Cell Walls of *Streptococcus pyogenes*, Type 14. C Polysaccharide–Peptidoglycan and G Polysaccharide–Peptidoglycan Complexes*

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ABSTRACT: About 20% of the organic phosphorus present in cell walls of *Streptococcus pyogenes* occurs as *N*-acetylmuramic acid 6-phosphate residues (50 mμequiv/mg of walls). These groups link about 10% of the peptidoglycan subunits to a hitherto unrecognized polysaccharide. This polymer was isolated after degradation of trypsin-treated walls with *Streptomyces* F_1 endo-*N*-acetylmuramidase; it consists of disaccharide peptide monomer (50 mμmoles/mg of walls) linked through a phosphodiester bridge to a polymer containing, per peptide monomer, five to six D-glucose residues, one glucosamine residue, and four to five unidentified hexosamine compounds. This polymer is designated as G polysaccharide.

About 70% of the organically bound phosphorus in the cell walls (150 mμequiv/mg of walls) is present in the form of phosphodiester groups in the interior of the rhamnose-containing C polysaccharide. The linkages of the C-polysaccharide chains to the peptidoglycan are visualized as bridges of trirhamnose groupings, each bearing one β-linked *N*-acetylglucosamine residue, bound at the reducing end, either directly or through an as yet undetermined intervening molecule, to C-4 of an *N*-acetylmuramic acid residue. After degradation of walls by endo-*N*-acetylmuramidase, the C-polysaccharide chains remain bound to one *N*-acetylmuramic acid residue. This *N*-acetylmuramic acid residue is substituted by one tetrapeptide monomer which in turn is linked *via* a di-L-alanyl bridge to another disaccharide peptide monomer. A structure for the cell walls of *Streptococcus pyogenes* is proposed that takes into account all of the recognized covalent linkages among the constituent polymers.

The cell walls of *Streptococcus pyogenes* are known to be composed of at least three polymers of different types: the rigid, insoluble peptidoglycan network, the group-specific C polysaccharide, and the type-specific M, R, and T proteins. The peptidoglycan consists, at least in part, of N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine peptide subunits branched on glycan strands of β-1,4-linked, alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues (Muñoz *et al.*, 1966a). Some of the peptide subunits are cross-linked through dipeptides, L-alanyl-L-alanine, extending from the ε-amino group of lysine of one peptide to the C-terminal group of D-alanine of another (Muñoz *et al.*, 1966a). The C polysaccharide contains α-1,3-linked L-rhamnose (Estrada-Parra *et al.*, 1963; Heymann *et al.*, 1963) and terminal *N*-acetylglucosamine β-1,3-linked to rhamnose (Heidelberger and McCarty, 1959; McCarty, 1956; Krause, 1963; Heymann *et al.*, 1964; see also McCarty and Morse (1964) for a general review on Gram-positive bacterial antigens). A portion of the rhamnose units is also substituted in the 2 position (Heymann *et al.*, 1963) and some of the glucosamine residues may not be terminal. The structures of the M protein, which is probably related to the virulence of the microorganism, and of the R and T proteins are still largely unknown. These components can be removed by treatment with suitable enzymes without affecting the viability of the microorganism or the insolubility and electron-microscopic appearance of isolated walls.

Peptidoglycan and C polysaccharide have long been suspected to be covalently associated (McCarty, 1952; Salton, 1953). Recently, various phosphoesters: muramic acid 6-phosphate, glycerol phosphate, and glyceryl rhamnoside phosphate, were isolated from *S. pyogenes* cell walls (Heymann *et al.*, 1967). It was proposed that such groupings bridged the peptidoglycan and the C polysaccharide. However, the possibility that some phosphate ester groupings might occur in the interior of the C polysaccharide polymer was recognized. Liu and Gotschlich (1967) reached similar conclusions in their study of *Diplococcus pneumoniae* polysaccharide. Moreover, they showed that in a series of selected bacteria, muramic acid 6-phosphate represented as much as 7–39% of the total muramic acid residues.

In the present paper, we are dealing with the covalent linkages that tie the peptidoglycan to the other poly-

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mers in *S. pyogenes* cell walls. It will be shown that the muramic acid 6-phosphate residues link about 10% of the disaccharide peptide subunits of the peptidoglycan to a polysaccharide polymer which had not been recognized heretofore (part II). Phosphorus is not involved in the covalent bonds which tie about 35% of the *N*-acetylmuramyl peptide subunits to the C-polysaccharide moiety, but in the interior of the C polysaccharide, 70% of the wall organic phosphorus occurs as phosphodiester (part III).

Material and Methods

Analytical Methods. Identification and measurement of N-terminal groups and free amino acids (by the FDNB[1] technique), of C-terminal groups (by the hydrazinolysis technique), and of D- and L-alanine (by the enzymatic procedure) have been described (Ghuysen *et al.*, 1966). Determination of reducing power by the Park–Johnson method, measurement of acetamido sugars using the Morgan–Elson reaction after 7 or 30 min of heating at 100° in 1% borate, measurement of amino sugars (after acid hydrolysis) employing the same procedure (7 min of heating) after chemical acetylation, and specific determination of glucosamine with yeast D-glucosamine 6-phosphate *N*-acetylase have also been described (Ghuysen *et al.*, 1966). Analysis of total organic phosphorus and of inorganic phosphorus was performed according to Lowry *et al.* (1954). Rhamnose estimation was carried out according to Dische and Shettles (1948); it was also performed according to the micromodification described by Heymann *et al.* (1967). Free D-glucose after adequate acid hydrolysis was characterized and determined using the Glucostat reagent obtained from Worthington Biochemical Corp., Freehold, N. J. Periodate oxidation and reduction with $NaBH_4$ were performed as described by Tipper *et al.* (1965) and Leyh-Bouille *et al.* (1966).

Paper Electrophoresis. Electrophoresis was carried out on Whatman 3MM paper (40 × 40 cm) in an Electrorheophor Pleuger (Antwerp, Belgium) apparatus at pH 5.0 in pyridine–acetic acid–water (4:2:1000) or at pH 2.5 in 0.1 N acetic acid buffer.

Paper Chromatography. Chromatography was carried out by the descending technique on Whatman No. 1 paper employing the following solvents: (I) isobutyric acid–0.5 N ammonia (5:3), (II) 1-butanol–pyridine–water (6:4:3), (III) 1-butanol–acetic acid–water (3:1:1), and (IV) pyridine–water (4:1).

Detection on Paper. Oligosaccharides (mainly disaccharides) from peptidoglycan were detected by fluorescence after the paper had been dipped in a 0.5 N NaOH solution made up in ethanol–1-propanol (6:4) and heated for 10 min at 120° (Sharon, 1964). Amino sugars, amino acids, and free amino groups were detected with ninhydrin spray (0.5% in isopropyl alcohol–water, 9:1). Sugars, amino sugars, and glycerol were detected with alkaline silver nitrate.

