# Structure of the Peptidoglycan from Spores of *Bacillus subtilis*†

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ABSTRACT: Peptidoglycan from spores of *Bacillus subtilis* was isolated by digestion of the cortex and germ cell wall in integuments of heat inactivated spores with lysozyme. The glycan component consisted of long chains of alternating *N*-acetylglucosamine and muramic acid, probably all β-(1→4) linked. Half of the muramic acid residues were present as muramic δ-lactam. The remainder of the residues was N-acetylated, and their side-chain carboxyls were substituted with either a peptide (35%) or L-alanine (18%). Peptide monomers had the structure L-alanyl-D-γ-glutamyl-*meso*-diaminopimelyl-D-alanine. Octapeptide dimers, which may serve to cross-link the glycan chains, were present in the molecular ratio of 1:3 monomers. Muramolactam glycosidic bonds were resistant to hydrolysis by lysozyme, and therefore the principal products of digestion were tetrasaccharides containing an internal muramolactam and a reducing terminal *N*-acetylmuramic acid residue. Teichoic acid and polysaccharides present in vegetative cell walls of *B. subtilis* were not found associated with peptidoglycan in the spores. Spore peptidoglycan in *B. subtilis* differed considerably in structure from the peptidoglycan of vegetative cells.

Peptidoglycan in bacterial spores is found in the cortex and germ cell wall. These layers surround the cytoplasmic membrane and cytoplasm and are themselves enclosed within the spore coats. The cortex appears to have a major role in determining the high resistance of spores to heating (Murrell and Warth, 1965) and in maintaining their dormancy. Spore peptidoglycan differs considerably in composition and probably in function from the peptidoglycans of vegetative cell walls (Warth *et al.*, 1963; Warth, 1965). Differences in the structure of peptidoglycan between cells and spores imply the existence of alternative biosynthetic mechanisms. Control of the expression of these alternatives is of interest in the study of the differentiation of vegetative cells to spores.

In this paper, the chemical structure of peptidoglycan from spores of *Bacillus subtilis* is investigated. For comparison, the structure of the cell wall peptidoglycan of the same strain has been studied (Warth and Strominger, 1971). Several major differences have been found. In the spore, many muramic acid residues are present as muramic δ-lactam, others are N-acetylated and substituted with either an L-alanine or a peptide. The degree of cross-linking of peptides is low. These features were also present in four other species (Warth, 1968) and in *Bacillus sphaericus* (Tipper, 1969) and may be common to all spores. Evidence for the muramic lactam structure has been presented (Warth and Strominger, 1969).

## Materials and Methods

*Spore Peptidoglycan.* Spores of *B. subtilis* (Porton strain) were heat inactivated, disrupted, and digested with lysozyme as described previously (Warth and Strominger, 1969). Typically, the deproteinized lysozyme digest contained at least 97% of the muramic acid, 93% of the Dpm, and 87% of the glucosamine present in the unfractionated disrupted spore suspension.

*Analytical Methods.* Amino groups, reducing groups, acetylhexosamine, D- and L-alanine and N- and C-terminal amino acids were determined by the methods given by Ghuysen *et al.* (1966). Acetyl groups were determined by the method of Ludoweig and Dorfman (1960) on a smaller scale, and Edman degradation used the conditions of Konigsberg and Hill (1962). Procedures for the identification of reducing end groups by reduction with [$^3$H]$NaBH_4$, for the determination of the degree of cross-linking of peptides by dinitrophenylation, and modified conditions for N-terminal estimation were described previously (Warth and Strominger, 1971). Amino acids, amino sugars, and ammonia were determined using a Beckman Model 120C amino acid analyzer. Amino sugar results were corrected for the hydrolytic loss (approximately 25% in 16 hr) found from a study of the kinetics of hydrolysis of a tetrasaccharide from spore peptidoglycan. The proportion of L and D isomers of glutamic acid was determined by incubation with L-glutamic decarboxylase (1 mg/ml of an acetone powder of *Escherichia coli* obtained from Sigma Chemical Co., St. Louis) in 0.3 M pyridine acetate (pH 5.0) for 3 hr at 37°. γ-Aminobutyric acid and glutamic acid were separated by paper electrophoresis and estimated with ninhydrin (Kay *et al.*, 1956). Ribitol, glucose, and phosphate were estimated as before (Warth and Strominger, 1971).

*Chromatography and Electrophoresis.* Oligosaccharides were chromatographed on silica gel G thin-layer plates using methyl ethyl ketone–acetic acid–water (4:1:1, v/v) as solvent, and were detected by spraying with 30% $H_2SO_4$ and heating at 140°. Peptides were separated with isobutyric acid–triethylamine–water (40:1:6, v/v) on silica gel G and detected with ninhydrin. High-voltage paper electrophoresis used pyridine–acetate buffers at 40 V/cm (Warth and Strominger, 1971). Oligosaccharides were detected on paper by fluorescence after heating with NaOH (Sharon and Seifter, 1964).

