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Structure of the Peptidoglycan from Vegetative Cell Walls of *Bacillus subtilis**

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ABSTRACT: Autolysis of *Bacillus subtilis* cell walls cleaved the muramyl-L-alanine bond of the peptidoglycan and yielded peptides, an acetylmuramylacetylglucosamine glycan and a teichoic acid–polysaccharide complex. The glycan had a number-average chain length of 23 hexosamine residues terminating at the reducing end with *N*-acetylglucosamine. The major peptides present were L-alanyl-D-γ-glutamyl-(L)-*meso*-diaminopimelyl-(D)-amide (monomer) and a heptapeptide (dimer) which consisted of a tetrapeptide: L-alanyl-D-γ-glutamyl-(L)-*meso*-diaminopimelyl-(D)-amide, (L)-D-alanine, linked through a D-alanyl-(D)-*meso*-diaminopimelyl bond to the tripeptide above. An undecapeptide triamide trimer was also found. Nine other peptides differed from the dimer and trimer in lacking an amide or L-alanyl-D-glutamic acid residues. The cell wall peptides differ from peptides in *Escherichia coli* and *Bacillus megaterium* cell walls and in *Bacillus subtilis* and other spores in having diaminopimelyl amides and in lacking carboxyl-terminal D-alanine.

Peptidoglycans typically consist of an alternating polymer of β-(1–4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid. Each muramic acid residue bears a short peptide side chain which may be linked to other peptides to form a structural polymer (Ghuysen, 1968). In the *Bacillaceae* and *Clostridium* vegetative cells contain peptidoglycan in their cell walls and in spores it forms the cortex and germ cell wall (Warth *et al.*, 1963). We have shown (Warth and Strominger, 1969; Warth, 1968) that the structure of spore peptidoglycan differs in several ways from that of typical gram-positive bacterial cell walls. To gain further insight into the functional and biosynthetic significance of these differences we sought to determine the structure of peptidoglycan in cell walls and spores of a single species, *Bacillus subtilis*.

Previous studies on the cell wall of *B. subtilis* 168 have shown it to contain a very active autolytic amidase which cleaved muramyl-L-alanine bonds to give a mixture of peptides, glycan, and teichoic acid–polysaccharide complexes (Young *et al.*, 1964; Young, 1966). Peptides and other components were isolated but no structural information was obtained. In this work the cell walls of the Porton strain of *B. subtilis* were degraded by the autolytic amidase and the structures of 12 peptides elucidated.

Materials and Methods

Cell Walls (small preparation). *Bacillus subtilis* (Porton strain) was obtained from Dr. W. G. Murrell, C.S.I.R.O. Division of Food Preservation, Ryde, Australia. Cells were grown on antibiotic medium no. 3 (Difco) in flasks at 37° to late exponential phase. Ice was quickly added to the culture to prevent lysis, and the cells were centrifuged and washed with 0.2 M KCl–0.1 M potassium phosphate (pH 6.5), 0.05 M KCl, and 0.05 M potassium phosphate. Cell walls were prepared by shaking cells with glass beads for 1 min in the Nossal disintegrator in 0.1 M potassium phosphate buffer (pH 6.5). The walls were separated from glass beads by filtration, and were washed with pH 6.5 buffer twice and water five times.

Preparation of Peptides from a Cell Wall Autolysate. Cells were grown rapidly ($\mu = 1.6$) on antibiotic media no. 3 in continuous culture in a Biogen (American Sterilizer Co.). A tendency of these cells to lyze was overcome by rapid chilling of the effluent cell suspension, followed immediately by centrifugation and after one wash with water, freezing of the cell pellet. The cells (17 g) were disrupted by blending with glass beads in 0.2 M phosphate (pH 6.0, 450 ml) for 20 min at 0–10°. The walls were separated from unbroken cells and

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debris by differential centrifugation in 0.2 M sodium phosphate (six times) and water (once). The yield of cell walls was 3.5 g.

A suspension of the cell walls in 150 ml of water was titrated to pH 9.1 and the pH maintained by periodic additions of 0.2 N NaOH during 4-hr incubation at 37°. After centrifugation the supernatant was concentrated and excess sodium phosphates were removed by crystallization at −5°. The autolysate was adjusted to pH 7.0 with acetic acid, diluted to 100 ml, and applied to an Ecteola-cellulose column. The column (100 × 3 cm) had been packed and equilibrated in 0.05 M lithium acetate (pH 7.0) and then washed with water. Elution was with a concave gradient formed between 0.05 M lithium acetate (pH 7.0, 1 l.) and 0.5 M lithium acetate (pH 4.0) containing 0.2 M LiCl (1 l.) followed by a gradient between 1 l. of the latter buffer and 1 M LiCl. The peak containing nearly all the peptides (500–800 ml) was concentrated to near dryness and most of the lithium salts and free alanine extracted with 12 ml of acetone–ethanol (1:9, v/v). No peptides containing Dpm[1] were extracted. The precipitate was dissolved in water and the pH adjusted to 2.7 before application to the A-15 column.

Preparation of Peptides by Lysozyme and Amidase Digestion of Cell Walls. Cells were disrupted for 1 min in the Nossal cell disintegrator in 0.1 M sodium phosphate (pH 6.0) at 0–5°. The cell walls were washed quickly with buffer (once) and water (three times) and then heated at 100° for 20 min to inactivate the autolytic enzyme. After two more washes with water the walls (20 mg/ml) were solubilized with lysozyme (0.3 mg/ml) at pH 6.8 for 3 hr at 37°. An equal volume of *Streptomyces* amidase (Ghuysen *et al.*, 1962) in 0.03 M pyridine–acetate buffer (pH 5.4) was added and the solution incubated for 4 hr at 37°. The amidase preparation, a gift of Dr. J.-M. Ghuysen (University of Liege, Belgium), completely hydrolyzed a tetrasaccharide–tetrapeptide substrate at 10 mM concentration under similar conditions. The cell wall digest was dialyzed against several small volumes of water. The diffusible fraction contained all the free peptides.

Preparation of DNP-amino Acids for Measurement of Optical Rotations. Boiled cell walls (41 mg) were solubilized with lysozyme (0.5 mg) in 2 ml of 0.01 M sodium phosphate (pH 6.0) for 2 hr and then dinitrophenylated with FDNB (160 mg) and triethylamine (0.4 ml) in 25 ml of 50% ethanol for 45 min at 60°. After evaporation of the reagents the DNP cell walls were hydrolyzed (6 N HCl, 107°, 16 hr), diluted with two volumes of water, and extracted successively with ether and 1-butanol saturated with 2 N HCl. The butanol extract in 1% $NaHCO_3$ was chromatographed on a DEAE-cellulose column, desalted on Sephadex G-10 (Bricas *et al.*, 1967), and mono-DNP-Dpm finally purified by preparative thin-layer chromatography. The final yield was 3 μmoles. The neutral dimer II (0.75 μmole) and standards of D-, L-, and DL-alanine (4 μmoles) were dinitrophenylated and hydrolyzed as above. DNP-alanine was purified from the ether extracts by preparative thin-layer chromatography using solvent I: yield, 0.7 μmoles. Mono-DNP-Dpm was extracted with 1-butanol saturated with 2 N HCl and purified by thin-layer chromatography.