Cell Walls. Cell walls of *S. pyogenes*, type 14, were prepared and trypsin treated as described earlier (Heymann *et al.*, 1963; Muñoz *et al.*, 1966a). The amino acid composition has been reported (Muñoz *et al.*, 1966a). One milligram of walls contained about 480 mμequiv of peptidoglycan subunits, 1450 mμmoles of rhamnose, 225 mμmoles of organic phosphorus, an undetermined amount of glycerol which has been previously identified (Heymann *et al.*, 1967), and about 250 mμmoles of D-glucose. It should be noted that the glucose oxidase–*o*-anisidine reaction (Glucostat) is not reliable when applied to acid hydrolysates of walls; even internal standards added after hydrolysis are not reliably detected or measured. However, when the estimation is performed on an acid hydrolysate of cell walls previously solubilized by the *Streptomyces* F_1 endo-*N*-acetylmuramidase (200 μg in 100 μl of 6 N HCl, 3 hr, 100°), about 120 mμmoles of glucose/mg of walls is found. The value of 250 mμmoles/mg of walls was calculated from the glucose content of a fraction (fraction S_3; *vide infra*) isolated from F_1 degraded cell walls and from the yield with which this fraction was obtained. Hydrolysis conditions for fraction S_3 were 2 N HCl, 2 hr, 100°. A glucose polymer could also be demonstrated in alkaline digests of *S. pyogenes* walls. Fractionation of such digests on Sephadex G-50 showed that both hexose and rhamnose polymers were being solubilized. With increasing time of exposure to alkali the hexose content of the excluded volume grew, whereas rhamnose was found increasingly in the fractionating volume. After drastic treatment (2 N NaOH, 17 hr, 100°) and filtration over Sephadex G-25 the excluded volume contained a polymer rich in hexose (Dische cysteine–H_2SO_4 reaction). The hexose was identified and measured as glucose by the glucostat reagent. The yield (100 mμmoles/mg) of walls was low, probably because of partial depolymerization. Nonetheless, the glucose polymer evidently is more resistant to degradation than the rhamnose polymer, and thus can be separated from the vast excess of methylpentose polymer present in the walls.

Enzymes. Streptomyces F_1 endo-N-acetylmuramidase (a lytic enzyme hydrolyzing linkages of *N*-acetylmuramic acid to *N*-acetylglucosamine in the glycan portion), *Streptomyces* aminopeptidase, and *Streptomyces N*-acetylmuramyl-L-alanine amidase (a nonlytic enzyme hydrolyzing the linkages between the *N*-acetylmuramic acid residues and the peptide subunits) have been described (Petit *et al.*, 1966; Muñoz *et al.*, 1966a,b). An *exo*-β-*N*-acetylglucosaminidase was prepared from pig epididymis according to Sanderson *et al.* (1962). D-Glucosamine 6-phosphate *N*-acetylase was prepared from yeast according to Brown (1962); this enzyme was a generous gift of Dr. R. Tinelli (Pasteur Institute, Paris). D-Glucose oxidase was purchased from Worthington Biochemical Corp., Freehold, N. J. Alkaline phosphatase from *Escherichia coli*, type III (10 mg/ml), was obtained from Sigma Chemical Co.

[1] Abbreviations used: FDNB, fluorodinitrobenzene; Glc, glucose; GlcN, glucosamine; HexN, hexosamine; Mur, muramic acid; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; *exo*-β-GlcNAcase, *exo*-β-*N*-acetylglucosaminidase.

Experimental Results

1. Degradation of Cell Walls by F_1 Enzyme to Fractions S_1, S_2, S_3, and S_4. Cell walls (100 mg) were treated at 37° with 2 mg of F_1 endo-*N*-acetylmuramidase in a final volume of 16 ml of 0.01 M K_2HPO_4 (pH 8.0). Complete clarification occurred after 5 hr, but the incubation was extended to 8 hr. The degraded products contained equivalent amounts of terminal N^{ϵ}-lysine and terminal NH_2-alanine which together amounted to about 200 mμmoles/mg of cell walls. These amino groups are native to the cell walls (Muñoz *et al.*, 1966a). About 280 mμmoles of peptide-substituted β-(1,4)-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides was detected among the degraded products, on the basis of the reducing power or of the Morgan–Elson reaction (30 min of heating in borate; ϵ_{585} 5700). This figure indicates that, seemingly, the glycan portion of the peptidoglycan moiety had been only partially split into disaccharide units (Muñoz *et al.*, 1966a,b). The solubilized cell walls were filtered, in water, on two columns (1.5 × 125 cm, each) of Sephadex G-50 (bead form) and Sephadex G-25 (bead form) connected in series (V_0 = 180 ml; $V_0 + V_i$ = 380 ml) (Figure 1A). Fractions S_1 (elution volume 175–200 ml), S_2 (elution volume 200–275 ml), S_3 (elution volume 275–310 ml), and S_4 (elution volume 340–400 ml) were freeze dried. Fraction S_2 contained about 70% of the organic phosphorus, 70% of the amino acids, 60% of the amino sugar residues, and 80% of the rhamnose present in the original cell walls, and also an undetermined amount of glycerol, but no detectable glucose. Fraction S_3 contained 20% of the organic phosphorus, 10% of the amino acids, 25% of the amino sugar residues, and all of the glucose encountered in this investigation, but no rhamnose or glycerol. Both fractions S_2 and S_3 were

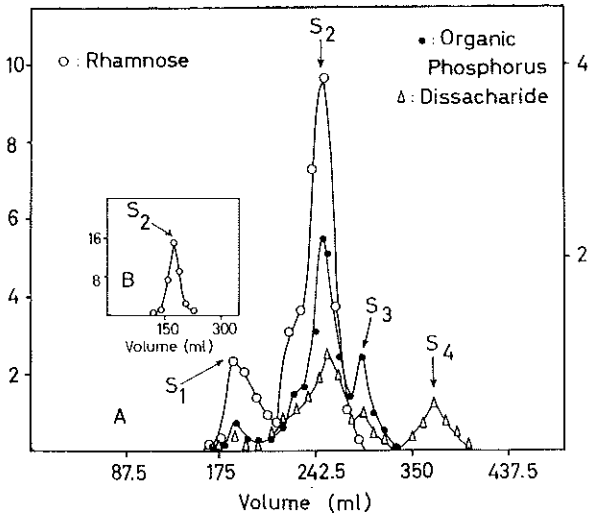

FIGURE 1: Gel filtration of *Streptococcus pyogenes* cell walls after solubilization with *Streptomyces* F_1 endo-*N*-acetylmuramidase. The soluble products from 100 mg of walls were filtered in water (A) or in 0.05 M LiCl (B) on two columns of Sephadex G-50 and Sephadex G-25 connected in series. Aliquots of 100 μl of each fraction were used to estimate rhamnose, organic phosphorus, and β-(1,4)-*N*-acetylglucosamine-*N*-acetylmuramic acid disaccharide (using the Morgan–Elson reaction with 30 min of heating in 1% borate and employing an ϵ_{585} of 9500 which is specific for disaccharide not substituted by peptide). Results are expressed in μmoles/3.5-ml fraction of effluent. The values for rhamnose are represented on the left ordinate, those for organic phosphorus and disaccharide on the right one. Reducing power, N-terminal groups, and total amino sugar residues were also estimated on aliquots of 20, 100, and 25 μl, respectively (not shown in the figure). Elution curves for these constituents were parallel to that for the disaccharide units.

TABLE 1: Chemical Composition of Fractions S_2 and S_3.[a]

	Fraction	
	S_2	S_3
Organic phosphorus	1	1
Amino acids		
Glutamic acid	2	1
Lysine	1.90	0.91
Total alanine	7.40	2.90
D-Alanine	2.20	1
L-Alanine	4.40	2.03
X	0.0	0.22[b]
Terminal amino groups		
NH_2-alanine	0.56	0.47
N^{ϵ}-Lysine	0.30	0.33
Terminal carboxyl groups[c]		
Alanine	0.96	1.09
L-Alanine liberated by aminopeptidase[d]	1.10	1.06
NH_2-alanine exposed by amidase[d]	1.70	0.75
Total amino sugars	5.90	7.60
Glucosamine	3.70	2.25
Muramic acid (see text)	2.0	1.05
Disaccharide β-(1,4)-GlcNAc-MurNAc (owing to Morgan–Elson reaction)	1.06	1.00
Disaccharide (owing to reducing power)	0.96	0.81
Glucose	0.0	5–6
Rhamnose	6.0	0.01

[a] Results are expressed in moles per mole of organic phosphorus. [b] Nonpeptidoglycan amino acids present as traces in the cell wall preparation (for complete amino acid composition of the walls, see Muñoz *et al.*, 1966a) were found in higher amounts in fraction S_3. [c] After correction on a basis of a 50% yield (as obtained with synthetic peptides used for controls). [d] For conditions of treatment see text and Muñoz *et al.* (1966a).