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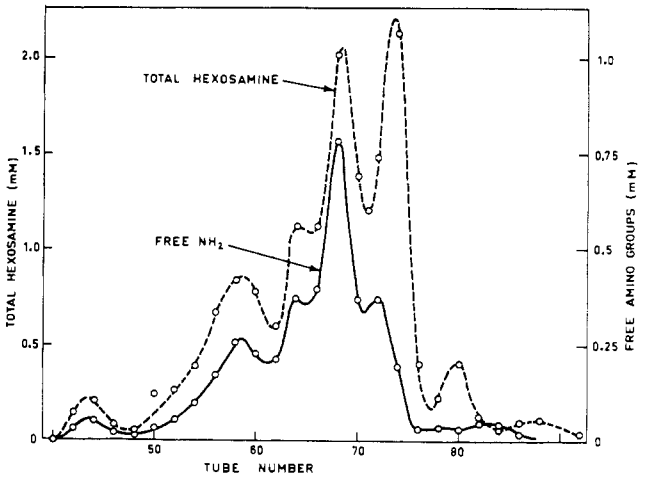


FIGURE 1: Gel filtration of the lysozyme digest of *B. subtilis* spore peptidoglycan. Two columns (85 × 1.4 cm), one containing Sephadex G-50 and the other Sephadex G-25, were connected in series, and equilibrated with 0.025 M pyridine–acetate (pH 4.7). The spore peptidoglycan applied to the column contained 63 μmoles of total MurN. Fractions of 4.5 ml were collected. Fully excluded compounds were eluted at fraction 42 and NaCl at fraction 98.

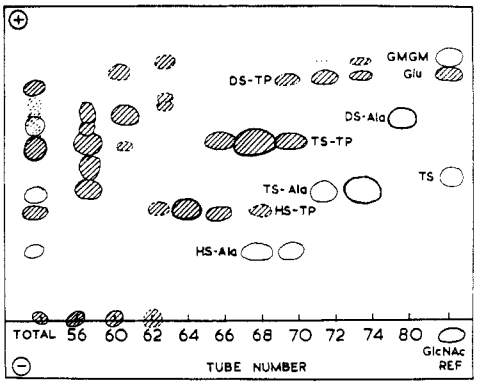


FIGURE 2: Paper electrophoresis of some fractions from the Sephadex column (Figure 1). Spots were detected by fluorescence after spraying with NaOH (Sharon and Seifter, 1964). Spots that also reacted with ninhydrin are shown hatched. The electrophoresis was run in pyridine–acetate buffer (pH 3.9) at 50 V/cm for 2.5 hr. 150 μl of each fraction was applied to the paper. References used were: GMGM, GlcNAc-MurNAc-GlcNAc-MurNAc; Glu, glutamic acid; TS, tetrasaccharide prepared by digestion of TS-TP with muramyl-L-alanine amidase, GlcNAc.

J. M. Ghuysen (University of Liege, Belgium); lysozyme was a crystalline preparation from Sigma Chemical Co. (St. Louis). D-Lactate dehydrogenase was given by Dr. D. Tipper (University of Wisconsin, Madison, Wis.).

*Preparation and Analysis of Partial Hydrolysis Products of Dinitrophenylated TS-TP.* TS-TP (200 nmoles) was dinitrophenylated with fluorodinitrobenzene and triethylamine (Jarvis and Strominger, 1967). The solution was evaporated *in vacuo*, and the product was hydrolyzed with 2 N HCl at 100° for 1.75 hr. After extraction with ether and evaporation, the hydrolysate was fractionated by paper electrophoresis at pH 3.9 for 2.5 hr at 37 V/cm. Products containing the DNP group and reacting with ninhydrin were eluted from the paper, dinitrophenylated, and hydrolyzed. N-Terminal amino acids derivatives were extracted with ether and identified by thin-layer chromatography. Part of the aqueous layer of the hydrolysate was chromatographed to identify mono-DNP-Dpm[1] and the remainder was dinitrophenylated, and the amino acids present were identified as their DNP derivatives.

*Digestion with Exo-β-N-acetylglucosaminidase.* The mixture containing 20 nmoles of oligosaccharide, 0.5 μl of the 20–40% acetone precipitate fraction of pig epididymal β-acetylglucosaminidase (Findlay and Levvy, 1960), and 2.5 μg of serum albumin in 25 μl of 0.05 M sodium acetate (pH 4.2) was incubated at 40° for 3 hr. Cold 2 N perchloric acid (10 μl) was added to the reaction mixture and after 5 min was neutralized with 2 N KOH. The mixture was then cooled and centrifuged. Acetylhexosamine was estimated, using a heating time of 3 min, on 25-μl samples of the supernatant. Blanks were treated similarly, but the enzyme was omitted.

*Identification of Amino Sugars Having a Free Amino Group.* Samples containing 0.5-μmole total hexosamine or standards of GlcN and MurN (0.2 μmole) were dissolved in 0.2 ml of water. Solid $NaHCO_3$ and 0.2 ml of 0.1 M fluorodinitrobenzene in ethanol were added, and the suspension was shaken in the dark for 16 hr. After acidification with HCl and evaporation, the samples were hydrolyzed with 2 N HCl at 100° for 2 hr. DNP-amino sugars were extracted with 1-butanol and excess dinitrophenol was removed by vacuum sublimation over NaOH.

DNP-MurN was separated by paper electrophoresis at pH 3.9 and DNP-GlcN by thin-layer chromatography on silica gel G developed with chloroform–methanol–acetic acid (85:14:1, v/v). Samples were applied in 95% methyl ethyl ketone.

## Results

*Isolation and Composition of Compounds from Lysozyme-Digested Spore Peptidoglycan.* Gel filtration of the lysozyme digest gave a complex profile of reducing and amino groups (Figure 1). Fractions 62–82 each contained one or two components which were separable by paper electrophoresis (Figure 2) and thin-layer chromatography. More excluded fractions contained complex mixtures and several minor components. Each fraction between 62 and 82 was subjected to preparative paper electrophoresis at pH 3.9 and six major oligosaccharide components were obtained. Their nomenclature is based upon their composition (Table I). Thus DS-Ala (disaccharide-alanine), TS-Ala (tetrasaccharide-alanine), and HS-Ala (hexasaccharide-alanine) contained respectively, one, two, and three residues each of glucosamine and muramic acid and one L-alanine residue. DS-TP, TS-TP, and HS-TP contained the elements of a tetrapeptide comprised of L-alanine, glutamic acid, Dpm, and D-alanine plus two, four, or six hexosamine residues. A minor compound, DS-Trp, contained equimolar amounts of glucosamine, muramic acid, L-alanine, glutamic acid, and Dpm, and appeared to be a disaccharide tripeptide.

