The Chain Length of the Glycans in Bacterial Cell Walls

By J. BARRIE WARD

National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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The glycan chain length of peptidoglycan was measured by reduction with NaB^3H_4 and isolation of the resulting muramitol, indicative of the length of the chains as biosynthesized, and glucosaminol, which measured the length of the chains after rupture by endo- β -*N*-acetylglucosaminidases. Measurement of the non-reducing terminal *N*-acetylglucosamine by Smith degradation confirmed the result.

The peptidoglycan fraction of bacterial cell walls has been shown to be a polymer containing *N*-acetylmuramic acid and *N*-acetylglucosamine together with a few specific amino acids. The hexosamines are linked glycosidically in a linear polysaccharide, the biosynthetic mechanism ensuring a strict alternation of the two amino sugars (Osborn, 1969). The average chain length of the glycan fractions has been determined in the walls of various bacteria and values of between 6 and 12 disaccharide units have been reported. However, glycan fractions have been found with lengths of 50 disaccharide units in *Arthrobacter crystallopoietes* and *Spirillum serpens* (Rogers, 1970). Where determined, glucosamine has been found as the major, and in some cases the only, reducing terminal group. Since this latter result would not be expected from the mechanism of biosynthesis of the peptidoglycan, the present investigation was undertaken to clarify the apparent contradiction.

Methods

Cell walls. Cell walls were prepared from *Bacillus licheniformis* 6346 *lyt*⁻ (Forsberg & Rogers, 1971) grown in a casein hydrolysate–salts medium (Hughes, 1970) and *Staphylococcus aureus* Copenhagen (obtained from Dr J. M. Ghuysen) grown in a tryptone–yeast extract–glucose medium. Cells were chilled in ice before being harvested and autolytic enzymes were inactivated by treatment with 4% (w/v) sodium dodecyl sulphate immediately after cell breakage. Subsequent isolation and purification of the cell walls was carried out as described previously (Forsberg & Ward, 1972). Wall preparations of *B. licheniformis* 6346 and peptidoglycan from *Bacillus subtilis* 168 *trp*.C2 were obtained from Dr. C. W. Forsberg and Mr. J. G. Pavlik of this Institute. The steps described above to minimize autolysis were always taken.

Reduction and periodate oxidation of samples. Samples of the cell-wall preparations containing 5–20 μmol of hexosamine were reduced with NaB^3H_4 (10 mCi/mmol; 0.1 M) in 12.5 mM-NaOH at 4°C for 6 h. After destruction of excess of reagent by acidification the reduced walls were hydrolysed in

4M-HCl for 4 h at 100°C and the HCl was then removed *in vacuo*. The hydrolysate was fractionated on a column (0.8 cm $\times$ 20 cm) of Dowex 50 (X2; 200–400 mesh) (Hughes, 1970). Hexosamines were determined by the method of Levy & McAllan (1959) and radioactivity was measured in a Packard liquid-scintillation spectrometer with a dioxan-based scintillator. Where appropriate, fractions were combined and the relative amounts of muramitol, muramic acid, glucosaminol and glucosamine were determined. In certain experiments the reduced walls were washed before hydrolysis to remove alaninol formed during the reduction process.

The non-reducing terminal of the glycan chain in wall preparations from *B. licheniformis* 6346 *lyt*⁻ was determined as radioactive glycerol. Wall preparations were oxidized in 25 mM-NaIO₄ overnight at 4°C. Excess of reagent was removed by centrifuging and washing the walls. They were then treated, as described above, with NaB^3H_4 . Excess of reagent was again removed by washing the walls. Glycerol was released by hydrolysis in 0.02M-HCl at room temperature for 16 h. Glycerol (1 μmol) was added to the hydrolysed sample as carrier and the walls were removed by centrifuging. The supernatant was removed and the walls were washed with distilled water. The supernatant and washings were pooled and the glycerol was purified by paper chromatography in solvent *A* (see below). After elution from the paper glycerol was determined enzymically (Wieland, 1963) and radioactivity was measured as described above. Losses occurring during this procedure were corrected from the recovery of the added glycerol.

After removal of the glycerol the reduced cell walls were hydrolysed and fractionated as described above. Identification of the pooled samples was based on paper chromatography and electrophoresis together with authentic standards.

Paper chromatography and electrophoresis. Paper chromatography was carried out on Whatman no. 1 paper with the following solvent systems: *A*, ethyl acetate–pyridine–water (5:2:7, by vol.); *B*, butan-1-ol–acetic acid–water (3:1:1, by vol.); *C*, butan-1-ol–pyridine–water (6:4:3, by vol.). Electrophoresis

was carried out at 40V/cm for 30 or 60min on Whatman no. 3MM paper in the following buffer systems: *D*, pyridine–acetic acid–water (6:46:323, by vol.), pH 3.5; *E*, pyridine–acetic acid–water (25:1:224, by vol.), pH 6.5.

