaB³H₄ and characterization of the radioactive sugar alcohol produced. In a typical experiment a sample (containing 4.0μmol of glucosamine after acid hydrolysis) of an oligosaccharide was treated with an excess of 1% (w/v) NaB³H₄ (0.4ml; approx. 2μCi/μmol) in 50mM-sodium borate buffer, pH 8.9, at 35°C overnight. Excess of reagent was destroyed with 12M-HCl (0.2ml). The acidified mixture was heated at 100°C for 4h, dried several times from water and methanol and finally dissolved in water (0.5ml). In some experiments, with larger amounts of material, excess of NaB³H₄ was destroyed by addition of 12M-HCl (0.2ml) and, after being dried, the reduced sample was dissolved in water and filtered through a column (2cm × 40cm) of Sephadex G-15. Fractions (2.0ml) were collected and hexosamine material was located in the void volume by Elson-Morgan analysis after acid hydrolysis. The peak fractions containing oligosaccharides were pooled, freeze-dried and dissolved in water. Suitable samples (usually containing up to 4μmol of glucosamine after acid hydrolysis) were hydrolysed and after removal of HCl were dissolved in water (0.5ml). Samples (0.4ml) were then taken for chromatography on Dowex 50 as described below. The NaB³H₄ reagent was standardized by reduction of glucosamine (2.8μmol) or muramic acid (2.0μmol) and isolation of the radioactive sugar alcohols by chromatography on Dowex 50. The recovery of [³H]-glucosaminol from the columns was checked in most experiments by periodate oxidation as described below. A yield of over 90% was usually obtained.

Separation of hexosamines on Dowex 50. Glucosamine, muramic acid and the corresponding sugar alcohols produced by reduction with NaBH₄ were separated by chromatography on Dowex 50. Hydrolysed samples containing up to 4.0μmol of glucosamine were applied to columns (0.84cm × 22cm) of Dowex 50 (X8; 200-400 mesh) equilibrated with 0.10M-pyridine-acetate buffer, pH 2.8 (Brendel, Roszel & Wheat, 1967). Fractions (2.0ml) were collected during elution of the column with 70ml of the same buffer. Then elution was continued with 0.133M-pyridine-acetate buffer, pH 3.85, and fractions (2.0ml) were collected. Portions (0.2ml) of the fractions were removed for counting of radioactivity and for analysis of hexosamine by the Elson-Morgan reaction (Gardell, 1958), modified to ensure an alkaline solution during treatment with acetylacetone. To samples (0.2ml) containing buffers at pH 2.8 and pH 3.85 were added 1.25M-Na₂CO₃ (0.3ml) and acetylacetone reagent (1.00ml). The mixtures were heated at 100°C for 45min and cooled, and the *p*-dimethylaminobenzaldehyde reagent (1.0ml) and ethanol (1.25ml) were added. The extinctions of the solutions were read at 530nm after 1h at room temperature. Standards of glucosamine and muramic acid were treated in the same way.

Periodate oxidation and determination of formaldehyde produced. Samples containing up to 0.4μmol of formaldehydogenic groups were dissolved in water (1.2ml). To each sample at room temperature was added 5M-H₂SO₄ (0.05ml) and 0.1M-NaIO₄ (0.25ml) (Renkonen, 1962). After various times portions (0.25ml) of the oxidation mixtures were withdrawn and added to tubes containing 10% (w/v) Na₂SO₃ (0.25ml) to stop oxidation. The formaldehyde produced by periodate oxidation was then measured with the chromotropic acid reagent [1g of sodium salt dissolved in 500ml of 48% (v/v) H₂SO₄] (Macfadyen, 1945). Chromotropic acid reagent (4.5ml) was added to each tube and heated at 100°C for 30min. The mixture was cooled, the iodine was discharged with 4.6% (w/v) thiourea (Frisell, Meech & Mackensie, 1954) (1.0ml) and the extinction at 570nm was read. The chromotropic acid reagent was standardized with a periodate oxidation mixture containing erythritol (0.2μmol). Under the conditions used for periodate oxidation, standard glucosaminol gave exactly 1mol.prop. of formaldehyde within 5min and only a very slow increase (5%) was obtained during the next hour. Evidently oxidation of the amino alcohol grouping was prevented by ionization of the amino group at the low pH used (Levitt, 1955; Cantley & Hough, 1960).