*Structure of the Peptide Component.* DS-TP, TS-TP, and HS-TP each contained C-terminal alanine, and the Dpm residue had a free amino group (Table I). Digestion with muramyl-L-alanine amidase cleaved a peptide from each oligosaccharide-peptide but had no effect on the oligosaccharide-alanine compounds. The peptides released were indistinguishable on thin-layer chromatography in two systems and on paper electrophoresis at pH 1.9 and 3.9.

Since each oligosaccharide-tetrapeptide appeared to con-

[1] Abbreviations used are: Dpm, 2,6-diaminopimelic acid; GlcN, D-glucosamine; MurN, muramic acid; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid.

TABLE I: Composition of Compounds from Lysozyme Digest of Spore Peptidoglycan.

| | Molar Ratio of | | | | | | |
|---|---|---|---|---|---|---|---|
| | DS-Ala | TS-Ala | HS-Ala | DS-TP | TS-TP | HS-TP | DS-TrP |
| Glucosamine | 1.03 | 2.24 | 3.26 | 1.25 | 2.15 | 3.36 | 1.17 |
| Muramic acid | 0.98 | 1.92 | 3.00 | 1.04 | 2.14 | 3.15 | 1.00 |
| L-Alanine | 0.99 | 1.03 | 0.91 | 1.12 | 1.09 | 1.12 | 1.03 |
| Glutamic acid | 0.02 | 0.02 | 0.07 | 1.13 | 1.09 | 1.05 | 1.15 |
| Diaminopimelic acid | 0.00 | 0.00 | 0.01 | 1.00 | 1.00 | 1.00 | 1.00 |
| D-Alanine | 0.01 | 0.10 | 0.06 | 0.94 | 1.01 | 1.00 | 0.20 |
| Total Alanine | 1.00 | 1.00 | 1.00 | 2.11 | 1.98 | 2.05 | 1.17 |
| N-Terminal Dpm | | | | 0.95 | 1.01 | 0.90 | 0.53 |
| Residual Dpm[a] | | | | 0.0 | 0.04 | 0.04 | 0.0 |
| C-Terminal alanine | 0.78 | 0.63 | 0.70 | 0.39 | 0.33 | 0.41 | 0.0 |
| C-Terminal Dpm | | | | *b* | *b* | *b* | 0.42 |

[a] Dpm remaining after dinitrophenylation and hydrolysis. [b] No spot visible on chromatograms.

TABLE II: Amino Acid and N-Terminal Amino Acid Composition of Some Acid Hydrolysis Products of Dinitrophenyl-TS-TP.[a]

| Product No. | $M_{Glu}$[b] | DNP Group | N-Terminal Amino Acid | Other Amino Acids | Sequence |
|---|---|---|---|---|---|
| I | 0.36 | − | Ala | Glu | Ala-Glu |
| II | 0.91 | + | Dpm | Ala | Dpm-Ala |
| III | 1.10 | + | Dpm | None | Dpm |
| IV | 1.28 | + | Ala | DNP-Dpm,Glu | Ala-(Glu,Dpm) |
| V | 1.51 | + | Glu | DNP-Dpm | Glu-Dpm |

[a] TS-TP was dinitrophenylated and hydrolyzed with 2 N HCl at 100° for 1.75 hr, and the products were separated by paper electrophoresis at pH 3.9. [b] Mobility at pH 3.9 relative to glutamic acid (1.00) and alanine (0.00).

tain the same tetrapeptide, the structure of this peptide was investigated in detail only for TS-TP. TS-TP was dinitrophenylated, and the product was partially hydrolyzed with 2 N HCl. Analysis of the products separated by paper electrophoresis indicated the partial sequences shown in Table II. Hydrolysis product I had N-terminal alanine and residual glutamic acid and therefore has the sequence Ala-Glu. It had the same mobility as L-alanyl-D-glutamic acid isolated from cell walls (Warth and Strominger, 1971). Product II contained N-terminal DNP-Dpm and residual alanine. Two structural isomers are possible where the alanine may be substituted on the Dpm carboxyl $\alpha$ or $\omega$ to the DNP group. Edman degradation gave free D-alanine as shown by paper electrophoresis at pH 1.9 before and after incubation with D-amino acid oxidase, thus showing that it was located on a carboxyl $\alpha$ to a free amino group in Dpm.

The partial sequences in Table II define the sequence L-Ala-Glu-Dpm-D-Ala, which is consistent with the presence of C-terminal alanine in TS-TP. The linkage between glutamic acid and Dpm was investigated by hydrazinolysis of TS-TP. Paper electrophoresis at pH 3.9 showed a ninhydrin-positive spot corresponding to the $\gamma$-hydrazide ($M_{Lys}$ = 0.05) but not the $\alpha$-hydrazide ($M_{Lys}$ = 0.33) of glutamic acid. Hence the structure of the tetrapeptide moiety is L-alanyl-D-$\gamma$-glutamyl-*meso*-diaminopimelyl-$\alpha$-D-alanine.

Digestion of DS-TrP with muramyl-L-alanine amidase yielded a peptide with a higher mobility at pH 3.9 than the tetrapeptide. It was isolated by paper electrophoresis and contained alanine, glutamic acid, and Dpm in the ratio 1.08:1.04:1.00. Only glutamic acid remained after dinitrophenylation and hydrolysis. DS-TrP had C-terminal Dpm indicating the sequence L-Ala-Glu-Dpm. The tripeptide had the same mobilities and $R_F$'s as the deamidation product of the peptide L-Ala-$\gamma$-D-Glu-(L)-*meso*-Dpm-(D)-amide from *B. subtilis* cell walls (Warth and Strominger, 1971).