Chromatography and Electrophoresis. Peptides were fractionated on columns (8.5 × 2.2 cm) of Beckman ion-exchange resin, type A 15. The columns were operated at 50° and eluted with 0.2 M pyridine–acetate pH 3.1 (pyridine (16 ml)–acetic acid (260 ml)–water, (724 ml)) for 80 ml followed by a linear gradient formed between 500 ml of this buffer and 500 ml of 2 M pyridine–acetate, pH 5.1 (pyridine (161 ml)–acetic acid (145 ml)–water (694 ml)). For rechromatography of neutral and acidic peptides the pH 5.1 buffer was replaced by 1 M pyridine–acetate (pH 4.35). After chromatography pyridine–acetate was removed by repeated evaporation from water. Gel filtrations were on columns (100 × 2.5 or 100 × 0.9 cm) of Sephadex G-25 fine (Pharmacia, Sweden) eluted with water.

High-voltage paper electrophoresis was carried out as described previously (Warth and Strominger, 1969) for 2–3 hr at 30–40 V/cm, using Whatman No. 3MM paper. For preparative work, guide strips equal to about 20% of the sample width were cut out and used for detection of the peptide bands with ninhydrin. Peptides were eluted from the remaining paper with 0.1 M acetic acid. Recoveries of the peptides were not corrected for the loss from guide strips or for samples removed from column fractions for assay. All thin-layer chromatography was carried out on silica gel G plates. Ether-soluble DNP-amino acids were chromatographed with chloroform–methanol–acetic acid (85:14:1, v/v) at 4° (system I) and on a duplicate plate with 1-butanol–1% ammonia (1:1, v/v) at room temperature (system II). System III (*tert*-amyl alcohol–chloroform–methanol–concentrated ammonia–water, 30:30:30:2:6, v/v) was used for mono-DNP-Dpm. Peptides were separated with isobutyric acid–triethylamine–water (100:7:43, v/v). Glycols were detected with the periodate–Schiff reagents (Buchanan *et al.*, 1950) after chromatography on Whatman No. 1 paper with isopropyl alcohol–5% boric acid (7:1, v/v). Hexoses were detected with aniline–phthalate after paper chromatography with ethyl acetate–pyridine–water (44:14:9, v/v).

Analytical Methods. The methods for determination of free amino groups, carboxyl-terminal amino acids, total hexosamine, acetylhexosamine, reducing power, and D- and L-alanines were as described by Ghuysen *et al.* (1966). For estimation of amino-terminal amino acids, peptides were dinitrophenylated by the procedure of Jarvis and Strominger (1967) and the DNP-amino acids estimated by thin-layer chromatography (Ghuysen *et al.*, 1966) after hydrolysis in 6 N HCl for 16 hr at 105°. Alanine which had been similarly dinitrophenylated, hydrolyzed, and chromatographed was used as the standard. Peptides usually gave yields of DNP-amino acids of 70–80% of the alanine standard. Edman degradations were carried out by the modifications of the Konigsberg and Hill (1962) procedure described by Tipper *et al.* (1967). In the first degradation cycle, phenylthiocarbamyl derivatives were cyclized with 4 N HCl at 37° for 2 hr. Trifluoroacetic acid was used as the cyclizing reagent in the second cycle. Amino acids, amino sugars, and ammonia were determined on a Beckman-Spinco Model 120C amino acid analyzer after hydrolysis in 6 N HCl at 105° for 16 hr. Amino sugar contents obtained from 16-hr hydrolysates were corrected for hydrolytic loss by multiplication of the glucosamine and galactosamine values by 1.32 and the muramic acid values by 1.38. Total phosphate was determined according to Lowry *et al.* (1954) and glucose and uronic acid by the cysteine–H_2SO_4 and the carbazole methods of Dische (1955). Acetyl groups were determined by the method of Ludoweig and Dorfman (1960) but on one-quarter the scale. Optical rotatory dispersion curves were recorded with a Carey Model 60 spectropolarimeter using 1-cm cells of 3-ml capacity.

Estimation of the Degree of Peptide Cross-Linking in Cell Walls. Samples containing 100 mμmoles of Dpm were dinitrophenylated by the method of Jarvis and Strominger (1967), evaporated dry, and hydrolyzed in 100 μl of 6 N HCl for 23 hr

[1] Abbreviations used are: Dpm, 2,6-diaminopimelic acid; FDNB, fluorodinitrobenzene; PTH, phenylthiohydantoyl.

TABLE I: Composition of *B. subtilis* Cell Walls, Glycan, and Teichoic Acid Complex.

	Cell Walls		Glycan	Teichoic Acid
	μmoles/g	Mole Ratio	(μmoles/g)	(μmoles/g)
Glucosamine	442	1.36	1140	260
Muramic acid	315	0.97	910	123
Alanine	1045	3.2	445	20
Glutamic acid	325	1.00	305	14
Diaminopimelic acid	324	1.00	307	11
Phosphate	1720	5.3	110	2500
Glucose	1230	3.8	0	1870
Galactosamine	103	0.32	0	93
Ribitol	+		ND[a]	+

[a] ND, not detected on paper chromatograms.

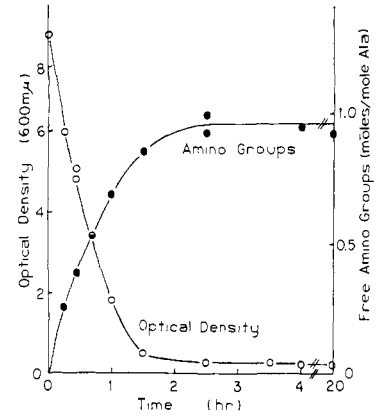

FIGURE 1: Kinetics of autolysis of *B. subtilis* cell walls. Cell walls (380 mg) were incubated in 25 ml of 0.05 M potassium carbonate buffer (pH 9.2) at 37°. Optical density at 600 mμ (○) was measured on a Zeiss Model PMQ spectrophotometer after appropriate dilution. Amino groups (●) were measured in the supernatant after centrifugation. The release of reducing power was insignificant.

at 105°. The hydrolysates were diluted with two volumes of water and extracted with ether (three times). Mono-DNP-Dpm and residual Dpm in the aqueous layer were each estimated on the amino acid analyzer. The pH of buffer B was adjusted to 4.06 with HCl. Use of the absorbance at 440 mμ rather than 570 mμ reduced the interference of leucine with mono-DNP-Dpm. Standards were similarly hydrolyzed Dpm and mono-DNP-Dpm. Results were corrected for the degree of dinitrophenylation (95 ± 1%) found by using purified peptides.