Results

Cell walls of *B. licheniformis* 6346 *lyt*[−] were treated with an excess of NaB³H₄ at 4°C for 6h. After removal of excess of reagent by acidification and washing the reduced cell walls were hydrolysed and the hydrolysate was fractionated on Dowex 50 (Fig. 1). Radioactivity was found in muramitol [2-amino-3-*O*-(*D*-1-carboxyethyl)-2-deoxy-*D*-glucitol] and to a smaller extent in glucosaminol. A peak of radioactivity was eluted unretarded from the Dowex 50 column and was found in control mixtures containing only acidified NaB³H₄ (Hughes, 1970). The amount of radioactivity found in this peak varied considerably and could be decreased by washing the reduced cell walls and removing acid and methyl borate *in vacuo*. A further peak of radioactivity was eluted from the column after glucosamine. This was identified as

alaninol by co-chromatography with authentic material in solvent *B* and electrophoresis in buffer system *E*. The alaninol was presumably derived from the esterified *D*-alanine substituted on the teichoic acid of *B. licheniformis* 6346 and *B. subtilis* 168 (Archibald & Baddiley, 1966). In fact much of the alaninol was removed during the washing of the reduced cell walls. In experiments where this washing was omitted and the alaninol was fractionated with the hydrolysate the yield was 0.24 μmol/mg of cell wall, corresponding to reduction of about 50% of the esterified alanine present in the walls. Unchanged glucosamine and muramic acid were also present, together with a small amount of muramic acid phosphate (less than 5% of the muramic acid present). Muramic acid phosphate was purified by electrophoresis and identified as muramic acid by chromatography in solvent *B* after dephosphorylation with alkaline phosphatase (EC 3.1.3.1).

The pooled samples of [1-³H]muramitol and [1-³H]glucosaminol produced by reduction followed by hydrolysis of the cell walls of *B. licheniformis* and *B. subtilis* were identical with authentic samples as shown by chromatography in solvents *B* and *C* and

Fig. 1. Fractionation of free reducing groups and hexosamines in hydrolysed cell walls of *B. licheniformis* 6346 *lyt*[−] 94

Samples containing 5–20 μmol of hexosamine were hydrolysed in 4M-HCl (1.0ml) at 100°C for 4h. The acid was removed *in vacuo* and the hydrolysate was applied to a column (0.8cm × 20cm) of Dowex 50 (X2; 200–400 mesh) equilibrated in 0.1M-pyridine–acetate buffer, pH 2.8. Fractions (2.0ml) were eluted with the same buffer (70ml total) and then with 0.133M-pyridine–acetate buffer, pH 3.85. Samples (0.1 ml) of the fractions were analysed for radioactivity (○) and hexosamines (●). Key: 1, [³H]muramitol; 2, muramic acid; 3, [³H]glucosaminol; 4, glucosamine; 5, alaninol.

electrophoresis in buffers *D* and *E*. The average chain length of the cell-wall polysaccharides was calculated from the incorporation of ³H into muramitol. Values for various cell-wall preparations of the bacilli ranged from 96 to 139 disaccharide units (Table 1). In addition, ³H was incorporated into glucosaminol in all wall preparations examined. Average chain lengths determined from the ratio of total amino sugar alcohol to half the total hexosamine gave values of 45–79 disaccharide units in the bacillary cell walls. Similar experiments were also carried out on cell walls of *Staph. aureus* Copenhagen. The average chain length of the polysaccharides in this organism, again determined by the incorporation of ³H into muramitol, was 176 disaccharide units. The ratio of half the total amount of amino sugar alcohol to the amount of muramic acid present gave an average value for the polysaccharide chains of 8.9 disaccharide units. This method of calculation was used because the staphylococcal cell wall contains an excess of glucosamine, presumably derived from the *N*-acetylglucosamine substituents of the ribitol teichoic acid (Sanderson *et al.*, 1962). The ratio of glucosamine to muramic acid in the cell wall preparation examined was 1.72.

Further experiments on cell walls of *B. licheniformis* 6346 *lyt*[−] strains measured the incorporation of ³H into glycerol derived by Smith degradation from the non-reducing terminal glucosamine of the polysaccharides. Such experiments are possible in these organisms, previously shown to be deficient in phosphoglucumutase (EC 2.7.5.1), because they do not contain glycosyl substituents in their cell walls (Forsberg *et al.*, 1973). The results obtained for an average chain length of the polysaccharides were in good agreement with those determined from the incorporation of ³H into muramitol (Table 1).