*Identification of the Terminal Residues of the Oligosaccharides.* Reduction with [$^3$H]NaBH$_4$ indicated that muramic acid was the reducing-terminal residue in all six compounds, as was expected for lysozyme digestion products. In addition to $C_1$-reduced muramic acid (2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxyglucitol), the tetra- and hexasaccharides gave other tritated products which were shown to be formed by reduction of the lactyl side chain in muramic lactam residues (Warth and Strominger, 1969; Warth, 1968). The reducing powers of the compounds relative to GlcNAc ranged from 1.5 for the disaccharides to 1.2 for HS-Ala (Table III), indicative of one terminal muramic acid per alanine or peptide (Ghuysen *et al.*, 1966). In the Morgan–Elson reaction, DS-Ala, DS-TP, and the disaccharide (DS) formed by amidase digestion of DS-TP had a similar molar extinction coefficient to the disaccharide *O*-$\beta$-D-GlcNac-(1→4)-D-MurNAc prepared from *Micrococcus lysodeikticus* cell walls and

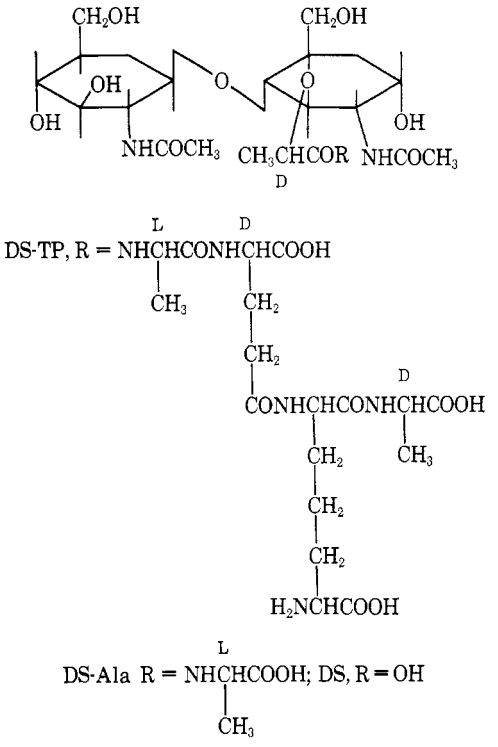

FIGURE 3: Structure of disaccharides from *B. subtilis* spore peptidoglycan.

characteristic of the 1→4-linked disaccharide (Sharon *et al.*, 1966). Tetra- and hexasaccharides gave little color in this reaction.

All compounds were digested with exo-β-*N*-acetylglucosaminidase, releasing approximately 2 moles of acetylhexosamine from the disaccharides and one from tetra- and hexasaccharides (Table III). GlcNAc was identified as one of the products in all cases by thin-layer chromatography and paper electrophoresis. DS and the disaccharide from *M. lysodeikticus* also formed MurNAc. The second product from each of the other compounds had the electrophoretic mobility expected for its parent compound lacking a GlcNAc residue. Hence all the spore compounds had β-D-GlcNAc at the nonreducing end.

*Linkage between the Oligosaccharide and the Peptide or* L-*Alanine Components.* Although the tetra- and hexasaccharides contained two and three residues, respectively, of muramic acid, only one peptide unit or L-alanine was present in each case. The reaction of muramic acid with alkali offers a means of characterizing the side-chain substituent of the reducing terminal muramic acid. Under alkaline conditions, muramic acid and its derivatives which have a free reducing group undergo a β-elimination reaction and release the lactyl side chain (Tipper, 1968).

None of the spore compounds eliminated free D-lactic acid when treated with alkali, but after further acid hydrolysis, each yielded 1 mole of lactate (Table IV). Therefore the carboxyl group of the reducing terminal muramic acid residues must be substituted. Paper electrophoresis of the alkali degradation products of DS-TP, TS-TP, and HS-TP showed a single ninhydrin-positive compound ($M_{Glu}$ = 1.40 at pH 3.9). It was isolated from TS-TP in 82% yield by paper electrophoresis and contained: D-lactic acid, alanine, glutamic acid, and Dpm in the ratio: 1.15:1.95:1.02:1.00. No amino sugars were present, and Dpm was the only residue with an amino

TABLE III: Some Reactions of Spore Oligosaccharides. Morgan–Elson Reaction, Reducing Power, and Acetylglucosamine Released after Digestion with Exo-β-*N*-acetylglucosaminidase.[a]

| Compound | Morgan–Elson Reaction | Reducing Power | Acetylhexosamine Released by β-*N*-Acetylglucosaminidase |
|---|---|---|---|
| | Relative Molar Extinction | | |
| Spore samples | | | |
| GlcNAc | 1.00 | 1.00 | 0.91 |
| DS-Ala | 0.57 | 1.52 | 1.78 |
| DS-TP | 0.56 | 1.55 | 1.48 |
| DS | 0.47 | 1.28 | |
| TS-Ala | 0.03 | 1.32 | 0.78 |
| TS-TP | 0.03 | 1.39 | 0.73 |
| TS | 0.03 | 1.28 | |
| HS-Ala | 0.02 | 1.2 | 0.70 |
| HS-TP | 0.02 | 1.48 | |
| References | | | |
| GlcNAc-MurNAc[b] | 0.52 | 1.5 | |
| GlcNAc-MurNAc-GlcNAc-MurNAc[b] | 0.10 | 1.4 | |

[a] The Morgan–Elson reaction, using a heating time of 30 min, and reducing power determination were carried out according to Ghuysen *et al.* (1966). GlcNAc was used as the standard. Digestion with exo-β-*N*-acetylglucosaminidase released GlcNAc which was determined by the Morgan–Elson reaction using a heating time of 3 min as described in Methods. Disaccharides gave a MurNAc derivative as well as GlcNAc. Molar concentrations of spore compounds were calculated on the basis of 1 L-alanine or tetrapeptide per mole (Table I). [b] From cell walls of *Micrococcus lysodeikticus*.

group free. The formation of lactyl-tetrapeptide as the only ninhydrin-positive product shows that all the peptides were substituted on the side chain of the reducing terminal muramic acid.