Identification of the Reducing End Group of the Glycan by Reduction with [^3H]NaBH$_4$. The samples containing 5–20 mμmoles of reducing end group and references of GlcNAc and muramic acid were reduced with 25 μl of unbuffered 0.2 M [^{3}H]NaBH$_4$ (50 mCi/mmole) at room temperature. After 16 hr the samples were acidified with 6 μl of 2 N HCl and evaporated dry. For the controls, the NaBH$_4$ was acidified before addition to the samples. All samples were hydrolyzed with 6 N HCl (40 μl) for 4 hr at 105° and evaporated from water to remove HCl and from methanol twice to remove borate. Reduction products, including glucosaminitol, galactosaminitol, C-1-reduced muramic acid, and side-chain-reduced muramic acid (from muramic lactam) were separated using the following systems: chromatography on Whatman No. 1 paper with 1-butanol–acetic acid–water (5:2:2, v/v) for 40 hr or with ethyl–pyridine–water (44:14:9, v/v), paper electrophoresis at pH 3.9, and paper electrophoresis at pH 3.9 of the N-acetylated products. Radioactivity was located by scanning strips in a Vanguard Autoscanner Model 880. Sections containing activity and corresponding sections from controls were cut out and eluted with 1% acetic acid. The eluate was evaporated, dissolved in 0.1 ml of water, 10 ml of dioxane scintillator was added, and then counted.

Results

Composition of Cell Walls. Equimolar amounts of muramic acid, glutamic acid, and Dpm were present (Table I). Thus each muramic acid could be substituted by a peptide. The major component in the walls of this strain of *B. subtilis* was teichoic acid which contained glucose and ribitol but no glycerol. The presence of galactosamine and of glucosamine in excess of muramic acid suggests a third type of polymer. No uronic acids were detected. The total acetyl content was equivalent to the total amino sugar. The analysis accounted for 102% of the dry weight of the walls assuming that the ribitol content was equal to the total phosphate.

Autolysis. On incubation of the cell wall suspension at pH 9.2 the optical density fell to 3% of its initial value within 4 hr (Figure 1), while free amino groups equivalent to 95% of the total alanine or 3 equiv/Dpm residue was released. An insignificant amount of reducing power was present. Young *et al.* (1964) showed that autolysis of *B. subtilis* cell walls involved cleavage of the muramyl-L-alanine bond joining the cell wall peptides to the glycan. The free amino groups that were solubilized during autolysis would include the amino-terminal L-alanine residues generated by the action of the autolytic enzyme together with Dpm amino groups on soluble peptides and D-alanine formed by alkaline hydrolysis of the labile ester linked D-alanine of teichoic acid.

Fractionation on Ecteola-cellulose. After partial desalting on Sephadex G-25 the autolysate was chromatographed on an Ecteola-cellulose column (Figure 2). Three major peaks were separated. Nearly all the free amino groups eluted were in an initial unabsorbed peak (fractions 8–10) that contained no phosphorus and little saccharide. Paper chromatography and

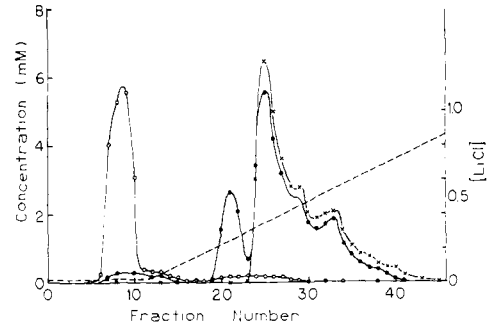

FIGURE 2: Fractionation of the cell wall autolysate on Ecteola-cellulose. The column (1.5 × 27 cm) of Ecteola-cellulose (Cellex E) (0.24 mequiv/g) was equilibrated with 0.01 M lithium acetate (pH 6.0) and eluted with a linear gradient formed by mixing 1 M LiCl (250 ml) into 0.01 M lithium acetate (250 ml). Fractions were assayed for amino groups (○), phosphate (×), and after hydrolysis with 2 N HCl for 2 hr at 100°, for reducing power (●).

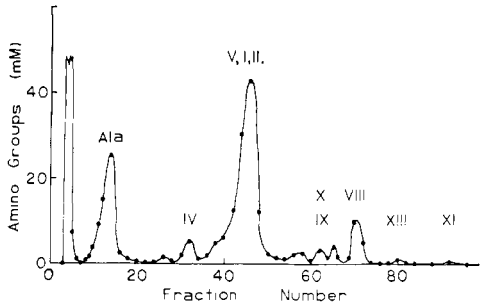

FIGURE 3: Chromatography of peptides on Beckman-type A15 cation-exchange resin. Fractionation of the cell wall autolysate on Ecteola-cellulose separated a peak containing nearly all the peptides from other cell wall degradation products (see Methods). The peptide peak was concentrated, extracted with ethanol–acetone to remove excess lithuum salts, and acidified to pH 2.7 with formic acid before application to the column. The column was eluted with pyridine–acetate buffers as described in Methods. Fractions of 7 ml were collected and amino groups were measured after hydrolysis of a sample with 6 N HCl for 16 hr at 105°. Roman numerals refer to the peptides present in each peak (see Table II, Figure 6).

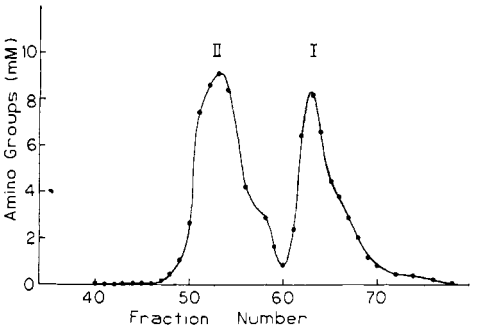

FIGURE 4: Separation of peptides I and II by gel filtration on Sephadex G-25. Fractions 34–52 from ion-exchange chromatography contained peptides I and II plus a small amount of V (Figure 3). Peptide V was separated from I and II by rechromatography on the A-15 column using a less steep gradient. Fractions of 5 ml were collected and assayed for free amino groups. Fully excluded compounds were eluted at fraction 34 (V_0).

electrophoresis showed the presence of much free alanine together with a number of acidic, neutral, and basic peptides. Fractions 19–23 contained polysaccharide together with small amounts of free amino groups and no phosphorus. All of the phosphorus and much of the saccharide was present in a complex peak immediately following in fractions 24–40. The fractionation pattern was very similar to that obtained from Ecteola-cellulose chromatography of a *Myxobacterium* AL-1 enzyme digest of *Staphylococcus aureus* cell walls (Tipper *et al.*, 1967) where peaks of peptide, glycan, and teichoic acid were separated.

Analysis of the Glycan and Teichoic Acid Complex. The teichoic acid complex and the glycan preparation were precipitated from fractions 24 to 40 and 19 to 23, respectively, with ethanol. Cell wall autolysate from 317 mg of cell walls yielded 77 mg of teichoic acid complex and 57 mg of glycan. Glucose and phosphate analysis of the teichoic acid complex (Table I) indicated a content of 90% glucosyl–polyribitol phosphate, assuming equimolar amounts of phosphate and ribitol. Galactosamine and excess glucosamine made up 5% of the weight and muramic acid plus glucosamine another 5%. One peptide subunit was present per ten muramic acid residues.

The glycan preparation contained no glucose or galactosamine but one phosphate was present for every twenty amino sugars and one peptide for every three muramic acid residues (Table I). The glycan contained 50% of the cell wall muramic acid and 17% of the Dpm. Another 24% of the cell wall muramic acid was in the teichoic acid complex.