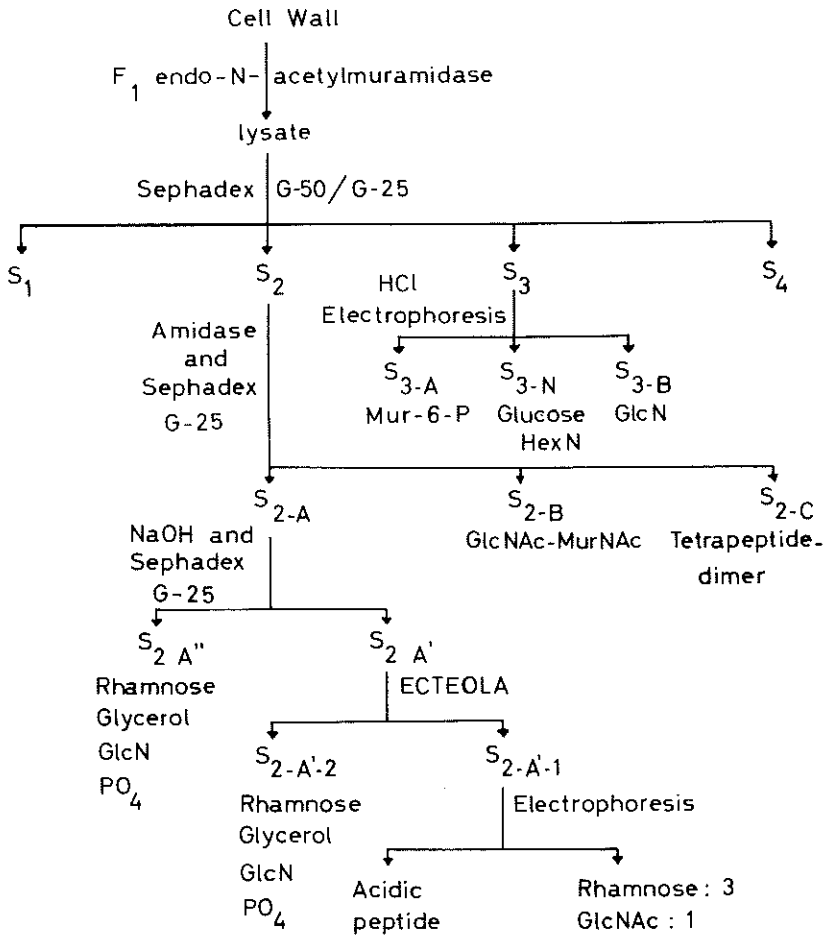

FIGURE 2: Degradation of cell walls of *S. pyogenes*. For abbreviations, see footnote 1.

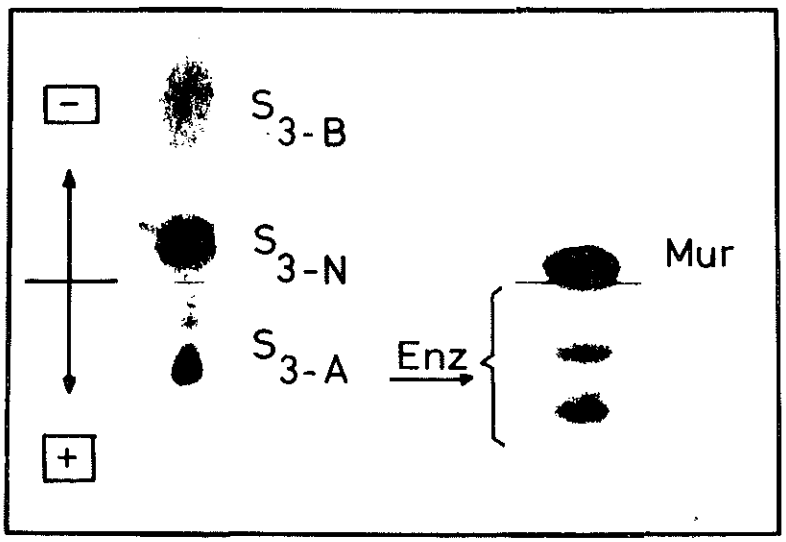

FIGURE 3: Paper electrophoresis at pH 5.0 of the acid-degraded complex S_3. The material was spotted at the center of the sheet. For conditions of electrophoresis, see text. The acidic compound S_{3-A} was extracted from the paper and treated with alkaline phosphomonoesterase (Enz). The mixture was again submitted to paper electrophoresis under the same conditions as above (right side of the figure). Ninhydrin was used for detection. Mur = muramic acid. Mur marks the region of electrophoretic immobility.

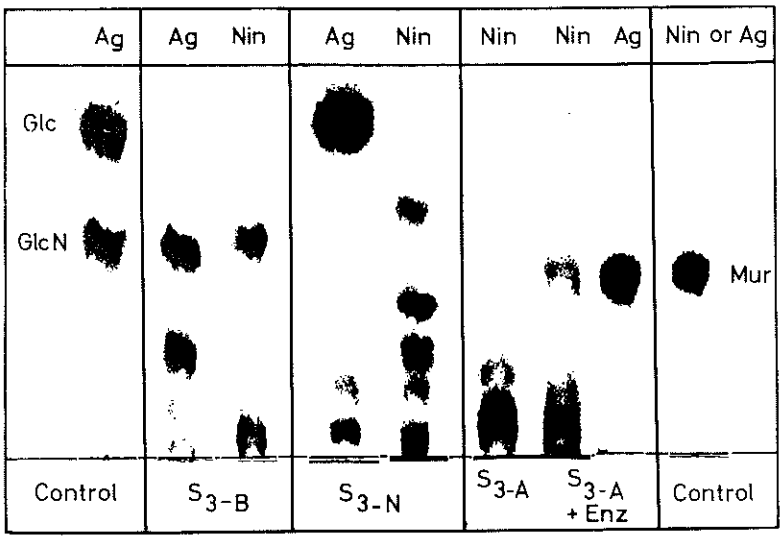

FIGURE 4: Paper chromatography in solvent II of S_{3-A}, S_{3-N}, and S_{3-B} fractions obtained by preparative electrophoresis (Figure 3) of the acid-degraded S_3 complex. Ninhydrin (Nin) and silver nitrate (Ag) were used for detection. S_{3-A} + Enz represents fraction S_{3-A} after treatment with alkaline phosphomonoesterase (see text). Muramic acid was liberated (Mur). Glucose (Glc), glucosamine (GlcN), and muramic acid served as standards.

found to be immobile on paper chromatography in solvent I and homogeneous on paper electrophoresis. They were cationic at pH 2.5 and neutral at pH 5.0. Traces of positively charged, ninhydrin-reactive materials were detected in fraction S_3 at pH 5.0.

The elution volumes of fractions S_2 and S_3 are not suitable for molecular weight estimations because fraction S_2, for example, is excluded from the double Sephadex column when it is applied in 0.05 N LiCl solution instead of in water (Figure 1B). Thus the results shown in Figure 1A must be due at least in part to retardation phenomena other than gel filtration.

Fraction S_1 (Figure 1A) was excluded from the gel even in aqueous solution; it contained about 20% of the total rhamnose and 15% of peptidoglycan components. Fraction S_4 (Figure 1A) was virtually free of peptide components. It represents only 20% of glycan fragments of the peptidoglycan, mainly in the form of free disaccharides. The flow sheet (Figure 2) outlines further processing of the fractions.