This experiment does not determine which of the muramic acid residues in TS-Ala or HS-Ala are substituted by the C-terminal D-alanine. However the results of [$^3$H]NaBH$_4$ reduction (Warth and Strominger, 1969; Warth, 1968) showed that the internal muramic acid residues were present as the δ-lactam, so alanine must also be substituted on the side-chain carboxyl of the reducing terminal muramic acid.

*Structure of the Compounds Isolated from Spore Peptidoglycan.* DISACCHARIDES. The above data establish the structures (Figure 3) of the disaccharides. Each contained one residue of MurNAc at the reducing terminal and one β-GlcNAc at the nonreducing terminal. The color yield in the Morgan–Elson reaction showed that the linkage was 1→4. Digestion of DS-TP with muramyl-L-alanine amidase and elimination of the lactyl side chain of the muramic acid with alkali demonstrate that the tetrapeptide in DS-TP and the L-alanine in DS-Ala were linked to the muramyl carboxyl by an L-alanylamide bond. Analyses of more highly purified preparations of DS and DS-Ala (Table V) establish the respec-

TABLE IV: Alkaline Elimination of D-Lactic Acid from Spore Compounds.[a]

| | Moles/Mole of | | | | | |
|---|---|---|---|---|---|---|
| | DS-Ala | TS-Ala | HS-Ala | DS-TP | TS-TP | HS-TP |
| D-Lactic acid (free) | 0.05 | 0.03 | 0.01 | 0.00 | 0.01 | 0.01 |
| D-Lactic acid (after hydrolysis) | 1.02 | 0.95 | 1.03 | 1.02 | 1.1 | 1.2 |

[a] Each compound (100 nmoles) was treated with 0.03 M NaOH (60 μl) for 1 hr. Part of the mixture (26 μl) was acidified with concentrated HCl to 4 N and hydrolyzed at 105° for 8 hr. Another part (26 μl) was acidified with 0.1 N HCl (13 μl) and kept at 0°. HCl was removed by evaporation *in vacuo* and D-lactic acid assayed using D-lactic dehydrogenase (Tipper, 1968).

tive empirical formulae $GlcN_1MurN_1Ac_2$ and $GlcN_1MurN_1Ac_2Ala_1$.

TETRASACCHARIDES. Hydrolysis of the spore tetrasaccharides with 6 N HCl at 100° formed a basic intermediate product ($M_{GlcN}$ = 0.60 at pH 3.9). It was isolated by paper electrophoresis and gel filtration after hydrolysis of TS-Ala for 30 min, and contained muramic acid and glucosamine in the mole ratio of 1.10:1.00, but contained no acetyl groups or alanine. Reduction with [$^3$H]$NaBH_4$ gave 0.73 mole of tritium-labeled glucosaminitol per mole of glucosamine. DNP-glucosamine and DNP-muramic acid were identified after dinitrophenylation and hydrolysis, showing that the amino groups of both sugars were free. A comparison of the electrophoretic mobilities at pH 1.9 and 3.9 of the compound to those of *O*-β-D-GlcN-(1→4)-D-GlcN showed that the muramyl carboxyl group was also free. N-Acetylation gave *O*-β-D-MurNAc-(1→4)-D-GlcNAc, which was identified by paper electrophoresis and by color yields given in the Morgan–Elson and reducing group determinations (Table VI).

A yield of 14.4 μmoles of the disaccharide was obtained from 28 μmoles of TS-Ala. Spore hexasaccharides, but not disaccharides or the tetrasaccharide from *M. lysodeikticus*, formed the same product. The high yield of disaccharide obtained can be explained if the muramyl 2-amino group adjacent to the resistant muramyl-glycoside bond was positively charged, as would result from rapid acid hydrolysis of muramic δ-lactam (Warth and Strominger, 1969; Warth, 1968). The isolation of *O*-β-D-MurN-(1→4)-D-GlcN from hydrolysates of the tetrapeptides demonstrates the sequence: GlcN-MurN-GlcN-MurN and characterizes the MurN-GlcN bond as β-(1→4).

Except for the nature of the two GlcN-MurN glycosidic bonds, the data here and that reported previously for the muramic lactam and N-acetylation of the internal glucosamine (Warth and Strominger, 1969), establishes the structure for TS-Ala shown in Figure 4. TS-TP differs from TS-Ala only in containing the tetrapeptide (Figure 3) in place of L-alanine. The link from the nonreducing terminal GlcNAc could be β-(1→4) or β-(1→6), and the glycosidic bond to the reducing terminal MurNAc could be α- or β-(1→4). It is most likely that both are β-(1→4) as in *Staphylococcus aureus* (Tipper *et al.*, 1967) and *M. lysodeikticus* (Leyh-Bouille *et al.*, 1966).