Discussion

Some of the earlier concepts of the structure of peptidoglycan of bacterial cell walls were that the polysaccharides making up the structural backbone were very long (Rogers, 1962; Weidel & Pelzer, 1964). Subsequent analysis of the isolated glycan fraction from a number of bacterial strains did not confirm this view, since the average chain length found was relatively short although the oligosaccharides, where examined, showed considerable heterogeneity (Krulwich *et al.*, 1967; Tipper *et al.*, 1967; Hughes, 1970). One major problem in the interpretation of these results is the presence in all organisms examined of autolytic glycosidases capable of splitting bonds in the polysaccharides. Tipper (1969) suggested that relatively little autolysis takes place during preparation of cell walls provided that reasonable precautions are taken to maintain a low temperature. However, many of the determinations of oligosaccharide chain length have been made on wall preparations that had

Table 1. Composition of reduced cell walls and estimated length of the polysaccharide chains from various bacteria

Micro-organism	Composition (mol.prop.)					Estimated average chain length		
	Glucosamine	Muramic acid	Glucosaminol	Muramitol	Glycerol	From muramitol/ muramic acid ratio	From glycerol/ glucosamine ratio	From total amino sugar alcohol/hexosamine ratio
<i>B. licheniformis</i> 6346 <i>lyt</i> ⁻ 94								
Prep. 1	1.00	0.91	—	0.0066	—	137.9	—	—
Prep. 2	1.00	1.27	0.0052	0.0091	0.0074	139.5	135.1	79.4
<i>B. licheniformis</i> 6346 <i>lyt</i> ⁻ 5	1.00	1.21	—	0.0106	0.0076	114	131.6	—
<i>B. licheniformis</i> 6346	1.00	1.18	0.0138	0.0103	—	114.5	—	45.2
<i>B. subtilis</i> 168	1.00	0.99	0.0103	0.0081	—	96.1	—	54.1
<i>Staph. aureus</i> Copenhagen	1.00	0.582	0.0618	0.0033	—	176.4	—	8.9

Experimental details are given in the text. Fractions from chromatography of hydrolysates on Dowex 50 were pooled, where appropriate. The proportions of glucosaminol, muramitol, muramic acid and glycerol are given in molar proportions relative to glucosamine. Average chain lengths were calculated as disaccharide units from the ratios given. The value for *Staph. aureus* was calculated from the ratio of total amino sugar alcohol to muramic acid because of the presence of excess of glucosamine, as described in the text.

been allowed to autolyse at higher temperatures (Hughes, 1970, 1971; Warth & Strominger, 1971). Thus the finding of relatively short chains terminating in reducing glucosamine presumably reflects the action of an endo- β -*N*-acetylglucosaminidase during this period of autolysis. Such an enzyme has been described among the autolytic activities of all the organisms examined (Hughes, 1970; Brown & Young, 1970; Tipper, 1969).

The results obtained with *B. licheniformis* 6346, the lytic-negative mutants obtained from it and *B. subtilis* suggest that the length of the polysaccharide chains *in vivo* is much longer than that previously reported. The presence of [^3H]muramitol in hydrolysates of oligosaccharides of *B. subtilis* and *Bacillus cereus* treated with NaB^3H_4 has been reported (Hughes, 1971; Warth & Strominger, 1971). In *B. subtilis* the ratio of 1 reducing muramic acid to 250 hexosamine residues was found, a value in close agreement with the average chain lengths obtained in this present study.

The lytic-negative mutants isolated from *B. licheniformis* 6346, in addition to lacking the major autolytic enzyme, namely *N*-acetylmuramyl-L-alanine amidase, are also deficient in phosphoglucomutase (Forsberg *et al.*, 1973). Thus they are unable to synthesize either glycosylated teichoic acid or teichuronic acid. This enabled a check of the proposed glycan chain length, determined from the ratio of muramitol to muramic acid, by measuring the ratio of glycerol from the non-reducing glucosamine terminal of the glycan chain to the total glucosamine. Any non-reducing terminal *N*-acetylmuramic acid residues liberated by the action of endo- β -*N*-acetylglucosaminidase would not be attacked by periodate and thus would not yield [^3H]glycerol. The two values obtained by these independent methods were in good agreement.

The present results distinguish between the average length of the glycan chains as biosynthesized, indicated by the ratio of muramitol to muramic acid, and the average final length of the glycan chains, indicated by the proportion of total amino sugar alcohol. The latter value probably represents the situation *in vivo*, although the splitting of a few glycosidic linkages during preparation of cell walls cannot be ruled out. In this context the average chain length found in *Staph. aureus* of 17.8 hexosamine residues compares closely with the value of 18 previously reported (Tipper *et al.*, 1967) for a purified preparation of the glycan fraction from this organism. Similar values for the chain length in *Staphylococcus lactis* were recently reported (Archibald *et al.*, 1973), but the ratio of muramitol to muramic acid was much

higher than any of the values reported here. These differences in average chain lengths as distinct from the biosynthetic chain length may reflect a difference in the structural organization of the cell walls of cocci and bacilli. The methods described above would facilitate a determination of the effect of growth conditions, and in synchronous cultures the age of the cell, on the size of polysaccharides in the cell wall.

The results correspond to a length for the biosynthesized glycan chains of 100–200 nm based on a length of 1.03 nm for a single disaccharide unit (Kelemen & Rogers, 1971) and an average final chain length of 8.7–77 nm. These chains would be too long to be arranged radially through the peptidoglycan layer. However, the results cannot distinguish between a longitudinal and a circumferential arrangement.

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