The average chain length of the polysaccharides in the glycan fraction was estimated by determination of the formaldehyde produced during periodate oxidation. In order to achieve an appreciable rate of periodate oxidation the samples were first reduced with $NaBH_4$ (Tipper *et al.*, 1967). Formaldehyde production was complete within 30 min and corresponded to an average chain length of 24.6 hexosamine residues.

The identity of the reducing terminal residue was shown by reduction with [^{3}H]$NaBH_4$. A major radioactive component corresponding to glucosaminitol, and a weaker component corresponding in position to C-1-reduced muramic acid were detected in the hydrolysates. Galactosaminitol was not present. A chain length of 23 hexosamine residues was calculated from the radioactivity in glucosaminitol. Muramic acid was terminal less than once for every 250 hexosamines present.

Large-Scale Isolation of Cell Wall Peptides. A large batch of cell walls (3.5 g) was prepared and allowed to autolyze by adjustment of the pH to 9.2. The optical density fell to one-half its initial value in 30 min and was stationary at 23% of the initial value by 4 hr. Addition of the autolysate to boiled cell walls caused rapid lysis showing that the autolytic enzyme remained active. On centrifugation, the autolysate gave a grey sediment (0.25 g) which contained protein amino acids but insignificant amounts of Dpm or glucosamine. Peptides were separated from other cell wall polymers on an Ecteola-cellulose column. Nearly all the peptides emerged in a large unabsorbed peak (500–800 ml) which was almost free of hexosamines and contained no phosphate. Immediately following, was a much smaller peptide peak (833–926 ml). Hexosamine-containing material was eluted as a series of peaks between 900 and 2000 ml. A teichoic acid–polysaccharide complex was eluted around 3000 ml and contained glucosamine, galactosamine, and muramic acid but less than 1 peptide unit/20 muramic acid residues.

Fractionation of Peptides. The major peptide peak from the Ecteola-cellulose column was fractionated on a column of cation-exchange resin (Figure 3). Fractions 90–93 and 80–83 contained the minor basic peptides XIII and XI, respectively, which were further purified by high-voltage paper electrophoresis. The basic peptide VIII was obtained from fractions 70 to 72. It was desalted by gel filtration on a column (100 × 1 cm) of Sephadex G-15. Fractions 64–66 contained only free lysine (30 μmoles). Paper electrophoresis of fractions 61–63 separated the basic peptide X from neutral IX. Fractions 53–59 contained several minor peptides which were not further investigated. The major peak from the column (fractions 34–52) and the initial breakthrough peak (fractions 3–5) each contained several peptides. Fractions 29–33 contained peptide IV but more substantial amounts of IV were obtained after further chromatography of fractions 3–5. Fractions 9–18 contained only free alanine. This is produced by alkaline hydrolysis of the ester-linked alanine of teichoic acid during autolysis of the cell walls.

Rechromatography of fractions 34–52 on the same column, but using a more shallow gradient, separated a minor acidic peptide V from a large peak containing neutral peptides I and

TABLE II: Electrophoretic and Chromatographic Data and Yield of Cell Wall Peptides.

Peptide	M_{Glu},[a] pH 6.4	M_{Glu},[a] pH 3.9	M_{Ala},[b] pH 1.9	R_F[c] of Solvent IV	Net Yield (μmoles of Dpm)
I	−0.01	−0.19	1.05	0.41	116
II	−0.02	−0.19	0.99	0.31	260
III	−0.02	−0.19	0.99	0.22	12
IV	0.35	0.39	0.84	0.22	76
V	0.32	0.20	0.97	0.22	9
VI	0.26	0.25	0.85	0.15	6
VII	0.23	0.13	0.94	0.15	17
VIII	−0.44	−0.84	1.15	0.41	58
IX	0.00	−0.28	1.10	0.15	4
X	−0.32	−0.65	1.05	0.30	5
XI	−0.62	−1.11	1.16	0.41	1
XII	0.68	0.39	0.83	0.54	40[d]
XIII	−0.74	−1.28	1.28	0.56	2

[a] Mobility relative to glutamic acid. High-voltage paper electrophoresis using pyridine acetate buffers. Zero mobility was arbitrarily assigned to alanine. [b] Mobility measured from the origin relative to alanine. [c] Thin-layer chromatography with isobutyric acid–triethylamine–water (100:7:43, v/v) on silica gel G. [d] This peptide contained no Dpm.

II. Peptide V required further purification by paper electrophoresis. The major peptides I and II were separated by gel filtration on Sephadex G-25 (Figure 4). Peptide I was obtained from fractions 61–71, and peptide II was obtained after removal of a minor contaminant from fractions 46–59 by paper electrophoresis.

A further yield of acidic and neutral peptides was obtained from the breakthrough peak (fractions 3–5, Figure 3) by rechromatography. Peptide IV and two minor acidic peptides VI and VII were eluted in fractions 49–53, 55–57, and 73–74, respectively, and further purified by paper electrophoresis. Fractions 82–87 contained neutral peptides. Gel filtration on Sephadex G-25 readily separated peptides III and II. Peptide I was not present.

The minor peak of peptide material from the Ecteola-cellulose column contained peptide XII and glycan. Peptide XII was purified by two passages through the Sephadex G-25 column. Seventy-five per cent of the cell wall Dpm was recovered in the peptide preparations I–XIII. Neutral peptides I and II accounted for 67% of the recovered Dpm (Table II) and peptides IV and VIII for another 23%. The chromatographic and electrophoretic properties of the peptides were determined in four systems (Table II). No impurities were detected except for small contaminants in peptides VI and XI. Samples containing 100 mμmoles of free amino groups were applied and impurities of 1–2 mμmoles were detectable.

Analysis of the Peptides. Peptide I contained equimolar amounts of L-alanine, glutamic acid, Dpm, and ammonia (Table III). The alanine was at the amino terminal and the Dpm had one amino group free (Table IV). After dinitrophenylation and hydrolysis, glutamic acid was the only remaining amino acid. I therefore is a tripeptide amide. The absence of a carboxyl-terminal amino acid as determined by hydrazinolysis, in this and most of the other peptides (Table IV), suggests that the carboxyl-terminal residues are amidated.

TABLE III: Amino Acid Composition of Cell Wall Peptides.

	Mole Ratios					
Peptide	Total Ala	L-Ala	D-Ala	Glutamic Acid	Dpm	Ammonia
I	0.95	0.94	0.01	1.02	1.00	1.12
II	2.86	1.89	0.91	1.99	2.00	2.09
III	5.44	3.02	1.98	3.34	3.00	2.94
IV	2.79	1.86	0.83	1.93	2.00	1.02
V	2.78	1.76	0.92	1.93	2.00	1.36
VI	5.03			3.33	3.00	1.48
VII	4.56			2.83	3.00	1.73
VIII	1.79	0.98	0.96	0.97	2.00	1.91
IX	1.95			1.00	2.00	0.95
X	3.52			1.96	3.00	2.94
XI	2.82	1.12	2.21	1.07	3.00	3.03
XII	1.00	0.96	0.01	1.06	0.00	
XIII	1.00	0.05	0.98	0.03	1.00	1.20

Peptide II appeared to be a dimer, containing approximately 2 moles of L-alanine, glutamic acid, Dpm, and ammonia to 1 of D-alanine. Two alanine and one Dpm amino groups were free. This result was confirmed by analysis of the purified DNP derivative which contained glutamic acid, Dpm, ammonia, and alanine in the ratio 1.87:1.00:2.06:1.02. Two isomeric acidic peptides IV and V differed in composition from II in containing only one amide function. Dpm was carboxyl terminal in peptide V.