HEXASACCHARIDES. HS-Ala and HS-TP had the same terminal residues as TS-Ala and TS-TP and contained at least one *O*-β-D-MurN-(1→4)-D-GlcN unit. Their other properties (Warth, 1968; Warth and Strominger, 1969) suggested that they were identical with the respective tetrasaccharides but contained an additional *O*-β-D-GlcNAc-(1→4)-D-Muramolactam unit.

*Composition and Structure of Spore Peptidoglycan.* The composition of the spore peptidoglycan preparation (Table VII) indicated that 35% of the muramic carboxyls were substituted with a peptide and a further 18% were substituted with L-alanine, leaving approximately 47% available for lactam formation. Likewise the total acetyl content was sufficient for N-acetylation of all the glucosamine and half the muramic acid residues. Dinitrophenylation and hydrolysis of spore peptidoglycan showed no muramic acid and only small amounts of glucosamine with an amino group free. Therefore it is likely that approximately half the total muramic acid was present as muramolactam. The average muramolactam con-

TABLE V: Composition of Spore Disaccharides.

| | μmoles/mg of | |
|---|---|---|
| | DS | DS-Ala |
| Alanine | | 1.85 |
| Glucosamine | 1.99 | 1.71 |
| Muramic acid | 1.98 | 1.73 |
| Acetyl | 3.78 | 3.59 |
| Calculated | 2.01[a] | 1.76[b] |

[a] Calculated for the formula: $GlcNAc_1MurNAc_1$, mol wt 496. [b] Calculated for the formula: $GlcNAc_1MurNAc_1Ala_1$, mol wt 567.

TABLE VI: Properties of the Bis-*N*-acetyl Derivative of an Acid Hydrolysis Product of TS-Ala.

| | Product (Bis-*N*-acetyl) | M-G[a] | G-M[b] | GlcNAc |
|---|---|---|---|---|
| Reducing power | 0.65 | 0.73 | 1.5 | 1.00 |
| Morgan–Elson reaction | 0.11 | 0.10 | 0.52 | 1.00 |
| Mobility at pH 3.9 relative to MurNAc | 0.64 | 0.63 | 0.73 | 0.00 |
| $R_F$[c] | 0.55 | 0.55 | 0.56 | 0.64 |

[a] *O*-β-D-MurNAc-(1→4)-D-GlcNAc obtained from lysostaphin digestion of *Staphylococcus aureus* cell walls (Tipper and Strominger, 1966; Tipper *et al.*, 1971). [b] *O*-β-D-GlcNAc-(1→4)-D-MurNAc obtained from *M. lysodeikticus* cell walls (Leyh-Bouille *et al.*, 1966). [c] $R_F$ in methyl ethyl ketone–acetic acid–water (4:1:1, v/v) on silica gel G.

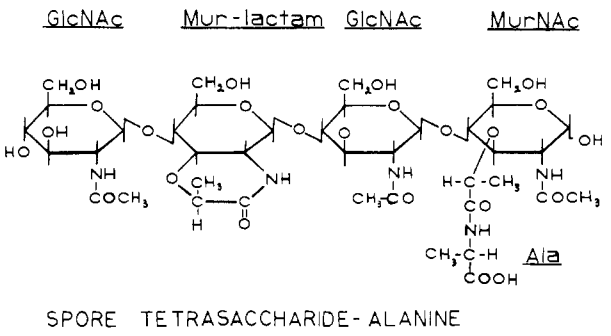


FIGURE 4: Structure proposed for TS-Ala.

tent of the compounds isolated was also near 50%. In contrast to the spore preparation, all of the amino sugars in *B. subtilis* cell walls appeared to be N-acetylated and a peptide unit was present for each muramic acid (Table VII).

The spore peptidoglycan preparation did not contain detectable amounts of ribitol or galactosamine and glucose and phosphate were present in very small amounts. Nearly all the glutamic acid (94%) was present as the D isomer and Dpm was the meso form.

In order to get a more representative sample of spore peptides than were present in the isolated oligosaccharide-peptides, the unfractionated lysozyme digest of spore peptidoglycan was digested with muramyl-L-alanine amidase. Gel filtration (Figure 5) resolved a major peak of amino groups at 70 ml (peptide 1) from a smaller peak at 58 ml (peptide 2). A small amount of amino groups eluted at the exclusion volume (51 ml) contained no detectable peptides. Thin layer chromatography and the elution pattern of reducing power showed that nearly all the oligosaccharides were hexa-, tetra-, or disaccharides with the major peak at 65 ml containing TS-Ala and TS.

Peptides 1 and 2 were separated from oligosaccharides by preparative paper electrophoresis at pH 1.9. Peptide 1 had the

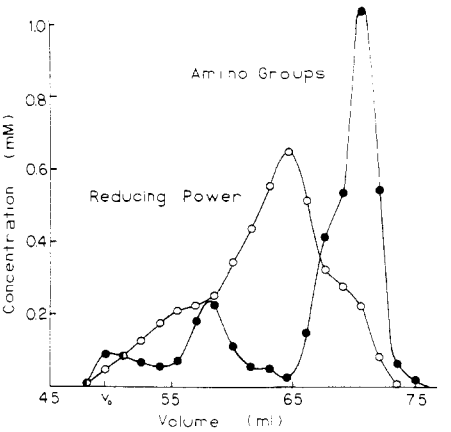


FIGURE 5: Gel filtration of peptides and oligosaccharides from amidase treatment of spore peptidoglycan lysozyme digest. Spore peptidoglycan (2.0 ml) containing 4.6 μmoles of Dpm was incubated with 0.1 M pyridine–acetate buffer (pH 5.4) (50 μl) and muramyl-L-alanine amidase (20 μl) at 37° for 14 hr. The reaction mixture was concentrated to 0.5 ml and applied to a column (100 × 1.3 cm) of superfine Sephadex G-25 and eluted with water. Six fractions of 1.5 ml were collected per hour and assayed for free amino groups (●) and reducing power (○).

same mobilities and $R_F$'s in four systems as the peptide from TS-TP. Only small amounts of the tripeptide from DS-TrP were present, and amidated peptides such as those found in cell walls of *B. subtilis* were not detected. Analysis of peptide 1 (Table VIII) and the isolation of $N^{\epsilon}$-DNP-Dpm-D-alanine from a partial hydrolysate of its DNP derivative identify it as the same tetrapeptide which was previously isolated.