II. Fraction S_3. Location of Muramic Acid 6-Phosphate Residues. The Peptidoglycan G–Polysaccharide Complex. ANALYTICAL DATA. Table I presents the composition of fraction S_3, determination of N- and C-terminal groups as well as the results of degradation by aminopeptidase and *N*-acetylmuramyl-L-alanine amidase. Evidently S_3 contains one tetrapeptide residue, N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine, of established structure (Muñoz *et al.*, 1966a) per atom of phosphorus, and about 50% of these residues bear uncross-linked L-alanyl-L-alanine dipeptide at the N^{ϵ}-lysine position, relative to glutamic acid taken as unity. Complex S_3 gave a Morgan–Elson reaction (100°, 1% borate, 30 min, ϵ 5700; Muñoz *et al.*, 1966a,b) indicative of the presence of one peptide-substituted disaccharide, GlcNac-β-1,4-MurNAc, and exhibited reducing power equivalent to 0.81 mole of disaccharide (Ghuysen *et al.*, 1966). Again relative to glutamic acid, 7.6 moles of amino sugar was found of which 2.25 was glucosamine (enzymatic assay); muramic acid was demonstrated qualitatively by two-dimensional chromatography in solvents III and IV. Hydrolysis for these determinations was done with 6 N HCl at 100° for 6 hr; 3 N acid acting for 3 hr gave incomplete hydrolysis. Analogous analyses performed after reduction of S_3 with $NaBH_4$[2] showed that only one hexosamine unit had disappeared and that this consisted entirely of muramic acid. Thus we can account for the muramic acid in S_3 and one of the glucosamine units as forming the disaccharide peptide signalled by the reducing power and the Morgan–Elson reaction.

ACID HYDROLYSIS OF FRACTION S_3. The phosphorus in S_3 became phosphomonoesterase labile on treatment with 2 N HCl at 100° for 2 hr. The reaction was followed by withdrawing aliquots of 50 μl containing 50 mμ-equiv of phosphate; the samples were neutralized and treated with 5 μg of alkaline phosphatase for 2 hr at 37° in a final volume of 100 μl of 0.02 M Tris-HCl buffer (pH 9.5), and then inorganic phosphorus was measured.

Preparative electrophoresis at pH 5.0 (20 v/cm, 45 min) of such an acid hydrolysate gave an acidic (S_{3-A}), a neutral (S_{3-N}), and a basic (S_{3-B}) fraction (Figure 3). The acid material S_{3-A} contained phos-

[2] Fraction S_3 or fraction S_2 (200 mμequiv of either) was dissolved in 100 μl of water and mixed with 100 μl of 0.5 M $NaBH_4$ solution. The solution was maintained at 25° for 4 hr, acidified with acetic acid, and freeze dried. Boric acid was removed by repeated freeze drying in the presence of methanol.

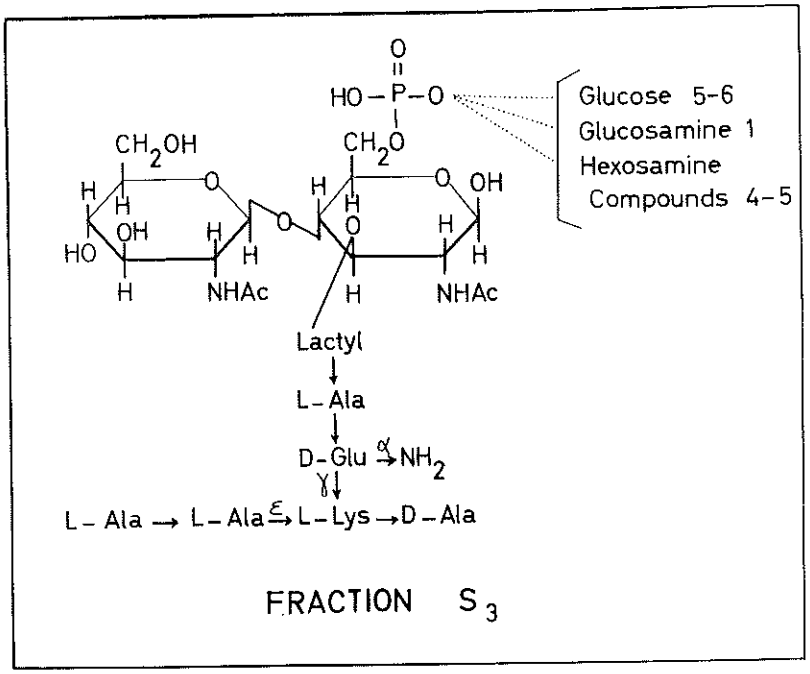

FIGURE 5: Proposed structure for the main complex present in fraction S_3. A disaccharide peptide monomer is linked through a phosphodiester bridge to a polysaccharide composed of glucose, glucosamine, and other unidentified hexosamine compounds (G polysaccharide).

phorus and hexosamine in a ratio of 1:1 (Morgan Elson after chemical acetylation) and gave muramic acid upon treatment with phosphomonoesterase, as shown in Figures 3 and 4 and also by chromatography in solvent III. Fraction S_{3-A} thus contains, or is, undoubtedly the muramic acid 6-phosphate that had been identified in previous work (Heymann *et al.*, 1967; Liu and Gotschlich, 1967).

Fraction S_{3-N} contained D-glucose as shown by chromatography in solvent II (Figure 4) and solvent III; the hexose was measured with glucose oxidase reagent. Similarly, fraction S_{3-B} was shown to contain D-glucosamine which was determined specifically with the *N*-acetylase.

Fraction S_{3-N} contained not only glucose but also a number of reducing, ninhydrin-positive substances (Figure 4) which are not glucosamine or muramic acid, are uncharged at pH 5, and migrate more slowly than glucosamine. They were not studied further; their chromatographic properties suggest (Wheat, 1966) that they may be aminohexuronic acids.

STRUCTURE OF FRACTION S_3. When amidase acts upon S_3, nearly one new terminal NH_2 group of alanine appears, per glutamic acid residue, but no free disaccharide GlcNAc–MurNAc is formed. Consequently, this disaccharide must still be bound to another constituent of S_3 by means of a linkage not involving C-1 of the muramic acid phosphate. Since the presence of disubstituted orthophosphate in S_3 is evident from the foregoing, we suggest that the glucose–glucosamine–hexosamine polymer is linked to the disaccharide peptide through a phosphodiester bond emanating from C-6 of the muramic acid moiety. It is also proposed that this hitherto unrecognized glucose-containing polymer be designated as G polysaccharide. The proposal is represented in the accompanying structural formula (Figure 5). Walls contain, per milligram, about 10% of the peptidoglycan subunits in this form, which corresponds to 20% of the total phosphorus or to 50 mμequiv of 6-phosphorylated disaccharide/mg of walls.

III. Fraction S_2. The Peptidoglycan–C-Polysaccharide Complex. ANALYTICAL DATA. The quantitative composition and N- and C-terminal group analyses before and after treatment with aminopeptidase or *N*-acetylmuramyl-L-alanine amidase are presented in Table I. The tetrapeptide subunits are present preponderantly as dimers, linked by L-alanyl-L-alanine bridges, of which one-half is free at the N terminus.

The total hexosamine content of S_2 in acid hydrolysates (3 N HCl, 3 hr, 100°) is 5.9 moles/2 moles of glutamic acid, as measured by the 7-min Morgan–Elson test. The enzymatic procedure showed 3.7 moles of this to be glucosamine. The only other hexosamine appearing on two-dimensional chromatograms with solvents II and III was muramic acid. The amount of glucosamine was not affected by $NaBH_4$ treatment of the complex S_2. However, 2 moles of hexosamine did disappear, per peptide dimer, on $NaBH_4$ reduction, and simultaneously the muramic acid spot disappeared from the paper chromatograms.

Thus the complex S_2 contains muramic and glutamic acids in equimolar amounts. However, one-half of the muramic acid differs in reactivity from the remainder.

Reducing power and Morgan–Elson reaction (30 min of heating) indicate the presence of one β-(1,4)-GlcNAc-MurNAc unit per peptide dimer, and this could be isolated by paper chromatography in solvents II or III after amidase action on complex S_2.

This disaccharide is completely liberated under conditions routinely used for amidase incubations, *i.e.*, 15 μg of enzyme, 2 hr at 37°; concurrently 1.3 equiv of NH_2 groups of alanine was exposed. Under more drastic conditions, however, more alanine amino groups were exposed, and the kinetics of amidase action was seen to be biphasic. To this end, aliquots of S_2 containing 40 mμequiv of glutamic acid were treated at 37° with 15–45 μg of amidase in a final volume of 100 μl of 0.03 M acetate buffer, pH 5.4, for 2- and 4-hr periods. Even the most extensive treatment applied in this study gave rise to no more than 1.7 moles of new alanine amino groups. Moreover, the appearance of these amino groups was not accompanied by the liberation of disaccharide over and beyond the 1 mole released during the rapid phase of amidase action.

Thus, one of the two muramic acid residues present in fraction S_2 fails to exert reducing power toward ferricyanide, does not give a Morgan–Elson test, has an amide link to L-alanine which is poorly susceptible to amidase action, but still must possess an unsubstituted hydroxyl group at C-1 which is attacked by $NaBH_4$. Since amidase action depends on the presence of an *N*-acetyl group at C-2 (Mirelman and Sharon, 1966), the susceptibility to amidase, even though reduced, shows that the residue in question must be an *N*-acetylmuramyl group. The properties discussed are, accordingly, not affected by attempts at chemical acetylation. The "odd" muramic acid residue is not the 6-phosphate because acid hydrolysis conditions known to be suitable for liberation of this ester (see part II; fraction S_3) produced no detectable amount of it.

We conclude that the "odd" residue of muramic acid must be substituted in a fashion not hitherto encountered, and that the substitution probably involves the link to the rhamnose portion of S_2. Further pertinent evidence will be presented below.

The 3.7 moles of glucosamine/2 moles of glutamic acid can be partitioned as follows: 1 mole belongs to the disaccharide GlcNAc-MurNAc which is readily released by amidase; the remaining, 2.7 moles, is probably in the rhamnose portion of S_2, 2.7 moles of GlcNAc/6 rhamnose residues, a ratio in agreement with the composition reported for formamide-extracted C polysaccharide (Heymann *et al.*, 1963). Of these GlcNAc residues, 1.6 moles/6 rhamnose units is released by the action of exo-β-*N*-acetylglucosaminidase. To that end, an aliquot of S_2 containing 40 mμequiv of aminohexose was treated for 15 hr at 37° with 5 μl of the enzyme solution in a final volume of 50 μl of 0.01 M citrate buffer at pH 4.2. The 7-min Morgan–Elson reaction showed the presence, per 2 moles of glutamic acid, of 3.6 moles of free *N*-acetylhexosamine, which contains, of course, 2 moles resulting from cleavage of the peptide-bound disaccharide GlcNAc-MurNAc. Only GlcNAc but no MurNAc was demonstrated on paper chromatograms. Thus of the 3.7 equiv of GlcNAc present/2 moles of glutamic acid in S_2, 2.6 is released by exo-β-*N*-acetylglucosaminidase. The remaining, nonsusceptible mole may be in an unaccessible position of the rhamnose portion.

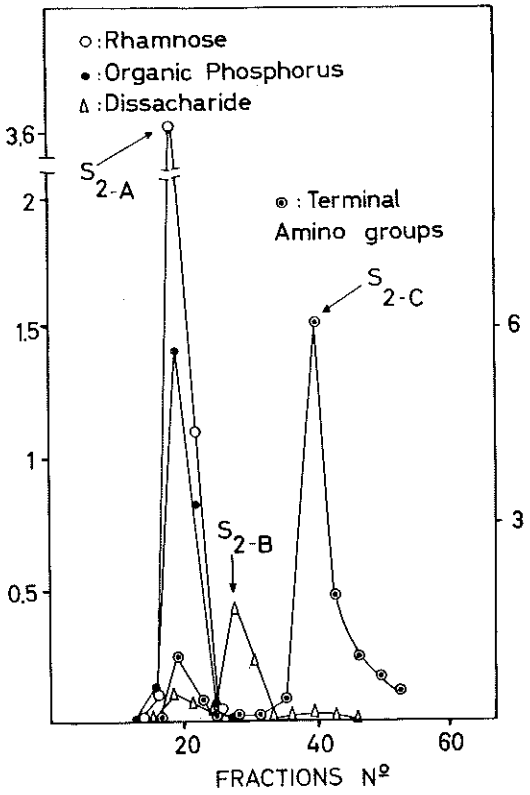

FIGURE 6: Gel filtration on Sephadex G-25 (bead form) of the amidase-treated S_2 fraction (Figure 1). Aliquots of 100 μl of each fraction were used to estimate bound rhamnose, organic phosphate, N-terminal groups, and β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide (using the Morgan–Elson reaction with 30 min of heating in 1% borate and employing an ϵ_{585} of 9500 which is specific for a disaccharide unsubstituted by peptide). Results are expressed in μmoles/3.5-ml fraction. Values for rhamnose, organic phosphorus, and disaccharide are represented on the left ordinate; those for N-terminal groups on the right one.

PREPARATIVE DEGRADATION OF COMPLEX S_2 BY *N*-ACETYLMURAMYL-L-ALANINE AMIDASE. *Preparation of fraction S_{2-A}.* The course of the fractionation following this, and the ensuing alkaline, degradation is set forth on the flow sheet (Figure 2). The enzymatic degradation is, in part, repetitive and confirmatory of material just presented. Complex S_2 (12 μequiv of glutamic acid) was treated for 4 hr at 37° with 7 mg of amidase in a final volume of 25 ml of 0.03 M acetate buffer (pH 5.4). New terminal-amino groups of alanine appeared to the extent of 1.7 moles/2 moles of glutamic

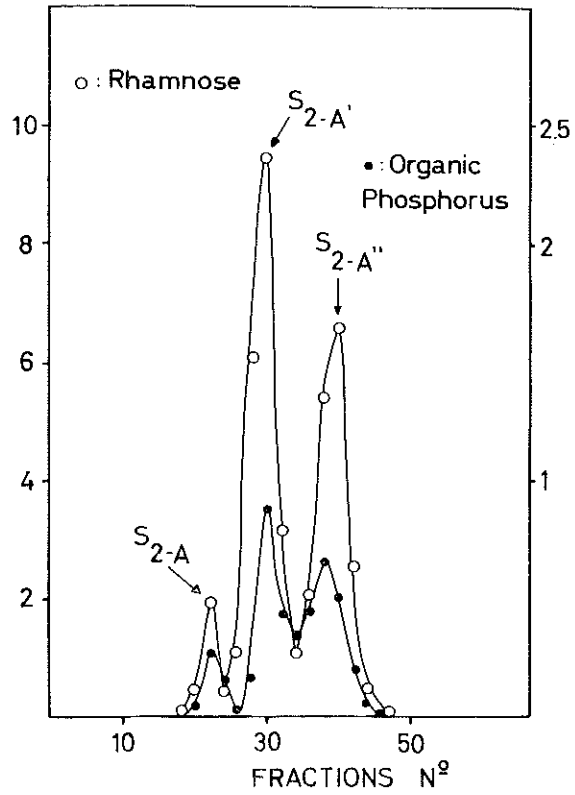

FIGURE 7: Gel filtration on Sephadex G-25 (bead form) (same column as in Figure 6) of the alkali-treated fraction S_{2-A} (see Figure 6). Results are expressed in μmoles/3.5-ml fraction. Aliquots of 50 μl were used to estimate rhamnose (left ordinate) and of 100 μl for organic phosphate (right ordinate).

acid. The degraded products were applied to a column of Sephadex G-25, bead form (1.5 × 100 cm; V_0 = 75 ml; $V_0 + V_i$ = 160 ml). Elution with water yielded fractions S_{2-A}, S_{2-B}, and S_{2-C}, as shown on the elution curve (Figure 6) and the flow sheet (Figure 2). The recovery was 85–90% on the basis of each of the parameters measured.

Fraction S_{2-C} was the free dimeric peptide, *i.e.*, two N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine monomers linked through an L-alanyl-L-alanine bridge. This structure was fully established by the usual procedures: amino acid composition, N- and C-terminal groups, and liberation of N-terminal L-alanine residues with concomitant appearance of new N-terminal glutamic acid residues.

Fraction S_{2-B} consisted preponderantly of the disaccharide (GlcNAc-β-1,4-MurNAc) which was characterized analytically by paper chromatography, cleavage by exo-β-*N*-acetylglucosaminidase, and Morgan–Elson reaction. In solvent III on chromatography, traces of a substance appeared migrating like β-(1,4)-*N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid (Tipper *et al.*, 1965), and exo-β-*N*-acetylglucosaminidase gave, besides the expected products, traces of a compound with the R_F of *N*-6-*O*-diacetylmuramic acid.

Fraction S_{2-A} was chromatographically immobile in solvent I and was practically nondetectable by ninhydrin or alcoholic alkali spray; it was located by analysis of strip eluates. Moreover, S_{2-A} was practically nonreducing and Morgan–Elson negative, despite attempts at acetylation of possible free NH_2 groups. The yields in fractions S_{2-B} and S_{2-C} and the composition of fraction S_{2-A} showed that amidase had removed 1 mole of disaccharide GlcNAc-MurNAc/2 moles of glutamic acid in S_2, but only about 50% of the bonds between peptide alanine and the "odd" *N*-acetylmuramic acid residue had been split. Accordingly, fraction S_2 still contained approximately one peptide dimer per two atoms of phosphorus, and it still contained, relative to phosphorus, 6 rhamnose units, 3.8 residues of hexosamine of which 2.7 residues were glucosamine, glycerol, and muramic acid. Of the hexosamines, only muramic acid was eliminated by $NaBH_4$ reduction, and exo-β-*N*-acetylglucosaminidase liberated 2.08 moles of *N*-acetylglucosamine, again relative to phosphate. The phosphorus appeared in the form of phosphomonoesters after acid hydrolysis (3 N HCl, 3 hr, 100°) but muramic acid 6-phosphate was not among them.

PREPARATIVE ALKALINE DEGRADATION OF FRACTION S_{2-A}. Fraction S_{2-A} now consisted of the rhamnose polymer moiety attached to its anchor point in the peptidoglycan, presumed to be the "odd" *N*-acetylmuramic acid residue. This latter residue was still partially substituted by peptide owing to incomplete amidase action. In order to remove the peptide chains without further expenditure of massive quantities of amidase, recourse was taken in the recently described β-elimination of 3-*O* substituents from *N*-acetylmuramic acid derivatives (Ghuysen *et al.*, 1967a).

The conditions were chosen so as to ensure not only β elimination but also complete conversion of the phosphate groups to monoesters: 2 N NaOH at 55° for 5 hr. The neutralized digest was applied to the Sephadex column described in the preceding section and eluted with water. The flow sheet (Figure 2) and Figure 7 show that two fractions appeared, $S_{2-A'}$ and $S_{2-A''}$, besides 5–10% of early material which is most likely undegraded complex S_{2-A}. Subfractions $S_{2-A'}$ and $S_{2-A''}$ no longer contained muramic acid, as was to be expected, but both of them contained rhamnose, glycerol, glucosamine, and phosphomonoester.

Subfraction $S_{2-A'}$ was further processed by ion-exchange chromatography on a column of ECTEOLA-cellulose (2 × 40 cm). The exchanger in the free base form was equilibrated with 0.5 M LiCl and washed chloride free. Water failed to elute fraction $S_{2-A'}$ from the column, but a LiCl gradient gave rise to two elution peaks, $S_{2-A'-1}$ and $S_{2-A''-2}$ (Figure 8 and flow sheet, Figure 2).

The first peak, $S_{2-A'-1}$, was further split by paper electrophoresis at pH 5.0 (20 v/cm, 45 min) into an acidic peptide and a neutral polymer. The peptide was not further studied; it is most likely the peptide

dimer, or a fragment of it, bearing the lactyl group split from the *N*-acetylmuramic acid residue.[3] The neutral polymer was essentially phosphate free and contained rhamnose and *N*-acetylglucosamine in the ratio 3:1. The *N*-acetylglucosamine was completely released by exo-β-*N*-acetylglucosaminidase and identified as such. Reduction with $NaBH_4$ did not affect the amount of rhamnose or of *N*-acetylglucosamine in the neutral polymer, but periodate destroyed about 30% of the rhamnose and 70% of the *N*-acetylglucosamine. The *N*-acetylglucosamine must be terminal since it is released by exo-*N*-acetylglucosaminidase. The absence of $NaBH_4$-reducible rhamnose no doubt stems from persistence of a link, probably glycosidic in nature, that ties the rhamnose polymer to the degraded muramic acid (or to an as yet undetected intervening molecule).

The trirhamnose block is formulated with α-1,3 linkage on the basis of the periodate results and of earlier reports (Estrada-Parra *et al.*, 1963; Heymann *et al.*, 1963, 1964). The residue of *N*-acetylglucosamine might be attached at C-2 or C-4 of one of the three residues, and in view of the finding of 2,3-disubstituted rhamnose in C polysaccharide (Heymann *et al.*, 1963) one would favor position 2. The periodate data also make it unlikely that the trirhamnose-*N*-acetylglucosamine block occurs as a repeating unit, because in that case less than one-third of the rhamnose would be destroyed by the oxidizing agent. The group A biose and group A triose, which contain *N*-acetylglucosamine in β-1,3 linkage attached to rhamnose or to rhamnosyl-α-1,3-rhamnose, could not have arisen from the fragment discussed here, but would have come from other portions of the C polysaccharide (Heymann *et al.*, 1964). These other portions are represented, at least in part, by fractions $S_{2-A'-2}$ (Figure 8) and $S_{2-A''}$ (Figure 7); at present it is known that these fractions contain phosphomonoester groups, rhamnose, glucosamine, and glycerol.

STRUCTURE OF COMPLEX S_2. The observations set forth lead us to a representation of complex S_2 that appears in Figure 9. About 70% of the peptide subunits of *S. pyogenes* walls is present as di-L-Ala-linked dimers, *i.e.*, 175 mμequiv of dimer/mg of walls. F_1 endo-*N*-acetylmuramidase completely splits the glycan backbone and from each of the peptide dimers one disaccharide, GlcNAc-MurNAc, is readily released by amidase. The other L-alanine amino terminus of the peptide dimer is attached to an *N*-acetylmuramic acid residue by a linkage poorly susceptible to amidase. This *N*-acetylmuramic acid residue, which is not muramic acid 6-phosphate, is the anchor point of the linkage that ties the C polysaccharide to the peptidoglycan. *N*-Acetylmuramic acid may be directly linked to rhamnose, or there may be an intervening molecule. The *N*-acetylglucosamine-substituted trirhamnose block appears as an attractive possibility for a bridge between the peptidoglycan and the bulk of the C polysaccharide. An internal position for the block $Rham_3$-GlcNAc is preferred because if it were externally located at a nonreducing end of the C-polysaccharide–peptidoglycan complex, the last residue should be periodate labile; this was not found to be so by Estrada-Parra *et al.* (1963). The remainder of the C polysaccharide contains the previously described constituents, rhamnose, glucosamine, glycerol, and phosphate, and is linked to the trirhamnose block by an alkali-labile bond, possibly phosphodiester. About 70% of the total cell wall phosphate is found in the alkali-dissociable portion of the C polysaccharide.

It should be understood that the rhamnose–glucosamine–glycerol phosphate moiety appearing in Figure 9 as part of complex S_2 is representative of the C-

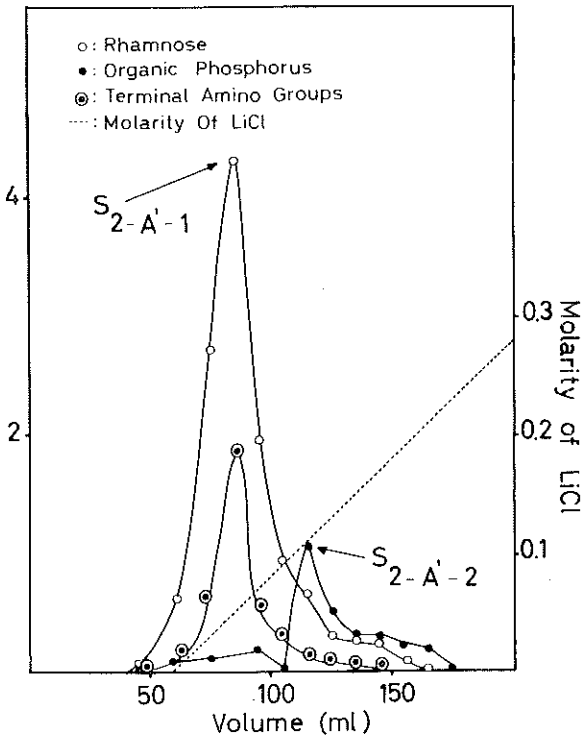

FIGURE 8: Purification of the alkali-treated fraction S_{2-A}' (see Figures 6 and 7) on ECTEOLA-cellulose. S_{2-A}' (15 mg) was applied, in water, to a 2 × 40 cm column of ECTEOLA prepared as described in the text. Water (150 ml) was passed through, and then a gradient of increasing LiCl concentration: mixing vessel, 150 ml of water; reservoir, 150 ml of 0.5 M LiCl (pH 5). Aliquots of 100 μl were used to determine terminal amino groups and organic phosphate. Bound rhamnose was determined on 200-μl aliquots. Determination of total amino sugars on 40-μl aliquots parallel those of bound rhamnose. Results are expressed in μmoles/5-ml fraction of effluent.

[3] The acidic property of the peptide compound(s) might result from a deamidation of the isoglutamine residue under the strong alkaline conditions which were used for the degradation. Treatment under identical conditions of the isolated *S. pyogenes* disaccharide peptide monomer (Muñoz *et al.*, 1966a) also produced an acidic peptide material.

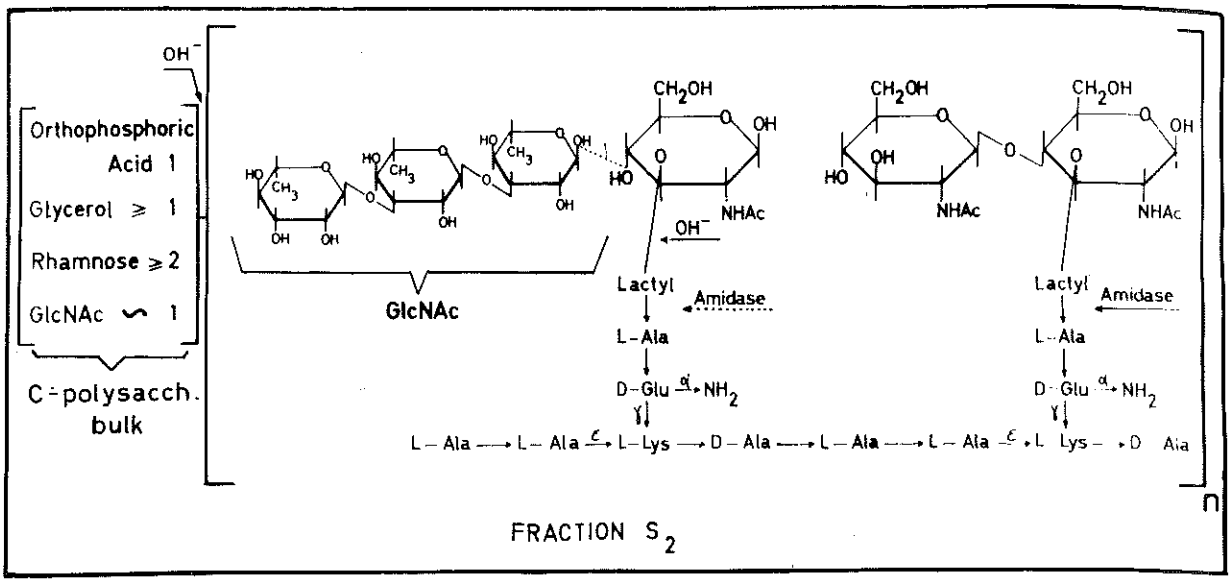

FIGURE 9: Proposed structure for the major polymer present in fraction S_2. A dimeric peptide unit is substituted at one of the amino termini by a β-(1,4)-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide and at the other terminus by an *N*-acetylmuramic acid residue glycosidically linked to a trirhamnosyl fragment of the C polysaccharide. One *N*-acetylglucosamine residue (GlcNAc) is attached by a β-glycosidic bond to the trirhamnosyl block. Phosphate groups occur within the inner portions of the C-polysaccharide moiety.

polysaccharide bulk to which the peptidoglycan subunit dimers are linked. The composition appearing in Figure 9 reflects the stoichiometry of the constituents, calculated relative to 2 moles of glutamic acid. The figure does not necessarily imply that there are repeating units of that composition, nor does it have any other structural implications regarding the bulk of the C-polysaccharide.

Discussion

The present studies (see structure of complexes S_3 and S_2 in parts II and III of the Experimental Section) led us to propose for a major part of the cell walls of *S. pyogenes* a structure of the type represented in Figure 10. Short glycan strands are composed of five *N*-acetylhexosamine residues with the sequence MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc. The *N*-acetylmuramic acid residues at the nonreducing end of these chains are glycosidically linked at C-4, either directly or through an as yet undetermined compound, to the reducing ends of tri-α-1,3-L-rhamnosyl fragments which extend from the C-polysaccharide bulk. *N*-Acetylglucosamine residues are attached through β-glycosidic linkages to the C-2 position within the trirhamnose units. Phosphodiester groupings, representing about 70% of the total phosphate residues of the walls, occur within the interior of the C-polysaccharide bulk as glycerol phosphate and glyceryl rhamnoside phosphate (Heymann *et al.*, 1967). In about 30% of the glycan strands, some *N*-acetylmuramic acid residues are substituted on C-6 by phosphate groups, and these link the peptidoglycan to a second polymer which contains glucose, glucosamine, and unidentified hexosamine compounds. It is proposed to designate this hitherto unrecognized polymer as G polysaccharide. The *N*-acetylmuramic acid 6-phosphate groupings represent about 20% of the total phosphate groups in the cell walls. Unsubstituted β-(1,4)-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides occur in about 70% of the glycan strands. The remaining *N*-acetylmuramic acid residues are substituted by tetrapeptides to which the sequence N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine was assigned (Muñoz *et al.*, 1966a). Most of the tetrapeptides are cross-linked by L-alanyl-L-alanine dipeptides extending from the N^{ϵ}-lysine residue of one peptide to the carboxyl group of the D-alanine residue of another (Muñoz *et al.*, 1966a). Thus, neighbouring glycan strands are cross-linked by decapeptide bridges. As shown in Figure 10, one decapeptide is present for every two glycan chains, linking the *N*-acetylmuramic acid residue at the nonreducing end of one chain to a similar residue in the interior of a second one.