RESULTS

Fractionation of autolysates of cell walls on DEAE-cellulose

B. subtilis cell walls. Walls prepared from exponentially growing cells became almost completely soluble after incubation at pH 8.9 and 35°C overnight. Fractionation of the solubilized material by column chromatography on DEAE-cellulose is shown in Fig. 2. A large ninhydrin-positive peak, A1, was eluted with 0.01M-pyridine-acetate buffer, pH 5.1. No hexosamine material, phosphorus or

Fig. 2. DEAE-cellulose chromatography of an autolysate of *B. subtilis* cell walls. Solubilized walls (280mg) were fractionated on a column (2cm × 40cm) of DEAE-cellulose. Elution was started with 0.01 M-pyridine-acetate buffer, pH 5.1 (150 ml) and then a gradient was established as described in the Methods section. Fractions (5.0 ml) were collected and analysed for free amino groups (—), for glucose (○—○) and after acid hydrolysis for hexosamines (----). The fractions that were made by combining tubes are indicated by the arrows.

Table 1. Analysis of fractions obtained by chromatography of an autolysate of *B. subtilis* cell walls on DEAE-cellulose

Fractions from Fig. 2 were analysed as described in the Methods section. Fraction A1 also contained lysine (6μmol) as discussed in the text.

Substance	Fraction ...	Composition (μmol/fraction)				Total (μmol)
		A1	A2	A3	A4	
Diaminopimelic acid		52	16	5	1	74
Glutamic acid		60	17	4	1	82
Alanine		196	22	6	2	226
Glucosamine		0	7	43	25	75
Muramic acid		0	7	40	16	63
Galactosamine		0	0	0	34	34
Phosphorus		0	0	0	233	233
Glucose		0	0	0	206	206

glucose was detected (Table 1). Diaminopimelic acid, glutamic acid and alanine were present in the molar proportions 1.0:1.1:3.0 respectively. This fraction represents the peptides that are produced by cleavage of amide bonds between *N*-acetylmuramic acid and *L*-alanine residues in mucopeptide. Fraction A1 accounted for 70% of the total diaminopimelic acid and glutamic acid recovered from the column. About 80% of the remaining diaminopimelic acid was recovered in fraction A2. This peak also contained, after acid hydrolysis, glucosamine and muramic acid. The composition of the fraction suggested that a small proportion of the amide bonds of mucopeptide had not been hydrolysed by amidase during the autolysis.

Fraction A3 contained oligosaccharides produced by the action of the amidase on mucopeptide. Approximately equimolar amounts of glucosamine and muramic acid were detected after acid hydrolysis. No phosphorus or galactosamine were found. Fraction A3 contained less than 6% of the total amino acids and approx. 60% of the total glucosamine or muramic acid recovered from the column. The remaining glucosamine and muramic acid were obtained in material containing phosphorus and glucose that was eluted from the column in a broad peak with more concentrated buffers. The material was combined in fraction A4 for analysis. Fraction A4 contained only traces of diaminopimelic acid, indicating the absence of peptides. All of the galac-

Fig. 3. DEAE-cellulose chromatography of autolysed walls of *B. licheniformis*. ^{32}P -labelled walls (226 mg) were autolysed at pH 7.5 and the soluble material was fractionated on DEAE-cellulose. Elution with 0.01 M-pyridine-acetate buffer, pH 5.1 (100 ml), was followed by a salt gradient as described in the Methods section. Fractions were analysed for amino groups (—), glucose (○—○), total hexosamines after acid hydrolysis (----) and glucuronic acid (●—●). Fractions eluted after tube 70 also contained ^{32}P and hexosamines (not shown). The fractions made by pooling tubes are indicated by arrows.

Table 2. Analysis of fractions obtained by chromatography of an autolysate of *B. licheniformis* cell walls on DEAE-cellulose

Fractions from Fig. 3 were analysed as described in the Methods section.

Substance	Fraction ...	Composition ($\mu\text{mol}/\text{fraction}$)				Total (μmol)
		A1	A2	A3	A4	
Diaminopimelic acid		36	17	3	0.4	56
Glutamic acid		31	16	3	0.5	50
Alanine		78	23	5	10	116
Glucosamine		0	17	14	14	45
Muramic acid		0	13	13	10	36
Galactosamine		0	0	0	79	79
Glucuronic acid		0	0	0	93	93
Phosphorus		0	0	Trace	140	140
Glucose		0	0	0	132	132

tosamine, phosphorus and glucose contents present in *B. subtilis* walls were recovered in fraction A4. This fraction represents a complex of the teichoic acid and a part of the oligosaccharides of mucopeptide (Young *et al.* 1964). The yields of glucosamine and muramic acid in fraction A4 were 34% and 25% respectively of the totals recovered from the column. The very low amount of alanine in fraction A4 may be explained by the lability of the aminoacyl residues of teichoic acid to the alkaline conditions used for autolysis. This is also indicated by the

large molar excess of alanine to diaminopimelic acid in fraction A1 (Table 1).

B. licheniformis cell walls. A typical fractionation on DEAE-cellulose of the material solubilized by autolysis of *B. licheniformis* cell walls is shown in Fig. 3. Four fractions were consistently obtained with different batches of *B. licheniformis* walls. Analysis (Table 2) showed that these materials were analogous to those isolated from autolysates of *B. subtilis* cell walls and discussed above. About 67% of the peptides containing

diaminopimelic acid, glutamic acid and alanine were present in fraction A1. Similar yields of peptides were obtained in a number of experiments starting from different batches of walls. The yield of oligosaccharides in fraction A3 was more variable and accounted for about one-third to one-half of the total glucosamine and muramic acid recovered from the DEAE-cellulose column. This value is significantly lower than the corresponding yield obtained from *B. subtilis* walls (Table 1), and indicates that a greater proportion of the amide linkages in mucopeptide were not hydrolysed. Consequently a larger proportion of oligosaccharides was isolated combined with peptides in fraction A2. The walls used in the experiment shown in Fig. 3 were labelled with ^{32}P and at least 98% of the radioactive teichoic acids was isolated in a broad peak, A4, from the column. The teichuronic acid polymer that is also present in *B. licheniformis* cell walls (Janczura *et al.* 1961) was eluted from the column in the leading edge of the peak containing radioactivity. Fraction A4 contained glucosamine and muramic acid after hydrolysis, but only traces of amino acids. Fraction A4 was clearly a complex fraction containing teichoic acid, teichuronic acid and approx. 28% of the oligosaccharides derived from mucopeptide. The composition of fraction A4 (Table 2) is similar to that previously reported (Hughes *et al.* 1968) for a preparation obtained from autolysates of another batch of cell walls.

The rest of this paper is concerned with the components present in fractions A1, A2 and A3.

Properties of the peptides

A sample of fraction A1 from *B. subtilis* cell walls containing 41 μmol of equivalent diaminopimelic acid was applied to a column (2 cm $\times$ 140 cm) of Sephadex G-25. Material containing free amino groups was eluted in three well-defined peaks, P1, P2 and P3 (Fig. 4). The peak fractions were pooled as indicated and buffer salts were removed by freeze-drying. Chromatography on Sephadex G-25 of a sample of fraction A1 from *B. licheniformis* cell walls containing 11 μmol of diaminopimelic acid equivalents gave identical results. Analyses of the pooled fractions P1 and P2 are given in Table 3. The recovery of diaminopimelic acid from the column was over 80%.

Fractions P3, eluted last from Sephadex G-25, contained alanine, as shown by chromatography in solvents *A*, *B* and *D* and by electrophoresis in solvent *G*. In addition, a small amount of lysine was found in fraction P3 isolated from *B. subtilis* cell walls, and ammonium salts derived from the buffers used for autolysis were present. The free amino acids were presumably produced from the teichoic acids by alkaline hydrolysis during autolysis

Fig. 4. Chromatography of *B. subtilis* fraction A1 (Fig. 2) on Sephadex G-25. Fraction A1 containing 41 μmol of diaminopimelic acid residues dissolved in water (4.0 ml) was passed through a column (2 cm $\times$ 140 cm) of Sephadex G-25 and eluted with 0.1 M-pyridine-acetate buffer, pH 5.1. Fractions (2.5 ml) were collected and portions (0.1 ml) were analysed for free amino groups with ninhydrin reagent. The fractions made by pooling tubes are shown by blocks along the abscissa.

of the cell walls. Chromatography of fractions P2 isolated from *B. subtilis* or *B. licheniformis* gave a major compound in solvent *A* (R_{Dap} 1.12) and traces of other material (R_{Dap} 1.28). Fractions P2 contained equimolar amounts of diaminopimelic acid, glutamic acid, L-alanine and ammonia, and accounted for 38% (*B. subtilis*) and 36% (*B. licheniformis*) of the total diaminopimelic acid equivalents recovered from the columns. The small amount of faster-moving material was removed from *B. licheniformis* and *B. subtilis* peptides P2 by chromatography in solvent *A*. The amino acid composition of the purified major component was unchanged from that shown in Table 3. The ammonia contents were increased some 30%, probably owing to contamination by ammonium salts from the chromatographic solvent. After treatment with 1-fluoro-2,4-dinitrobenzene, all of the diaminopimelic acid and over 85% of the alanine was recovered as mono-DNP-diaminopimelic acid and DNP-alanine respectively. The content of glutamic acid was unchanged. The material present in peak P2 is therefore the monomer peptide L-Ala-D-Glu-meso-Dap.

Table 3. *Analysis of peptides isolated from B. subtilis and B. licheniformis cell walls after autolysis*

Fractions were obtained from Sephadex G-25 (Fig. 4) and analysed as described in the Methods section. N.D., Not determined.