Peptide III contained three residues of L-alanine, glutamic acid, Dpm, and ammonia to two of D-alanine. One Dpm and

TABLE IV: Amino- and Carboxyl-Terminal Residues in Cell Wall Peptides.[a]

	Amino Terminal			Carboxyl Terminal	
Peptide	Alanine	Bis-Dpm	Mono-Dpm	Amino Acid	Ratio
I	0.97	0.0	0.75		0
II	1.46	0.0	0.74		0
III	2.89	0.0	0.89		0
IV	1.56	0.0	0.89		0
V	1.65	0.0	0.72	Dpm	0.89
VI	2.3	0.0	0.86		0
VII	2.57	0.0	0.86		0
VIII	0.71	0.53	0.11		0
IX	0.89	0.72	0.06	Dpm	0.85
X	1.56	1.02	0.12		0
XI	0.94	0.69	0.75		0
XII	0.78	0.0	0.0	Glu	0.71
XIII	0.00	0.81	0.05	Ala	0.16

[a] Data are mole ratios based on total Dpm = 1, 2, or 3 (Table III).

TABLE V: Amino-Terminal Amino Acids after One Cycle of Edman Degradation.[a]

Peptide	Amino-Terminal Residue		
	Alanine	Glutamic Acid	Mono-Dpm
I control	0.98	0.02	0.75
I	0.02	0.76	0.00
II control	1.42	0.00	0.67
II	0.03	1.46	0.00
IV	0.00	1.46	0.00
V	0.00	1.50	0.00
VIII	0.85	0.74	0.00

[a] Results are expressed as moles per mole of starting material. No phenyl isothiocyanate was added to the controls.

three alanine residues had a free amino group. The purified DNP derivative contained 2.84 moles of glutamic acid to 2.00 moles of Dpm and 1.94 of alanine, indicating a trimer. The analytical data for peptides VI and VII suggests that they are isomeric trimers differing from III in having only two amide groups.

A basic peptide VIII differed in composition from the neutral dimer II in containing one less L-alanine and glutamic acid residues and in having bisamino-terminal Dpm and one amino-terminal alanine in place of monoamino-terminal Dpm and two terminal alanines. Peptide XII contained L-alanine and glutamic acid. All the alanine was amino terminal and the glutamic acid was carboxyl terminal. Its structure is L-alanyl-D-glutamic acid. Peptides IX, X, and XI were minor components whose composition suggests they resemble peptides VIII or III but have fewer amide or alanylglutaminyl residues.

Edman Degradation of Peptides I, II, IV, and V. In the first cycle of degradation the amino-terminal alanine residues were eliminated and an equal number of glutamic acid residues became amino terminal (Table V). Analysis of the isolated peptide products (Table VI) confirmed the loss of amino-terminal alanine and Dpm. The alanine remaining in peptides II and IV was the D isomer (Table VI) so the amino-terminal alanine in I, II, and IV was of the L configuration.

The degradation product isolated from each peptide had a uv spectrum characteristic of a phenylthiohydantoin (Frankel-Conrat *et al.*, 1955) with λ_{max} 269 mμ (ϵ 15,000), indicating that cyclization of the phenylthiocarbamyl-Dpm peptide had taken place and any substituent of the carboxyl group α to the free amino group of Dpm should have been eliminated. Estimation of ammonia released by Edman degradation where the amino group is α to a carboxamide was complicated by partial acid hydrolysis of the amide caused by the strong acid necessary to effect cyclization (Tipper *et al.*, 1967). In the case of peptide I the PTH peptide was anionic (M_{Glu} = 0.85 at pH 3.9) and therefore had lost the amide function. When phenyl isothiocyanate was omitted from the procedure, I was only slightly deamidated.

Edman degradation eliminated ammonia from peptide II but not from peptide IV. During degradation II lost 1.28 ammonia residues, while in the absence of Edman reagent 0.82 residue was lost due to acid hydrolysis. Peptide IV lost 0.12 residue during degradation compared to 0.44 in the control. Both II and IV formed an anionic (M_{Glu} = 0.56) PTH peptide. These were combined to provide adequate material for analyses. Approximately 2 residues of glutamic acid were present to 1 of Dpm, D-alanine (Table V), and PTH, and 1.15 of ammonia. A minor more anionic (M = 0.95) PTH peptide from peptides II and IV appeared to be derived from the major product by acid hydrolysis of the remaining amide. It had the same mobility as the PTH peptide from V. Peptides IV and V appear to differ from peptide II only in containing one less amide function. In II and V the side-chain carboxyl of Dpm adjacent to the free amino group is amidated, whereas in IV it is free. After a second cycle of degradation no amino-terminal amino acids were found (Table VI).

Edman Degradation of Peptide VIII. This peptide had three free groups, one from an alanine residue and two on one of the Dpm residues. These were removed after the first degradation cycle and one glutamic acid and one alanine amino-terminal residues appeared (Table V). Sixty per cent of the ammonia (1.2 residues) was eliminated. Paper electrophoresis of the products showed a major ninhydrin-positive, uv-negative, slightly anionic spot (M_{Glu} = 0.10 at pH 3.9). A weaker but more acidic spot (M_{Glu} = 0.75) probably represents the product lacking both amides, produced by nonspecific acid hydrolysis of the major product. Analysis of the major product (Table IV) eluted in 58% yield from the electrophorogram, showed one residue each of glutamic acid, Dpm, D-alanine, and ammonia with glutamic acid and D-alanine amino terminals. The product from Edman degradation of this tripeptide contained a monoamino-terminal Dpm residue (Table IV). The most likely sequences are L-Ala-Glu together with Dpm-amide-D-Ala-Dpm but the Edman degradation data are also consistent with L-Ala-D-Ala-Dpm and Dpm-amide-Glu. The latter possibility is very unlikely in view of the Ala-Glu sequences already demonstrated for peptides I, II, IV, V, and VII. Since mono- and not bisamino-terminal Dpm was formed after the second Edman degradation cycle, the two sequences must be joined through a glutamyl–Dpm bond.

Deamidation of Peptides by Acid Hydrolysis. The tripeptide amide I was converted by 10 N HCl at 25° into an acidic peptide (M_{Glu} = 0.79 at pH 3.9). The product was eluted from the paper electrophoresis strip and passed through a column of Sephadex G-15. It contained alanine, glutamic acid, Dpm, and ammonia in the ratio 1.05:1.01:1.00:0.07 and had alanine and Dpm at the amino terminal, and Dpm at the carboxyl terminal. Approximately half the peptide was deamidated in 30 hr and only traces of peptide-bond hydrolytic products were formed in this time.