It should be understood that Figure 10 represents only one of the possibilities considered here; the phosphorylated *N*-acetylmuramic acid residues need not be terminally located but could be in the interior of the glycan strands, and the chain length of 5 given for the glycan portion may well be an average resulting from the presence of longer or shorter chains.

The proposed network structure takes into account the nature of the products arising from the action of F_1 endo-*N*-acetylmuramidase, as well as the yields in which they are obtained, *i.e.*, complexes S_2 and S_3 and disaccharide units in fraction S_4. The seemingly

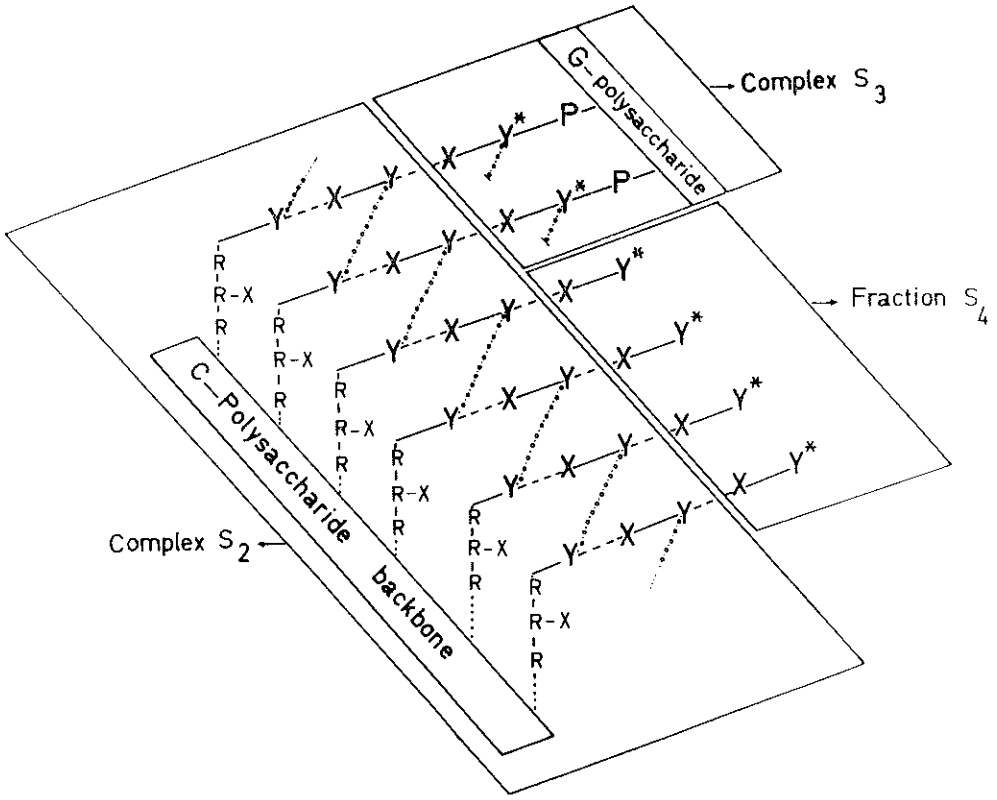

FIGURE 10: Tentative schematic structure of cell walls of *S. pyogenes*. (Only one alternative is shown.) X = *N*-acetylglucosamine; Y = *N*-acetylmuramic acid; Y* = *N*-acetylmuramic acid at the reducing end of the glycan chains; R = L-rhamnose; P = orthophosphoric acid; (– – – –) glycosidic linkage hydrolyzed by the *Streptomyces* F_1 endo-*N*-acetylmuramidase; (O–O–O–O) uncross-linked tetrapeptides; (O–O–O–O–O–O–O–O–O–O) decapeptide bridges resulting from the cross-linking of two tetrapeptides through L-Ala-L-Ala dipeptides. Hydrolysis by the F_1 enzyme liberates polymer S_2, polymer S_3, and disaccharide units in yields close to those experimentally found.

incomplete splitting of the glycan backbone which one observes when reducing power or Morgan–Elson color are used to measure the extent of hydrolysis (Muñoz *et al.*, 1966a,b) can now be understood in the light of the diminished reactivity of that *N*-acetylmuramic acid residue to which the C polysaccharide is linked. Moreover, the above structure provides evidence for the existence of a continuous peptidoglycan network in which only one peptide bridge occurs per two polysaccharide chains. Finally, it explains the poor yield of disaccharide peptide monomers produced by the sequential degradation of the walls with *Streptomyces* SA endopeptidase and *Streptomyces* F_1-endo-*N*-acetylmuramidase. According to the scheme of Figure 10 only 33% of the total tetrapeptide units present in the walls would be obtainable as disaccharide peptide monomers; the actual yield after purification was 16% (Muñoz *et al.*, 1966a).

The proposed structure does not take into account the material contained in fraction S_1 (Figure 1). As stated, this fraction contains only about 10% of the wall material. Preliminary assays have shown that it probably consists of C polysaccharide linked to tetramer peptidoglycan fragments, rather than to dimer fragments as in complex S_2.

According to the proposed structure, the wall peptidoglycan contains 1.5 *N*-acetylmuramic acid residues/*N*-acetylglucosamine residue. Experimental evidence for the existence of such a ratio has not yet been adduced because large amounts of glucosamine and other hexosamine compounds are present in the nonpeptidoglycan portions of the walls, and have not yet been cleanly dissociated from the peptidoglycan.

The existence in *S. pyogenes* cell walls of a polysaccharide complex distinct from C polysaccharide (*i.e.*, G polysaccharide in fraction S_3; see Figure 5) was not expected, although the occurrence of glucose in unspecified amounts has been reported for one strain of group A *Streptococcus* (Roberts and Stewart, 1961). The unidentified hexosamine residues in S_3 are neutral at pH 5 on paper electrophoresis and might be aminohexuronic acids or related compounds. It should be noted, as shown in Table I, that fraction S_3 has been preferentially enriched in the nonpeptidoglycan amino acids which were detected as trace contaminants in trypsin-treated cell walls. It is therefore suggested that

the G polysaccharide might be the moiety to which the proteins M, R, and T are linked in the native walls.

The structural interdependence of the C polysaccharide and the peptidoglycan in *S. pyogenes* cell walls, as described here and in previous reports (McCarty, 1952; Salton, 1953; Krause and McCarty, 1961), may be of biological importance. Recent studies (Panos and Cohen, 1966) suggested, for example, that the inability of stable L forms of group A *Streptococcus* to form polyrhamnose may be related to the lack of cell wall biosynthesis. Recent studies of the nonpeptidoglycan components of bacterial cell walls (for reviews, see Ghuysen *et al.*, 1967b; Lüderitz *et al.*, 1967), give increasing support to the view that these structures result from the covalent association among various polymeric components.

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