Substance	Peptide ...	<i>B. subtilis</i>				<i>B. licheniformis</i>			
		(μmol/fraction)		(mol. prop.)		(μmol/fraction)		(mol. prop.)	
		P1	P2	P1	P2	P1	P2	P1	P2
Diaminopimelic acid		20.3	13.1	1.00	1.00	6.1	3.3	1.00	1.00
Glutamic acid		19.2	12.5	0.96	0.96	6.1	3.5	1.00	1.04
Alanine		30.6	13.7	1.52	1.04	9.5	3.7	1.54	1.10
L-Alanine		23.8	15.5	1.16	1.19	5.8	3.9	0.94	1.07
D-Alanine		13.3	N.D.	0.56	—	3.0	N.D.	0.53	—
NH ₃		19.4	12.3	0.97	0.94	6.9	3.8	1.13	1.13

The first peak, P1, eluted from the columns of Sephadex G-25 gave a major compound (R_{Dap} 0.77) in solvent A and usually only traces of other material (R_{Dap} 0.54 and 1.28). A P1 fraction isolated from one batch of *B. licheniformis* walls contained a substantial amount of the slowly moving component (R_{Dap} 0.54). Diaminopimelic acid, glutamic acid, L-alanine and D-alanine were present in P1 in the approximate molar proportions 1:1:1:0.5 respectively (Table 3). The presence of D-alanine was expected for the cross-linked dimer peptide. Samples (1 μmol) of the peptides were purified further by chromatography in solvent A. The amino acid compositions of the purified dimer peptides were similar to those shown in Table 3. The proportions of diaminopimelic acid and alanine recovered after treatment of P1 from *B. subtilis* with 1-fluoro-2,4-dinitrobenzene were 51% and 42% respectively. After treatment of peptide P1 from *B. licheniformis* with 1-fluoro-2,4-dinitrobenzene, 56% and 40% respectively of the diaminopimelic acid and alanine were recovered. Theoretical values expected for the cross-linked dimer are 50% and 33% respectively. The contents of glutamic acid and D-alanine were unchanged after 1-fluoro-2,4-dinitrobenzene treatment.

The major peptides of P1 and P2 from *B. subtilis* and *B. licheniformis* were positively charged during electrophoresis at pH 3.6 and neutral at pH 6.5, because of the presence of amide groups. The slowly moving P1 peptide (R_{Dap} 0.54) from *B. licheniformis* was negatively charged at pH 6.5, indicating a loss of amide groups. After hydrazinolysis of the *B. subtilis* and *B. licheniformis* monomer peptide P2, only small amounts (3–15% of the total) of diaminopimelic acid residues were recovered as the free amino acid. In addition, γ-glutamylhydrazide was detected by electrophoresis at pH 3.6 and this substance could only have arisen from α-carboxyl-unsubstituted γ-glutamyl residues in the peptides. The amount of γ-glutamylhydrazide

liberated by hydrazinolysis of the tripeptides was not measured. The purified neutral peptide from *B. licheniformis* peptide P1 was kindly examined by Professor J.-M. Ghuyssen for its susceptibility to a purified L-alanine aminopeptidase from *Streptomyces* (Ghuyssen, 1968). The peptide was completely resistant to the enzyme. From experience with other peptides of established sequence it is known that the enzyme removes N-terminal L-alanyl residues from substrates only when these residues are attached to D-isoglutamyl residues. Peptides containing a free α-carboxyl group adjacent to the L-alanyl peptide bond, as in the L-alanyl-D-isoglutamyl sequence, are not cleaved.

From these results it was concluded that the main neutral peptide component present in the P1 fractions contained a free α-carboxyl group of glutamic acid and therefore the amide group was substituted on a carboxyl group of diaminopimelic acid.

Peptides in the A2 fractions. These fractions (Tables 1 and 2) contained 1 mol of L-alanine/mol of glutamic acid. Samples (containing 1 μmol of diaminopimelic acid) from several different preparations were treated with 1-fluoro-2,4-dinitrobenzene. After hydrolysis, 52–65% of the diaminopimelic acid residues were recovered as the free amino acid and the amount of N-terminal alanine was 40–44%.

Properties of the oligosaccharides

Fraction A3 isolated from autolysates of *B. licheniformis* walls by DEAE-cellulose chromatography contained a small proportion of amino acids (Table 2). The fraction (containing 14 μmol and 3 μmol of glucosamine and diaminopimelic acid respectively) was passed through a column (2 cm × 40 cm) of Dowex 50 (H⁺ form) and the oligosaccharides eluted with water contained 10.6 μmol and

9.8 μmol of glucosamine and muramic acid respectively and less than 0.05 mol.prop. of amino acids. Fraction A3 prepared from ^{32}P -labelled walls also contained radioactivity that was removed completely from the oligosaccharides by chromatography on a column (2 cm $\times$ 40 cm) of Sephadex G-15 run in 0.1 M-pyridine-acetate buffer, pH 5.1. At least half of the radioactivity was present as inorganic phosphate.

Electrophoresis of the purified oligosaccharides for 3 h in pyridine-acetate buffer, pH 3.6, followed by chlorination (Rydon & Smith, 1952) gave a major band moving 12 cm towards the anode and two minor bands moving 8.5 cm and 15 cm towards the anode. The disaccharide, 4-*O*- β -*N*-acetylglucosaminyl-*N*-acetylmuramic acid, and tetrasaccharide from *M. lysodeikticus* moved identically with the main oligosaccharide band during the electrophoresis. Chromatography of the oligosaccharide fraction from *B. licheniformis* in solvent *C* gave an elongated spot (R_{GlcNAc} 0.35–0.42, approx.) detected by chlorination. This compound moved more slowly than the *M. lysodeikticus* disaccharide (R_{GlcNAc} 0.82). Electrophoresis and chromatography of the *B. subtilis* A3 fraction, containing about 0.1 mol.prop. of amino acids (Table 1), gave a similar pattern to the purified *B. licheniformis* oligosaccharides.

Treatment with *exo*- β -*N*-acetylglucosaminidase. The enzyme released *N*-acetylhexosamine residues from the purified oligosaccharide fraction of *B. licheniformis* and from *M. lysodeikticus* tetrasaccharide (Fig. 5). *N*-Acetylglucosamine was identified by chromatography. Prolonged treatment of *M. lysodeikticus* tetrasaccharide released 0.51 mol of *N*-acetylglucosamine/mol of total glucosamine. In the same time 0.19 mol of *N*-acetylglucosamine/mol of total glucosamine was released from the purified oligosaccharide chains. This value gave a number-average chain length of 10–11 hexosamine residues for the oligosaccharides present in fraction A3 (Table 4). The oligosaccharide chains in *B. licheniformis* fraction A2 were somewhat shorter and contained about 6 hexosamine residues. The *B. subtilis* oligosaccharides present in fraction A3 isolated from DEAE-cellulose were also treated with β -*N*-acetylglucosaminidase. The enzyme released 0.22 mol of *N*-acetylglucosamine/mol of glucosamine, giving a number-average chain length of about 9–10 hexosamine residues.

Separation of *B. subtilis* fraction A3 on Sephadex G-50. The oligosaccharides were fractionated on the basis of size by chromatography on Sephadex G-50. They were eluted in an asymmetric peak with a pronounced bias towards lower-molecular-weight material (Fig. 6). Peak fractions were pooled and analysed. The overall yield of glucosamine from the column was 72% and each fraction

Fig. 5. Action of β -*N*-acetylglucosaminidase on *B. licheniformis* oligosaccharides and *M. lysodeikticus* tetrasaccharide. Samples of the oligosaccharides present in fraction A2 (▲) (containing 0.22 μmol of total glucosamine) and fraction A3 (■) (treated with Dowex 50 and containing 0.41 μmol of total glucosamine) and *M. lysodeikticus* tetrasaccharide (●) (0.041 μmol) were treated at pH 4.4 and 35°C with enzyme. *N*-Acetylglucosamine released was measured by the Morgan-Elson reagent.

contained about equimolar amounts of glucosamine and muramic acid after acid hydrolysis (Table 5). Each oligosaccharide fraction contained amino acids in very similar proportions to those found in the starting material. The difference in size of the oligosaccharide chains was not therefore due to cross-linking by residual peptide side chains. The number-average chain lengths determined by treatment with β -*N*-acetylglucosaminidase gave values of 21, 9 and 5 hexosamine residues in order of elution from the column. The weighted mean of these values (about 10 hexosamine residues) is identical with the result obtained with the unfractionated oligosaccharide material.

Reducing terminals of oligosaccharides. The purified oligosaccharide fraction from *B. licheniformis* was treated with an excess of sodium borohydride at 35°C overnight, hydrolysed and examined by chromatography on Dowex 50 (Fig. 7c). A large radioactive peak was found in the glucosaminol region from the column and no muramicitol [2-amino-3-*O*-(*D*-1-carboxyethyl)-2-deoxy-*D*-glucitol] was detected. Unchanged glucosamine and muramic acid were also present.

Table 4. Chain lengths of *B. licheniformis* and *B. subtilis* oligosaccharide fractions

B. licheniformis fraction A3 (Table 2) was passed through Dowex 50 (H^+ form) and the oligosaccharides, eluted with water, were analysed. Two preparations isolated from different batches of walls were used with very similar results. *B. licheniformis* fraction A2 (Table 2) and *B. subtilis* fraction A3 (Table 1) were analysed directly. Number-average chain lengths were estimated as follows: *a*, the proportion of *N*-acetylglucosamine released by *exo*- β -*N*-acetylglucosaminidase; *b* and *c*, the proportion of glucosamine converted into glucosaminol by $NaBH_4$ [glucosaminol was measured by incorporation of 3H (*b*) or periodate oxidation (*c*)]; *d*, periodate oxidation of $NaBH_4$ -reduced oligosaccharides. Details of experimental methods are given in the text. N.D., Not determined.

Method	Fraction ...	Chain length (hexosamine residues)		
		<i>B. licheniformis</i>		<i>B. subtilis</i>
		A2	A3	A3
<i>a</i>		6	10–11	9–10
<i>b</i>		8	14–17	15
<i>c</i>		7	13–15	N.D.
<i>d</i>		N.D.	14	N.D.

Fig. 6. Sephadex G-50 chromatography of *B. subtilis* oligosaccharides. A part (containing $17\mu\text{mol}$ of total glucosamine) of fraction A3 (Table 1) was passed through the column ($2\text{cm} \times 40\text{cm}$) in 0.1M -pyridine-acetate buffer, pH 5.1. Fractions (2ml) were collected and analysed for total hexosamines. The fractions made by pooling tubes are shown by blocks along the abscissa.

Glucosaminol was similarly identified after reduction of *B. licheniformis* fraction A2 and *B. subtilis* fraction A3, and in both cases no radioactivity was found in the muramicitol region.

Control experiments were carried out with *M. lysodeikticus* tetrasaccharide. Samples ($0.125\mu\text{mol}$)

of the tetrasaccharide were treated with an excess of sodium boro[3H]hydride before and after hydrolysis (4M -hydrochloric acid at 100°C for 4h). The products of reduction were separated on Dowex 50 as shown in Figs. 7(a) and 7(b). The reducing *N*-acetylmuramic acid residue of the tetrasaccharide was quantitatively converted into the sugar alcohol. The radioactivity found in the pooled muramicitol peak from Dowex 50 was doubled if borohydride reduction was carried out after total acid hydrolysis of the tetrasaccharide. Before hydrolysis no glucosaminol was formed, showing the complete stability of the glycosidic linkages to the conditions used for borohydride reduction. Some radioactivity was eluted unretarded from the columns and was present in control mixtures containing only acidified borohydride solutions. Presumably this material was due to incomplete removal of excess of 3H from the reduction mixtures or to a radioactive impurity in the reagent.

The [1-^3H]glucosaminol produced by reduction, then hydrolysis, of *B. licheniformis* and *B. subtilis* oligosaccharides was identical with authentic glucosaminol as shown by chromatography in solvents *B* and *D* and by electrophoresis in solvent *H*.

The number-average chain length of *B. licheniformis* oligosaccharides obtained from different preparations of fraction A3 was estimated (Nordin, Hasegawa, Smith & Kirkwood, 1966) from the extent of incorporation of 3H into glucosamine by treatment with sodium boro[3H]hydride. Values of 14–17 hexosamine residues were obtained. The corresponding values for the oligosaccharide chains present in *B. licheniformis* fraction A2 and *B. subtilis* fraction A3 were 8 and 15 hexosamine residues respectively. Glucosaminol, isolated from reduced then hydrolysed oligosaccharides by Dowex

Table 5. *Analysis of B. subtilis oligosaccharides isolated by Sephadex G-50 chromatography*

Fractions S1, S2 and S3 (Fig. 6) were analysed as described in the Methods section. Chain lengths were determined with exo- β -*N*-acetylglucosaminidase as described in the text.

Fraction	Molar ratios		Glucosamine (% of total)	Chain length (hexosamine residues)
	Muramic acid/ glucosamine	Diaminopimelic acid/ glucosamine		
S1	0.91	0.13	10	21
S2	0.92	0.09	37	9
S3	0.92	0.10	53	5
Starting material	0.93	0.12	—	9–10

50 chromatography (Fig. 7), was also estimated by the formaldehyde produced on periodate oxidation at low pH as described in the Methods section. From the relative proportions of glucosaminol to glucosaminol plus glucosamine (measured by the Elson-Morgan reaction) number-average chain lengths of 7 and 13–15 hexosamine residues were obtained for *B. licheniformis* fraction A2 and purified oligosaccharides prepared from *B. licheniformis* fraction A3 respectively (Table 4).

Periodate oxidation of reduced oligosaccharides. Periodate oxidation of borohydride-reduced oligosaccharides takes place preferentially at the acyclic terminals with little concomitant oxidation elsewhere in the molecule (Clancy & Whelan, 1959). The reducing terminals of *B. licheniformis* oligosaccharides prepared from fraction A3 were estimated by periodate oxidation after borohydride reduction. Periodate oxidizes the terminal *N*-acetylglucosaminol residue, producing 1 mol. prop. of formaldehyde (Tipper, Ghuyssen & Strominger, 1965). A sample of reduced *B. licheniformis* oligosaccharides (containing 1.3 μ mol of total glucosamine) rapidly produced formaldehyde on periodate oxidation. The amount formed within 30 min did not increase appreciably over the next 4 h. The number-average chain length was 14 hexosamine residues, in reasonable agreement with results (Table 4) obtained by incorporation of ^3H from sodium boro[^3H]hydride and by measurement of glucosaminol.

DISCUSSION

During autolysis of *B. subtilis* cell walls a small number of amide bonds in the mucopeptide were not hydrolysed by the amidase. In walls prepared from cells grown into exponential phase, when the activity of the autolytic amidase is highest (Young, 1966), the amount of diaminopimelic acid recovered in fraction A2 from DEAE-cellulose was about 21%. In several experiments with *B. licheniformis* walls prepared from exponential-phase cells the number

of unhydrolysed bonds appeared to be consistently larger. The amount of diaminopimelic acid recovered in fraction A2 varied from 24% to 38%. The A2 fractions contained peptide side chains, linked presumably through L-alanine residues to short glycan chains containing approximately equimolar amounts of the expected amino sugars. The survival of these linkages during autolysis is surprising and requires further work. Monomer, dimer and perhaps more highly cross-linked peptides were present, as judged from the amino acid composition and the content of *N*-terminal groups. Since *N*-terminal alanine residues were found, a proportion of the cross-linked peptides were presumably substituted only at one end with *N*-acetylmuramic acid residues.

It is of note that fraction A4 containing the teichoic acids and teichuronic acid always contained very few amino acids. This indicated an almost complete hydrolysis of the amide bonds substituting oligosaccharides that were covalently attached to acidic polymers in the intact cell wall. Autolysis of insoluble cell walls proceeds in two phases. First the enzyme attacks an insoluble substrate, releasing soluble material that may include the enzyme. Nothing is known at present of the rate at which the different wall polymers and the enzyme are solubilized during autolysis. The rates need not be the same. In a later stage of autolysis the soluble enzyme reacts with soluble substrates. The activity or stability of amidase may be different in these two situations. The efficiency with which peptide side chains were removed from the mucopeptide oligosaccharides linked to other polymers in the intact cell walls may reflect a close physical proximity of the enzyme with certain regions of mucopeptide that carry these acidic polymers. Indeed preparations of soluble amidase, purified extensively from *B. subtilis* Marburg strain 168 cells, contain teichoic acid that may be covalently attached to the enzyme (Brown, Frazer & Young, 1968). The rapid turnover of non-mcopeptide wall polymers observed in bacilli undergoing transition in a chemo-

Fig. 7. Dowex 50 chromatography of NaB^3H_4 reaction mixtures. (a) Tetrasaccharide from *M. lysodeikticus* ($0.125\text{ }\mu\text{mol}$) was hydrolysed in 4 M-HCl at 100°C for 4 h and dissolved in 0.1 M-borate buffer, pH 8.9 (0.2 ml). Then 2% (w/v) NaB^3H_4 (0.2 ml ; approx. $2\text{ }\mu\text{Ci}/\mu\text{mol}$) was added and the mixture kept at 35°C overnight. Excess of reagent was destroyed with 12 M-HCl (0.2 ml), and the reaction mixture was dried, dissolved in water (0.5 ml) and a portion (0.4 ml) was applied to the column. The column ($0.84\text{ cm}\times 22\text{ cm}$) was eluted with $0.1\text{ M-pyridine-acetate}$ buffer, pH 2.8, and 30 fractions (2.0 ml each) were collected. Then additional fractions (2.0 ml each) were eluted with $0.133\text{ M-pyridine-acetate}$ buffer, pH 3.85. Portions (0.2 ml) of the fractions were analysed for radioactivity. (b) As (a) except that reduction with NaB^3H_4 preceded acid hydrolysis. (c) *B. licheniformis* oligosaccharides (containing $4.0\text{ }\mu\text{mol}$ of glucosamine), after treatment with NaB^3H_4 , were hydrolysed and examined by chromatography as described in (a). Portions (0.2 ml) of the fractions were assayed for radioactivity (—) and for hexosamine material (----). 1, [^3H]-Muramicitol; 2, muramic acid; 3, [^3H]-glucosaminol; 4, glucosamine.

polymers without causing excessive damage to the rest of the mucopeptide could then take place by scission of relatively few amide bonds.

The mucopeptides of *B. licheniformis* and *B. subtilis* show a low degree of cross-linking between peptide side chains. In the particular wall preparations described, about one-third of the total diaminopimelic acid residues recovered as peptides or 26% of the total diaminopimelic acid of the autolysates was isolated in a monomer unit L-Ala-D-Glu-Dap. This value is similar to the yield (13–18%) of a monomer fragment, N-acetylglucosaminyl-(1→4)-N-acetylmuramic acid tripeptide, from lysozyme digests of heated *B. licheniformis* walls (Hughes, 1968b). The remaining diaminopimelic acid in the peptide fraction of autolysates was found in the dimer unit (L-Ala-D-Glu-Dap) $_2$ -D-Ala. The yield of diaminopimelic acid in the peptide fraction A1 was at least 70% and therefore the distribution probably represents quite closely the relative proportions of the two peptides in the intact mucopeptides. It should be mentioned that no peptide cross-bridges were hydrolysed during autolysis of *B. licheniformis* (J. S. Thompson & H. J. Rogers, personal communication) or *B. subtilis* (Young, 1966) cell walls in these experiments.

In *Escherichia coli* mucopeptide a part of the monomer and dimer peptides carries D-alanine residues at the C-terminals (Primosigh, Pelzer, Maas & Weidel, 1961; Weidel & Pelzer, 1964). These residues are removed to a variable extent by D-alanine carboxypeptidase activity during preparation of the cell walls (Leutgeb & Weidel, 1963). No units containing C-terminal D-alanine residues were detected in *B. licheniformis*, presumably because of the complete enzymic removal of these residues, and the peptides lacking C-terminal D-alanine residues were shown to be normal structural units of mucopeptide (Hughes, 1968b).

E. coli mucopeptide contains no amide groups (van Heijenoort *et al.* 1969), in contrast with the single amide group present in the side chains of *B. licheniformis* and *B. subtilis* mucopeptides. The resistance of the dimer peptide from *B. licheniformis* N.C.T.C. 6346 to L-alanine aminopeptidase, together with the results of hydrazinolysis, strongly suggests that the single amide group is substituted on diaminopimelic acid residues, as proposed previously (Hughes, 1968a). A similar assignment has been reported for *B. subtilis* mucopeptide (Warth & Strominger, 1968). In contrast, in the mucopeptide of another strain of *B. licheniformis* A.T.C.C. 9945, amide groups were reported to be substituted on α -carboxyl groups of D-glutamic acid residues (Mirelman & Sharon, 1968).

The oligosaccharide chains of *B. licheniformis* and *B. subtilis* mucopeptide appear to contain on

stat between Mg^{2+} limitation and PO_4^{3-} limitation (Ellwood & Tempest, 1969) may require positional integration with the amidase. Release of the acidic

average about 10 hexosamine residues, as determined by the β -*N*-acetylglucosaminidase assay. A value of 13 hexosamine residues for *B. subtilis* Marburg strain 168 oligosaccharides was obtained independently by determination of reducing power (Young *et al.* 1964). In common with other bacterial mucopeptides, the *B. subtilis* oligosaccharides were found by chromatography on Sephadex G-50 to be polydisperse. The release of *N*-acetylglucosamine from oligosaccharide fractions by exo- β -*N*-acetylglucosaminidase unequivocally places these residues at non-reducing ends of at least some of the chains. This is to be expected, since mucopeptide is built up by addition of *N*-acetylglucosaminyl-*N*-acetylmuramyl peptide units into growing strands. The finding of *N*-acetylglucosamine as apparently the only reducing terminal residue in the oligosaccharide chains is not consistent with this scheme. It could be argued that the oligosaccharides studied undergo degradation during normal growth of the bacteria or during preparation of the cell walls. Glycosidases are known to be present in a number of bacterial cell walls, including *B. subtilis* (Brown & Young, 1970) and *B. licheniformis* (J. S. Thompson & H. J. Rogers, unpublished work). The possibility cannot be ruled out that the glycosidase, presumably an endo- β -*N*-acetylglucosaminidase, hydrolysed a few bonds during preparation of the cell-wall fragments. However, in this process no new non-reducing end groups of *N*-acetylglucosamine could be formed, and the average chain lengths determined from the content of these residues would be maximum values and reflect the size of the undegraded molecules. In fact number-average chain lengths of the oligosaccharides as estimated from the content of reducing terminal groups were usually higher than the values obtained by using exo- β -*N*-acetylglucosaminidase. The reason for this discrepancy is not known. The occurrence of a low degree of branching in the oligosaccharide chains is unlikely because of the high chromatographic mobility of the isolated chains, and no evidence has been forthcoming as yet for a modified or blocked reducing end group in some of the oligosaccharide chains.

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