The analysis of peptide 2 (Table VIII) suggested that it was a dimer, consisting of two tetrapeptides linked through an alanyl-Dpm bond. Its behavior on chromatography and paper electrophoresis at pH 1.9 and 3.9, compared to heptapeptide dimers isolated from *B. subtilis* vegetative cell walls, was consistent with the proposed structure.

The degree of peptide cross-linking was determined by estimation of DNP-Dpm and Dpm on the amino acid analyzer after dinitrophenylation and hydrolysis of the unfractionated spore peptidoglycan (lysozyme digest). A free amino group was present in 81% of the total Dpm residues, which corresponds to 19% of the potential peptide cross-links. Peptides 1 and 2 were isolated in a relative molar yield of approximately 4:1 which corresponds to 16% cross-linking.

*Yields of Lysozyme Digestion Products.* The six compounds

TABLE VII: Composition of Spore and Vegetative Cell Peptidoglycans from *B. subtilis*.[a]

| | Mole Ratios of | |
|---|---|---|
| | Spore | Vegetative Cell |
| Glucosamine | 1.02 | 1.17 |
| Muramic acid | 1.00 | 0.94 |
| Acetyl | 1.50 | 2.04 |
| L-Alanine | 0.53 | 1.08 |
| Glutamic acid | 0.38 | 1.16 |
| *meso*-Dpm | 0.34 | 1.00 |
| D-Alanine | 0.29 | 1.55 |
| Total alanine | 0.97 | 3.16 |
| Phosphate | 0.04 | 3.43 |
| Ribitol | Not detected | ++++ |
| Glucose | 0.03 | 2.2 |
| Galactosamine | 0.00 | 0.3 |

[a] Spore peptidoglycan was prepared by lysozyme digestion of heat inactivated spore integuments and vegetative cell peptidoglycan by lysozyme digestion of heat inactivated cell walls.

TABLE VIII: Composition of Peptides Isolated after Lysozyme and Muramyl-L-alanine Amidase Digestion of Spore Peptidoglycan.

| | Mole Ratio of | |
|---|---|---|
| | Peptide 1 | Peptide 2 |
| Muramic acid | 0.00 | 0.00 |
| Alanine | 1.88 | 3.86 |
| Glutamic acid | 0.98 | 1.92 |
| Diaminopimelic acid | 1.00 | 2.00 |
| N Terminal | | |
| Alanine | 0.73 | 1.46 |
| Diaminopimelic acid | 0.85 | 0.67 |

isolated accounted for 60% of the muramic acid and 50% of the Dpm in the spore peptidoglycan. Each was substituted with a tetrapeptide or L-alanine and therefore would not be involved in cross-linking. Most of the cross-linking peptides in the remaining material would be expected to be a series of minor compounds of higher molecular weight. Indeed chromatography of the peptides produced by amidase digestion of this fraction (Figure 1, fractions 50–60) showed mainly the octapeptide dimer. Most of the potentially lysozyme-sensitive bonds appeared to have been hydrolyzed by the enzyme. The reducing power of the digest was 0.56 (as GlcNAc) per muramic acid residue, which is equivalent to 40% of the muramic glycosidic bonds or approximately 80% of the *N*-acetylmuramic glycosidic bonds.

The relative molecular yields of di-, tetra-, and hexasaccharide were approximately 1:3:1 in both the tetrapeptide and L-alanine series. This ratio is very different from the ratio of 4:2:1 expected if the lysozyme-resistant muramolactam residues were randomly distributed along the glycan chains. This result suggests that a substantial part of the spore glycan consisted of alternating GlcNAc-muramolactam and GlcNAc-MurNAc units and that extended regions free from muramolactam were not present. Quantitative interpretation of the yields is not possible since hydrolysis of cell wall oligosaccharides by lysozyme is known to involve transglycosylation, and the cell wall tetrasaccharide is hydrolyzed very slowly (Chipman *et al.*, 1968). The low yield of disaccharides was confirmed by the Morgan–Elson reaction on the unfractionated lysozyme digest which showed that less than 20% of the total saccharides present were disaccharides. Chromatography of the saccharide-alanine compounds after passage of the digest through Dowex 50 ($H^+$) showed mainly TS-Ala and HS-Ala, but higher oligomers, except for a possible trace of the octasaccharide, were not detected.

## Discussion

The tetrapeptide in *B. subtilis* spore petidoglycan has the same structure as peptides in *Escherichia coli and Bacillus megaterium* cell walls (van Heijenoort *et al.*, 1969; Diringer, 1968) and has recently been synthesized (Dezelee and Bricas, 1970). The position of the D and L centers of *meso*-Dpm was not determined for the spore peptide, but is probably the same as in cell walls of *B. subtilis* (Warth and Strominger, 1971), *B. megaterium* (Bricas *et al.*, 1967), and *E. coli* (Diringer and Jusic, 1966). Although the peptide structure is not unusual, it differs from the peptides in cell walls of the same strain. The cell wall peptides were amidated on the Dpm-D-carboxyl and lacked carboxyl-terminal D-alanine. More striking differences between vegetative and spore peptides were found in *B. sphaericus*. Cell walls contained $N^{\alpha}$-(L-alanyl-D-isoglutamyl)-$N^{\epsilon}$-($\beta$-D-isoasparaginyl)-L-lysine (Hungerer and Tipper, 1969), whereas the spore peptidoglycan (Tipper, 1969) was the same as in *B. subtilis* and in four other species (Warth, 1968). It appears that although cell wall peptides vary considerably between species (Ghuysen, 1968), spore peptidoglycans have generally a similar structure.