Hydrolysis of the heptapeptide diamide II formed two peptides with mobilities identical with the heptapeptide monoamides IV and V. These two hydrolysis products were eluted. The major product had identical mobilities at pH 1.9, 3.9, and 6.5 and R_F in the isobutyric acid system as peptide V and the minor product had the same mobilities and R_F as peptide IV. On more prolonged hydrolysis, these two products decreased in intensity and a more highly anionic (M_{Glu} = 0.77 at pH 3.9) product appeared. This product was eluted as above and contained: alanine, glutamic acid, Dpm, and ammonia in the ratio 2.71:1.91:2.00:0.14. The same nonamidated heptapeptide (M_{Glu} = 0.77) appeared to be formed by hydrolysis of the monoamidated heptapeptides IV and V.

The rate of deamidation of peptides I and V and of II to form IV was less than half that for the deamidation of IV and of II to form V. This is expected if the amide hydrolyzed in the first group is adjacent to a positively charged amino group and the second more rapidly hydrolyzed amide is adjacent to a

TABLE VI: Analysis of Products Isolated after One Cycle of Edman Degradation.

Original Peptide	Recov (%)	Mole Ratios: Total Analysis: Alanine: Total	Alanine: L	Alanine: D	Glutamic Acid	Dpm	Amino Terminal: Glutamic Acid	Amino Terminal: Alanine	Amino Terminal after 2nd Cycle of Degradation: Alanine	Glutamic Acid	Mono-Dpm
I	78	0.03			1.00	0.02	0.89	0.03	0.00	0.04	0.00
II + IV	50	0.94	0.07	0.85	2.05	1.00	1.62		0.01	0.07	0.07
V	28	1.04			2.37	1.00	1.72		0.05	0.07	0.03
VIII	51		0.00	1.14	1.08	1.00	1.08	1.15	0.09	0.15	0.60

peptide bond. The electrophoretic properties support this conclusion. Peptide IV was more anionic at pH 3.9 than V and therefore probably contains a more strongly acidic carboxyl group, adjacent to the free amino group. Peptides I and II were more strongly cationic at pH 3.9 than a tetrapeptide amide from *Corynebacterium diphtheriae* in which the glutamic acid α-carboxyl was amidated (Kato *et al.*, 1968), and therefore lack a strong carboxyl group and probably are amidated on the carboxyl adjacent to the free amino group.

Hydrolysis of the trimer, peptide III, gave spots corresponding to three peptides lacking one amide (M_{Glu} = 0.25, 0.15, and 0.09 at pH 3.9), three lacking two amides, and an acidic product (M_{Glu} = 0.77) probably lacking all three amides and having the same mobility as the fully deamidated dimer. Significant splitting of peptide bonds was also evident after 24 hr. Peptide VI corresponded in mobility to the most strongly acidic (M_{Glu} = 0.25) and the most slowly formed of the bis-amidated trimers and therefore, like peptide IV, has lost an amide from a carboxyl adjacent to a charged amino group. Peptide VII had the same mobility as the second (M_{Glu} = 0.15) trimer diamide and on hydrolysis gave spots corresponding to the fully acidic trimer and the fastest and slowest of the monoamidated trimers.

Hydrolysis of the basic peptide VIII gave two neutral products (M_{Glu} = −0.14 and −0.28 at pH 3.9) and one acidic product (M_{Glu} = 0.43), consistent with the loss of one and two amide residues, respectively. As above, the isomer containing the stronger acid was formed more slowly and hence had lost an amide adjacent to a free amino group. This product and the fully deamidated product, but not VII, gave a yellow color with acid ninhydrin, indicative of a Dpm residue having at least one carboxyl and two amino groups free (Bricas and Nicot, 1964). Peptide IX had the same mobility (M_{Glu} = 0.28) as the other hydrolysis product of VIII, and on hydrolysis gave a similar acidic product (M_{Glu} = 0.43).

Partial Hydrolysis of Dinitrophenyl Peptides. Peptides II, IV, and V were dinitrophenylated and isolated by thin-layer chromatography. Paper electrophoresis of the partial hydrolysis (3 N HCl 2 hr, 105°) products showed the same pattern from each DNP-peptide. The major ninhydrin-positive DNP-peptide product (M_{Glu} = 1.09 at pH 3.9) was eluted and contained mono-DNP-Dpm and alanine. Mono-DNP-Dpm was amino-terminal and Edman degradation released free alanine thus establishing that D-alanine is substituted on the Dpm carboxyl ϵ to the DNP group. Another product (M_{Glu} = 0.83), contained mono-DNP-Dpm, alanine, and Dpm. Dinitrophenylation and hydrolysis gave bis-DNP-Dpm and mono-DNP-Dpm. Since in peptides II and V the Dpm-carboxyl adjacent to the amino group has been shown to be amidated, this product supports the sequence Dpm-D-Ala-Dpm.

Optical Configuration of the Amino-Terminal Dpm and Alanine. Chromatography in system II of the bis-DNP derivative of Dpm prepared from cell walls or from purified peptides, gave a spot corresponding to the meso isomer (Jusic *et al.*, 1963). No DD or LL isomer was found. The mono-DNP derivative of Dpm was prepared by dinitrophenylation of cell walls and of peptide II. The molar optical rotation, $[M]_D$, of the cell wall product in glacial acetic acid was +250 ± 28°. Diringer and Jusic (1966) reported a value of +250° ± 25° for mono-DNP-(D)-*meso*-Dpm from *E. coli* peptidoglycan, while Bricas *et al.* (1967) found +238 ± 5° for the synthetic compound and +248 ± 6° for the compound isolated from a *B. megaterium* cell wall fraction.

The rotatory dispersion curve (Figure 5) showed a positive Cotton effect centered on 412 mμ and between 460 and 600 mμ followed the simplified Drude equation $[M] = K/(\lambda^2 - \lambda_0^2)$, where K was found to be = 0.434 deg × μ^2 and λ_0 = 412 mμ. Corresponding values reported for synthetic mono-DNP-(D)-*meso*-Dpm, were K = 0.416 ± 0.006 deg × μ^2 and λ_0 = 418 mμ (Bricas *et al.*, 1967). Mono-DPN-Dpm prepared from peptide II had a positive rotation and a dispersion curve similar to the cell wall product but the sample was too small for accurate measurement of rotation at 589 mμ.

The molar rotation at 460 mμ for the DNP-alanine prepared from the amino-terminal residues of peptide II was +2060 ± 120°. Reference DNP-L- and D-alanine gave $[M]_{460\ m\mu}$ = +1990 ± 40° and −2070 ± 60°, respectively.

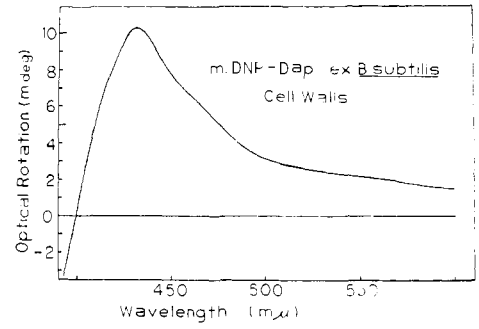

FIGURE 5: Optical rotatory dispersion curve of mono-DNP-Dpm prepared from *B. subtilis* cell walls. The concentration was 0.021% in acetic acid. Cuvet: 0.1 dm.

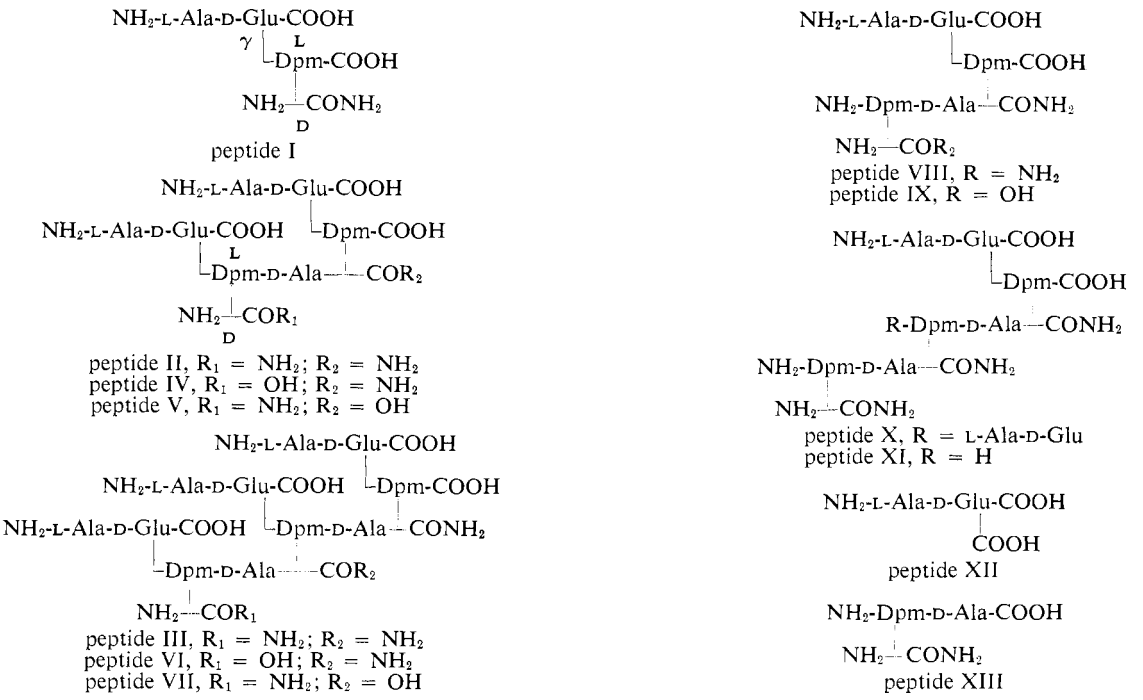

FIGURE 6: Structures proposed for the peptides derived from *B. subtilis* cell walls by autolysis. All horizontal sequences are linked by α-peptide bonds and links involving side-chain functions are denoted by vertical lines.

Proposed Structures for Cell Wall Peptides. Structures consistent with the data are shown in Figure 6. Glutamic acid is assumed to be present as the D isomer in view of its general occurrence in cell wall peptidoglycans. All the cell wall Dpm was the meso form and when one amino group was free this was at the center with D configuration.

In peptide I all of the alanine and Dpm had a free amino group and Edman degradation showed the sequences L-Ala-Glu and Dpm-amide. Paper electrophoresis at pH 3.9 of the hydrazinolysis products showed γ-glutamyl hydrazide, indicating that the sequences were linked *via* the γ-glutamyl carboxyl and that the α-carboxyl of glutamic was free. The location of the amide on the Dpm carboxyl adjacent to the free amino group is supported by the electrophoretic properties and rate of deamidation and by the lack of a carboxyl-terminal Dpm residue.

Edman degradation and amino-terminal analysis of peptide II revealed two L-Ala-Glu sequences and a Dpm-amide sequence. Hydrazinolysis showed that the γ-carboxyls of the glutamic acid residues were substituted, and the α-carboxyls free. Partial hydrolysis of the tri-DNP derivative of II showed the sequence N^{ϵ}-DNP-Dpm$^{\alpha}$—D-Ala, therefore one alanyl glutamic sequence must be substituted on each Dpm residue *via* a γ-glutamyl–Dpm bond. The absence of a carboxyl-terminal Dpm in II and its presence in V indicates that the non-amino-terminal Dpm is amidated. The foregoing evidence establishes the structure in Figure 6 unambiguously except for the orientation of the D and L assymetric centers of the second Dpm residue and the position of its amide substituent. These most probably are the same as shown for peptide I and the other Dpm in this molecule.

The composition and end-group data suggested that peptide III was a trimer with the same basic structure as the dimer II and monomer I. The chromatographic and electrophoretic properties of the three compounds and their derivatives were consistent with this. On Sephadex G-25, III was the most and I the least excluded.

Peptides IV and V resembled II in all their properties except those involving the amide groups. Partial deamidation of II by acid to yield compounds indistinguishable from IV and V, and deamidation of all three peptides to form apparently the same nonamidated dimer, confirmed that all three shared the same peptide structure. Edman degradation showed that peptide IV lacked the amide on the Dpm with the free amino group, and the presence of a carboxyl-terminal residue Dpm in peptide V showed that the other Dpm was not amidated in that isomer. These conclusions were supported by the slower rate of hydrolysis of the amide and the lack of a more strongly acidic carboxyl group in V.

The composition and amino-terminal data for peptides VI and VII suggested that they were trimers each lacking one amide. Both were formed by acid deamidation of the neutral trimer III and all three formed the same pattern of further hydrolysis products. Peptide VI was the most anionic of the three isomeric bis-amidated trimers and was formed at the slowest rate by hydrolysis of III. It therefore lacked the amide on the carboxyl α to the free Dpm amino group. Peptide VII had a free carboxyl of intermediate strength and lacked a carboxyl-terminal Dpm suggesting that it lacked the amide on the middle Dpm residue.

The structure proposed for the basic peptide VIII was based largely on the Edman degradation which indicated L-Ala-Glu-γ and Dpm (amide)-D-Ala-Dpm sequences. The two sequences could only be joined by a γ-glutamyl bond to an amino group of the second Dpm. The lack of a carboxyl-terminal meant that this Dpm residue was also amidated, presumably at the carboxyl α to the D-alanine. Peptide IX differed from VIII in lacking the amide on the carboxyl-terminal Dpm. The color reactions with ninhydrin of VIII and IX and their deamidated derivative provided further evidence for the amidation of the amino-terminal Dpm residue. The composition of the minor basic peptides X and XI suggested that these were trimers lacking one and two Ala-Glu sequences, respectively. Both had bisamino-terminal

Dpm and hence lacked the Ala-Glu on the first Dpm residue. Comparison of the mobility at pH 1.9 of peptide XI with those of other peptides suggested that a carboxyl of low p*K* was not present and that the Ala-Glu sequence was missing from the middle Dpm residue.

The structure of the dipeptides XII and XIII was shown by amino- and carboxyl-terminal analyses. Although the yield of carboxyl-terminal alanine from peptide XIII was very low, the structure, Dpm-amide-Ala, was consistent with the purple ninhydrin color of the compound.

Structure and Degree of Linking of Peptides in the Cell Wall. To check that the peptides isolated were representative of those in the cell wall and had not been modified, other than by the autolytic cleavage of the muramyl-L-alanine bond, peptides were isolated from heat-inactivated cell walls by digestion with lysozyme and muramyl-L-alanine amidase. Paper electrophoresis and thin-layer chromatography revealed no differences in the pattern of peptides between the diffusible fraction of this digest and the diffusible fraction of the autolysate. In particular, spots corresponding to peptides IV, VIII, and XII were present with similar intensities. The structures of peptides VIII and XII suggested that they may have been formed by cleavage of a glutamyl–Dpm bond during autolysis. Dinitrophenylation of intact cell walls and of the diffusible peptides from the autolysate showed that 6 and 5%, respectively, of the Dpm was bisamino terminal. The calculated content of bisamino-terminal Dpm from the yields of purified peptides (Table II) was 6%, mainly in peptide VIII. Peptide IV cannot have been formed from II by acid hydrolysis during purification since hydrolysis of II yielded principally the isomer V.

Peptide monomers were joined through alanyl–Dpm bonds to form dimers and trimers (Figure 6). Among the peptides isolated in this study an average of 41% of the possible D-alanyl–Dpm bonds were formed (Table II). Nonautolyzed cell walls and the diffusible fraction of autolyzed cell walls had 41 and 35%, respectively, of their Dpm unavailable for reaction with FDNB. This result, together with the absence of tetrapeptide amide in the autolysate shows that significant breakage of D-alanyl–Dpm bonds did not occur during autolysis. Because of the presence of peptide VIII which has a D-alanyl–Dpm bond but which cannot form a link between two glycan strands, the actual average degree of cross-linking in the cell wall should be reduced to 35%.

Discussion

The composition of the cell walls was consistent with all the amino sugars being N-acetylated and with all the muramic acid residues having a peptide substituent. Muramic δ-lactam, which was a major component of spore peptidoglycan in *B. subtilis* (Warth and Strominger, 1969) was not present. About half of the cell wall muramic acid was isolated, after autolysis, as a glycan which was free of 90% of its peptides. Most of the remainder was bound to a teichoic acid complex, which was the major component in these cell walls. The number average length of the polysaccharide chains in the glycan fraction was 23 hexosamine residues. Comparable values for glycans prepared from other cell walls range from around 100 in *Spirillum serpens* (Kolenbrander and Ensign, 1968) and *Arthrobacter crystallopoietes* rods (Krulwich *et al.*, 1967) to about 20 in *Staphylococcus aureus* (Tipper *et al.*, 1967) and *Lactobacillus casei* (Hungerer *et al.*, 1969). The presence of glucosamine as the major reducing-terminal residue would not be expected from the mechanism of biosynthesis of peptidoglycan (Anderson *et al.*, 1965), and probably results from the action of autolytic enzymes during growth. Indeed spores of *B. subtilis* contained an acetylglucosaminidase active at pH 5 bound to the cortex (A. D. Warth, unpublished results). Culture supernatants of *B. subtilis* contained an enzyme which lysed cell walls with the formation of reducing groups (Richmond, 1959). An enzyme, partly purified from the supernatant of a sporulated culture, degraded *B. subtilis* cell walls almost completely to disaccharides with reducing-terminal muramic acid (A. D. Warth, unpublished results). Evidently cell walls *in vivo* escape the action of this enzyme. Recently, Brown and Young (1970) have detected autolytic enzymes which form reducing groups in a mutant of *B. subtilis* 168.

The peptide structures, although so far unique in detail, closely resemble those of other peptidoglycans (Ghuysen, 1968). Cross-linking between peptide chains involved a single D-alanine residue and no additional amino acids. The average degree of peptide cross-linking was 30–35% both for the intact cell wall and the peptides recovered. These walls were much less cross-linked than *B. licheniformis* (Hughes, 1968a) *B. sphaericus* (Hungerer and Tipper, 1969), *B. stearothermophilus* (Grant and Wicken, 1970), or *B. thuringiensis* (Kingan and Ensign, 1968) walls appeared to be, but had more peptide cross-links than *B. megaterium* cell walls, in which nearly all the Dpm had a free amino group (Bricas *et al.*, 1967). In common with *E. coli* (Diringer and Jusic, 1966) and *B. megaterium* (Bricas *et al.*, 1968) the *meso*-Dpm was orientated with its L assymetric center as part of the γ-glutamyl-Dpm-D-alanine sequence and its D-amino group free. The experimental evidence was obtained only for the Dpm residues which had a free amino group.

Amidation of a Dpm carboxyl group has been reported for cell walls of *Corynebacterium diphtheriae* (Kato *et al.*, 1968), *L. plantarum* (Matsuda *et al.*, 1968), and *B. stearothermophilus* (Grant and Wicken, 1970). Two dissaccharide tripeptides were isolated from *B. licheniformis* (Mirelman and Sharon, 1968), an organism that is closely related to *B. subtilis*. The glutamyl α-carboxyl was amidated in one of these and free in the other. Similar compounds isolated by Hughes (1968b) from another *B. licheniformis* strain were compared to these and appeared to be identical.

The structures of the basic pentapeptide VIII and the acidic dipeptide XII suggest that they were formed from the neutral dimer II by cleavage of the glutamyl–Dpm bond adjacent to the D-alanine residue. Similar compounds were found by Grant and Wicken (1970) in *B. stearothermophilus* walls. The total recovery of alanylglutamic acid (peptide XII) was similar to the combined recovery of the peptides (VIII, IX, X, and XI) which appeared to have lost this unit. This cleavage did not appear to have occurred during autolysis. The enzyme responsible for this cleavage could have been the γ-glutamyl peptidase produced by *B. subtilis* (Williams *et al.*, 1955) and which released peptides from spore peptidoglycan (Strange and Thorne, 1957).

Peptidoglycan from spores of *B. subtilis* (Warth and Strominger, 1968, 1969) differed in structure from vegetative cell walls in several ways. Muramic δ-lactam and *N*-acetylmuramyl-L-alanine residues appear to be spore-specific modifications. In *B. subtilis* a D-alanine carboxypeptidase, a Dpm amidation system and teichoic acid synthesis are expressed during biosynthesis of the cell wall but not the spore.

Since this paper was submitted, two papers by Hughes have appeared (Hughes, 1970; Hughes *et al.*, 1970). They report for *B. subtilis* Marburg strain 168 and *B. licheniformis* a very similar fractionation of cell wall autolysate on DEAE-cellulose

and found an average chain length of the glycan fraction of 10 hexosamines, compared to 24 in the present work. Acetylglucosamine was the terminal residue at both ends. The peptide monomer was found to be amidated on the Dpm carboxyl and hence probably is identical with peptide I from our strain. The peptide dimer probably has the structure of peptide II.

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