Muramolactam and the single L-alanine substituent of *N*-acetylmuramic acid appear to be important features characteristic of spore peptidoglycan. Muramolactam has been found elsewhere only as a minor component from *M. lysodeikticus* cell walls (Hoshino *et al.*, 1972), and a glycopeptide containing acetylmuramylalanylamide has been isolated from streptococcal cell walls (Heymann *et al.*, 1964).

Spore cortex and germ cell wall differ dramatically in composition and chemical structure from vegetative cell walls in *B. subtilis*. Some of these differences may relate to differences between the properties appropriate to the functions of the cortex as opposed to the cell wall. The mechanical and hydration properties of the structures will be determined by the degree of cross-linking between polymer chains and the presence of ionized groups. Spore peptidoglycan was lightly cross-linked since only 40% of the peptide units participated in a cross-link and only 17% of the amino sugars had a peptide substituent. Cell walls (Ghuysen, 1968) including *B. subtilis* (Warth and Strominger, 1971; Hughes, 1970) typically have much greater cross-linking and would be less elastic. Spore peptidoglycan contains a large net negative charge (Warth, 1965) from free carboxyl groups on peptide and L-alanine residues. These properties are consistent with the requirements of the contractile cortex theory (Lewis *et al.*, 1960; Warth *et al.*, 1963). At low ionic strength the cortex would initially exist in an expanded, very hydrated state. An increase in ionic strength or the binding of a specific cation would contract the cortex about the spore protoplast, assisting with its dehydration and, hence, heat resistance. To date there has been no information as to whether such cation concentrations or binding exist in the spore. However, heat-resistance differences between species have been correlated significantly with the amount of cortical peptide (Murrell and Warth, 1965), and Vinter and Stastna (1967) showed polyvalent cations to cause a partial restoration of refractility in germinated spores.

No specific function for muramolactam at present can be proposed, except that it reduces cross-linking and spaces out the anionic side chains. The tendency to alternate regularly with MurNAc suggests that a regular local structure may exist in both the germ cell wall and the cortex. The absence from the spore of cell wall type peptides, teichoic acid, and polysaccharide further indicates that both the germ cell wall and the cortex are considerably modified from the vegetative structure.

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# A Natural Inhibitor of Sialyl Transferase and Its Possible Influence on This Enzyme Activity during Brain Development†

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ABSTRACT: The total activity of cytidine 5′-monophospho-*N*-acetylneuraminic acid lactosyl ceramide sialyl transferase of rat brain increases from the 8- to 40-day-old rat and then remains stable until, at least, the 60th day of life. Similar changes were observed with the activity in each of the different subcellular fractions that contain the enzyme. An inhibitor of the enzyme was found which increases with the age of the animal. It is present in the mitochondrial and microsomal fractions and in the cytosol. It is active on the enzyme from all of the subcellular particles. The inhibitor increases the $K_m$ value of lactosyl ceramide for the enzyme and it is probably the responsible factor for the increase of this value in the adult animal. Gel filtration experiments indicated that the molecular weight of the inhibitor is approximately 70,000–80,000.

The particulate enzyme CMP-NANAc[1] lactosyl ceramide sialyl transferase, or hematoside sialyl transferase, was found by Arce *et al.* (1966) in preparations from rat brain and independently by Kaufman *et al.* (1966) in embryonic chicken brain preparations. The reaction catalyzed by this enzyme is probably a part of the main pathway for the synthesis of the major gangliosides (Kaufman *et al.*, 1966) but this is somewhat controversial (Yip and Dain, 1969); for a complete discussion, see Arce *et al.* (1971).

The rate of synthesis of brain gangliosides *in vivo* increases from the 5th to the 15th day of the life of the rat and then decreases rather rapidly, being about 3-fold higher in the 15- than in the 20-day-old rat (Burton *et al.*, 1963; de Maccioni and Caputto, 1968). On the other hand, the total activity of particulate brain hematoside sialyl transferase measured *in vitro* at the optimal conditions, as will be seen in the present report, increases continually from the 8th to the 40th day of the life of the rat. This apparent contradiction between the results *in vitro* and those expected from the observations *in vivo* indicated that the enzyme is subjected to regulatory influences *in vivo*. In support of this preliminary conclusion a natural inhibitor of the transferase was found which increases as the rat becomes older and which had been partially eliminated from the enzymatic preparations used to determine the total activity.

## Materials and Methods

*Subcellular Organelles.* Mitochondrial, synaptosomal, microsomal, and supernatant fractions were prepared according to Eichberg *et al.* (1964). The fractions obtained by this method have been tested in our laboratories by electron microscopy and by enzymatic methods and they were found satisfactorily pure (for details, see Maccioni *et al.*, 1971).

*Enzyme Preparations.* Unless otherwise stated, 7 g of brain tissue from 15-day-old rats were dispersed in 16 ml of water in a glass homogenizer with Teflon pestle. The minced tissue was centrifuged successively at 800*g* for 10 min to eliminate cells and debris and at 10,000*g* for 20 min; the supernatant fraction was centrifuged again at 100,000*g* for 40 min. Each precipitate was washed once with 10 ml of water and then

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[1] Abbreviation used is: CMP-NANAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid.