Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. VII. Mode of Action of the Bacteriolytic Peptidase from *Myxobacter* and the Isolation of Intact Cell Wall Polysaccharides*

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ABSTRACT: The mode of action of the lytic peptidase isolated from culture filtrates of *Myxobacterium* strain AL-1 has been studied by kinetic analysis of end groups liberated during hydrolysis and by fractionation and analysis of the products of its action on isolated cell walls of *Staphylococcus aureus* and *Arthrobacter crystallopoietes*. The *Myxobacter* enzyme solubilizes these walls by a primary attack on their peptide cross-bridges (pentaglycine in the case of *S. aureus* and L-alanine in the case of *A. crystallopoietes*) which interlink peptidoglycan strands. These bridges are attacked at their linkage to the D-alanyl terminus of the basal tetrapeptide (L-Ala-γ-D-Glu(α-$CONH_2$)-L-Lys-D-Ala) which is found in both of these peptidoglycans. Hydrolysis of the *N*-acetylmuramyl-L-alanine linkages between the polysaccharide and the peptides occurs at a slower rate in both cases, with eventual liberation of small peptide fragments, undegraded polysaccharide, and a polysaccharide–teichoic acid complex. The periodate insensitivity and chemical analyses of the polysaccharides are consistent with the structure of alternating β-1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, predicted from previous analyses of disaccharides isolated from the *S. aureus* polysaccharide. Analyses show the polysaccharides to be polydisperse with average chain lengths of 19–25 hexosamine residues but containing molecules as short as 12 and as long as 70–100 residues.

The polysaccharide is relatively insensitive to egg white lysozyme and other endo-*N*-acetylmuramidases, but it is rapidly attacked by an endo-*N*-acetylglucosaminidase.

In the course of a study of the morphogenetic cycle of *Arthrobacter crystallopoietes*, an organism identified as a *Myxobacter* and designated as AL-1 was isolated which produced an extracellular enzyme capable of lysing cells or cell walls of the *Arthrobacter*. Further study indicated that this enzyme had a broader substrate specificity and was also able to lyse cell walls of *Staphylococcus aureus* and some other bacteria. The enzyme has been purified 632-fold to the point of homogeneity by physical criteria. The purified enzyme is also a proteinase; no separation of proteinase and cell wall lytic activities was detected during purification (Ensign and Wolfe, 1965, 1966).

Recent studies on the mode of action of enzymes able to lyse bacterial cell walls have shown that in every case the substrate is the cell wall peptidoglycan,[1] a polymer containing *N*-acetylglucosamine, *N*-acetylmuramic acid, and a few amino acids. This structure is found in all bacterial cell walls, although it is unlikely that its components are assembled in exactly the same way in different organisms.

The hexosamines, acetylglucosamine and acetylmuramic acid, are linked together in a polysaccharide in which the strict alternation of the two sugars is ensured by the biosynthetic mechanism (Anderson *et al.*, 1965). In *S. aureus*, all of the acetylmuramic acid residues of the polysaccharide are linked through their carboxyl group to peptide subunits which are themselves cross-linked by polyglycine bridges. It is generally assumed that all cell wall peptidoglycans are built on a similar pattern, each with a polysaccharide backbone linked in a three-dimensional mesh by a polypeptide of variable structure.

Although the disaccharides which comprise the poly-

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[1] From both the structural and biosynthetic points of view, the muramic acid containing polymer in the cell wall is a polysaccharide (glycan) substituted by peptide subunits. These peptides are cross-linked to a variable extent, subsequent to the polymerization of the polysaccharide. This polymer is, therefore, referred to as a peptidoglycan (a peptide-substituted polysaccharide). However, cleavage of the polysaccharide to disaccharides with an endoacetylmuramidase leaves a soluble polymer in which disaccharide units are attached to a polypeptide. This material is, therefore, a glycopeptide (an oligosaccharide-substituted polypeptide).

saccharide have been obtained from several microorganisms, the intact polysaccharide, free of peptide, has not previously been isolated. The purpose of the present paper is to describe the mode of action of the *Myxobacter* enzyme and to describe its use in isolating this polysaccharide from the cell walls of *S. aureus* and *A. crystallopoietes*. In addition, the isolation of the carbohydrate-free peptides resulting from this lysis will be described.

Materials and Methods

Cell walls. Cell walls of *S. aureus* and *A. crystallopoietes* (spherical form) were prepared as described previously (Ghuysen and Strominger, 1963; Ensign and Wolfe, 1965). The *S. aureus* cell walls were prepared from post-log-phase cells and care was taken during their mechanical disintegration to keep the temperature low, and the pH at about 5. After the first wash, they were heated for 20 min at pH 5 in a boiling water bath to inactivate autolytic enzymes. After isolation as an acetone-dried powder, the cell walls of *S. aureus* were digested for 2 hr with trypsin and RNase in 0.05 M phosphate buffer, pH 7.4, extensively washed with water, and lyophilized.

Enzymes. The purification of the *Myxobacter* enzyme has been described (Ensign and Wolfe, 1965, 1966). The B enzyme from *Chalaropsis* (Hash, 1963; Tipper *et al.*, 1964b), an endoacetylmuramidase, was a gift from Dr. J. Hash. The 32 and F1 acetylmuramidases from *Streptomyces albus* (Ghuysen *et al.*, 1962; Muñoz *et al.*, 1966a) were gifts from Dr. J.-M. Ghuysen, and the *Micrococcus* endoacetylglucosaminidase (π enzyme) (Browder *et al.*, 1965; Tipper and Strominger, 1966) was a gift from Mead Johnson and Co. Crystalline egg white lysozyme was a commercial preparation (Armour and Co.).

Ion-Exchange Chromatography and Gel Filtration. Column chromatography was carried out on ECTEOLA-cellulose (Bio-Rad Cellex E), CM-cellulose (Bio-Rad Cellex C), and Sephadex G-25 and G-50, fine grade (Pharmacia, Inc.). Columns of varying size were operated at room temperature. The ECTEOLA- and CM-cellulose columns were washed extensively with 0.5 M LiCl before use, and then washed with water until the eluate was salt free. Mixtures were applied in water or dilute buffer. Unretarded materials were eluted with several bed volumes of water; then retarded materials were eluted with a linear gradient of increasing concentration of LiCl up to 0.7 M. Sephadex columns were eluted with water.

Optical Rotation. A Rudolph Model 200 photoelectric polarimeter was used with a 1-dm microcell of 1.8-ml capacity and solutions at a concentration of 10 mg/ml.

Analytical Procedures. Total phosphate, reducing groups, NH_2- and COOH-terminal amino acids, amino sugars, and free amino groups were measured as previously described (Ghuysen and Strominger, 1963; Ghuysen *et al.*, 1966). Amino acids in acid hydrolysates were determined using a Beckman-Spinco amino acid analyzer, or by quantitative thin layer chromatography of the dinitrophenyl (DNP) derivatives as recently described (Ghuysen *et al.*, 1965, 1966). Glucosamine was measured using the specific glucosamine 6-phosphate *N*-acetylase of yeast (Lüderitz *et al.*, 1964).

Reduction, De-O-acetylation, and Periodate Oxidation of Polysaccharide Samples. Samples (2 μmoles of total hexosamines) of polysaccharide fractions were reduced for 6 hr at room temperature in fresh, unbuffered 0.2 M sodium borohydride solutions (30 μl). The solutions were then heated at 60° for 30 min. This procedure resulted in quantitative reduction and de-*O*-acetylation of 4-*O*-β-*N*-acetylglucosaminyl-*N*- and 6-*O*-diacetylmuramic acids (Tipper *et al.*, 1965). The solutions were then adjusted to pH 4.5 by the addition of 2.0 M acetic acid (6 μl). Unreduced controls contained identical polysaccharide samples in 36 μl of a mixture of five volumes of 0.2 M $NaBH_4$ with one volume of 2.0 M acetic acid, pH 4.5.

Aliquots (9 μl, 0.5 μmole of total hexosamines) of the polysaccharide solutions were oxidized in 20 μl of 0.01 M $NaIO_4$ at room temperature in the dark. Aliquots (1 μl) were diluted to 100 μl, and periodate consumption was measured by the decrease in absorbancy at 224 mμ, using the differential extinction coefficient for periodate and iodate of 8730 (Dixon and Lipkin, 1954), verified by controls containing known amounts of periodate, with and without excess ethylene glycol. Formaldehyde was measured in aliquots (2 μl). Oxidation of *N*-acetylglucosaminitol, under the same conditions, served as a standard for formaldehyde production.

Results

Determination of End Groups Liberated from Cell Walls and Other Substrates during Lysis. Cell walls of *S. aureus* (0.3 mg) and *A. crystallopoietes* (0.4 mg) were incubated with *Myxobacter* enzyme (9 μg) in 72 μl of 0.01 M sodium barbital buffer, pH 8.9, at 37°. Aliquots were removed at intervals for the determination of liberated reducing power and free amino groups. In neither case could significant release of reducing power be detected, while free amino groups were liberated in substantial amounts (Table I). NH_2-terminal amino acids were determined after incubation for 18 hr (Table I). The high initial NH_2-terminal alanine content of the *S. aureus* cell walls is due to the ester-linked D-alanine of their teichoic acid. Lysis of these walls was accompanied by the release of NH_2-terminal glycine and alanine (1.2 and 0.7 moles/mole of total glutamic acid, respectively). Lysis of *A. crystallopoietes* cell walls was accompanied by the release of 2.2 moles of NH_2-terminal alanine/mole of total glutamic acid.

Action of the *Chalaropsis* B (Tipper *et al.*, 1964b) or *Streptomyces* 32 (Ghuysen and Strominger, 1963) endoacetylmuramidase on the cell wall of *S. aureus* yields a soluble glycopeptide[1] in which disaccharide units are linked amidically to the L-alanine of the polypeptide (tetrapeptide units cross-linked by polyglycine bridges) (Ghuysen *et al.*, 1965). Action of the *Myxobacter* enzyme on this substrate resulted

TABLE I: NH_2-Terminal Amino Acids Produced by the Action of *Myxobacter* Enzyme on Various Substrates.[a]

Substrate		Alanine	Glycine	Total
A. crystallopoietes cell walls	Control	0.27	0	0.27
	Digested	2.47	0.05	2.52
	Δ	2.20	0.05	2.25
S. aureus cell walls	Control	0.80	0.17	0.97
	Digested	1.50	1.34	2.84
	Δ	0.70	1.17	1.87
S. aureus soluble glycopeptide obtained with *Chalaropsis* B enzyme	Control	0.15	0.08	0.23
	Digested	0.28	1.52	1.80
	Δ	0.13	1.44	1.57

[a] Cell wall controls were incubated without enzyme and gave identical analyses before and after 18-hr incubation. The values for NH_2-terminal glycine are minimal values (*cf.* legend to Figure 1). Data are expressed as moles per mole of total glutamic acid in the various substrates.

in the liberation of NH_2-terminal glycine at a rate similar to that from intact cell walls. However, the amount of NH_2-terminal alanine released from this substrate was extremely small (Table I).

Kinetics of Liberation of NH_2- and COOH-Terminal Groups from the Cell Walls of S. aureus and A. crystallopoietes. The native cell walls of *S. aureus* are not suitable for determination of liberated COOH-terminal amino acids because the ester-linked alanine of the teichoic acid is liberated as free alanine during hydrazinolysis, presumably by base-catalyzed hydrolysis. These ester-linked alanine residues were, therefore, removed by prior mild alkaline hydrolysis. *S. aureus* cell walls (2 g) were suspended in 0.1 M sodium pyrophosphate, pH 9.2, at 37°. At intervals, aliquots (16 μl) were removed and centrifuged, and the total free amino groups in the supernatant solutions was measured. A total of 0.33 mmole was liberated, one-half of this being released at about 20 min. After 6 hr, the cells were washed with water several times by centrifugation and lyophilized. Suspensions of these ester alanine-free *S. aureus* cell walls and of *A. crystallopoietes* cell walls (containing 0.27 and 0.40 μmole of total glutamic acid, respectively), each in 180 μl of 0.02 M sodium barbital buffer, pH 9.0, were digested with *Myxobacter* enzyme (20 μg). Analyses of these substrates are shown in Table II. At intervals, aliquots (22 μl) were removed and analyzed for free NH_2- and COOH-terminal amino acids (Figure 1) for the *S. aureus* digest and the *A. crystallopoietes* digest (Figure 2).

Determination of the Configuration of the Liberated COOH-Terminal Alanine. Aliquots (22 μl) of the above cell wall digests were taken after 24 hr, lyophilized, and subjected to hydrazinolysis. The free amino acids in the aqueous phase after extraction with benzaldehyde were fractionated on a column of Dowex 50 (H^+ form) as described by Ghuysen *et al.* (1966). The eluted alanine peak was assayed enzymatically for D- and L-alanine, and found in both cases to be almost entirely D-alanine. This indicates that the *Myxobacter* enzyme, in both cell walls, had hydrolyzed the peptide link between the COOH-terminal D-alanine of the tetrapeptide subunit (*cf.* Ghuysen *et al.*, 1965) and the NH-terminal end of the interpeptide bridge.

Determination of the Configuration of the NH_2-Terminal Alanine Liberated during Lysis of the Cell Walls The basic peptide fractions, whose isolation from cel

TABLE II: Composition of Cell Walls.[a]

	A. crystallopoietes	*S. aureus* (ester alanine free)
Lysine	0.89	1.0
Ammonia	1.2	1.1
Glutamic acid	1.0	1.0
Glycine	0.12	5.1
Alanine	2.4	2.1
Glucosamine	1.8	2.1
Galactosamine	0.31	0
Muramic acid	0.85	1.0

[a] Samples (2 mg) of cell walls were hydrolyzed in 4 N HCl (40 μl) for 8 hr at 100°. After dilution with the appropriate buffer, samples were applied directly to the columns of a Beckman-Spinco amino acid analyzer. The *S. aureus* walls contained 0.47 mμmole of total glutamic acid/mg. A correction was made for hydrolytic loss of muramic acid. The *A. crystallopoietes* cell walls contained 0.42 μmole of total glutamic acid/mg, and also contained about 0.03 μmole of aspartic acid, threonine, serine, leucine, histidine, and methionine/mg. Data are expressed as moles per mole of glutamic acid.

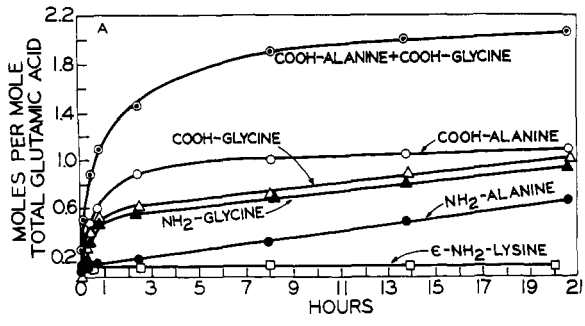


FIGURE 1: Kinetics of the release of NH_2-terminal and COOH-terminal amino acids during the lysis of ester alanine-free *S. aureus* cell walls by *Myxobacter* enzyme. Incubations were carried out as described in the text. Aliquots were acidified with acetic acid to pH 5, boiled to inactive the *Myxobacter* enzyme, and then digested with *Chalaropsis* B enzyme (0.5 mg) for 2 hr at 37°. This solubilized incompletely digested cell walls, and was necessary to ensure quantitative reaction in subsequent end-group determinations. The *Chalaropsis* B enzyme is devoid of peptidase activity (Tipper *et al.*, 1964b). The upper curve, the sum of COOH-terminal glycine and alanine, is presumed to be equal to the total bridge peptide bonds broken. This curve should, therefore, be identical with that for the release of NH_2-terminal glycine. In the procedure used for determination of NH_2-terminal amino acids, tetra- and triglycine yield only about 40 and 60%, respectively, of the theoretical amount of DNP-glycine. No correction for this was made in determining total NH_2-terminal glycine, and the plotted data are, therefore, only about 50% of the true values.

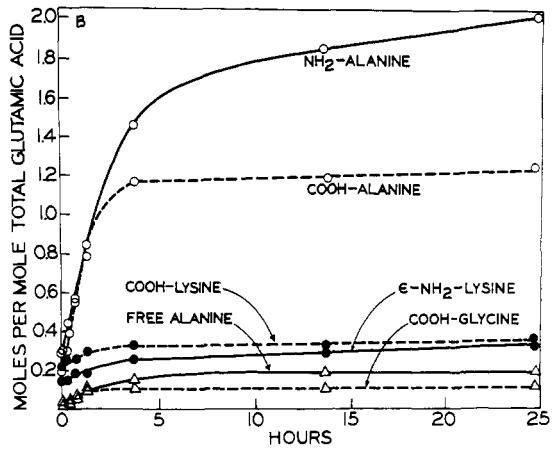


FIGURE 2. Kinetics of the release of NH_2-terminal and COOH-terminal amino acids during the lysis of *A. crystallopoietes* cell walls by *Myxobacter* enzyme. Incubation and determinations were carried out as described in Figure 1. The figures for COOH-alanine,[5] but not those for NH_2-alanine, include the 0.2 mole of free alanine liberated during the digestion.

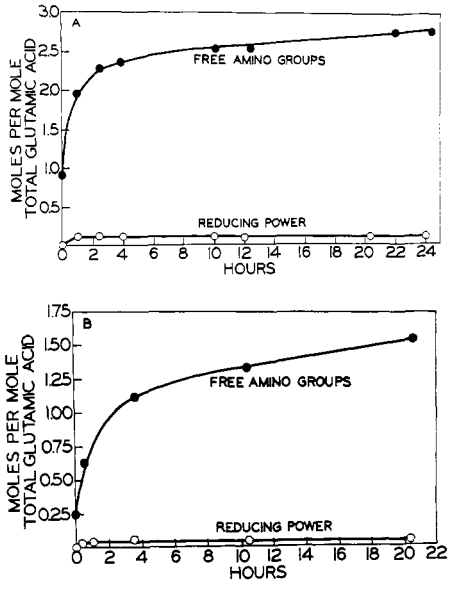


FIGURE 3: Release of total NH_2-terminal groups and reducing power during the digestion of untreated cell walls with *Myxobacter* enzyme. (A) Digestion of *S. aureus* cell walls (365 mg). (B) Digestion of *A. crystallopoietes* cell walls (307 mg). Details of the incubations are given in the text. Free amino groups were determined directly from aliquots (0.27 μl) of the incubation mixtures. After dinitrophenylation, the mixtures were hydrolyzed to solubilize all the peptide derivatives present. The high intrinsic content of free amino groups in the cell walls of *S. aureus* is due to ester-linked alanine. Reducing power was determined on supernatants of diluted aliquots (0.6 μl) after centrifugation to remove insoluble material.

walls lysed with the *Myxobacter* enzyme is described below, contain most of the lysine, glutamic acid, and alanine of the original cell walls (with the exception of the ester-linked D-alanine removed earlier). Samples (32 μg) of the basic peptides from both *S. aureus* and *A. crystallopoietes* cell walls were dinitrophenylated in 1% borate (20 μl) for 60 min at 60°, acidified with concentrated HCl (11 μl), and hydrolyzed for 9 hr at 95°. Identical samples of the peptides were similarly hydrolyzed without prior dinitrophenylation. The DNP derivatives were extracted from the hydrolysates with ether and quantitated by thin layer chromatography. The residues were lyophilized and aliquots were fractionated on Dowex 50 (H^+ form) as described in the previous section. Aliquots of the eluted alanine peak were analyzed enzymatically for D- and L-alanine. Other aliquots of the residues from ether extraction were analyzed by a Beckman-Spinco amino acid analyzer for total amino acids. The *S. aureus* peptide contained, per mole of glutamic acid, 2.16 moles of total alanine, 1.06 mole of D-alanine, and 1.08 mole of L-alanine. It also contained 1 mole of lysine and 1.8 moles of glycine. After dinitrophenylation it gave rise to 1.04 mole of DNP-glycine and 1.04 mole of

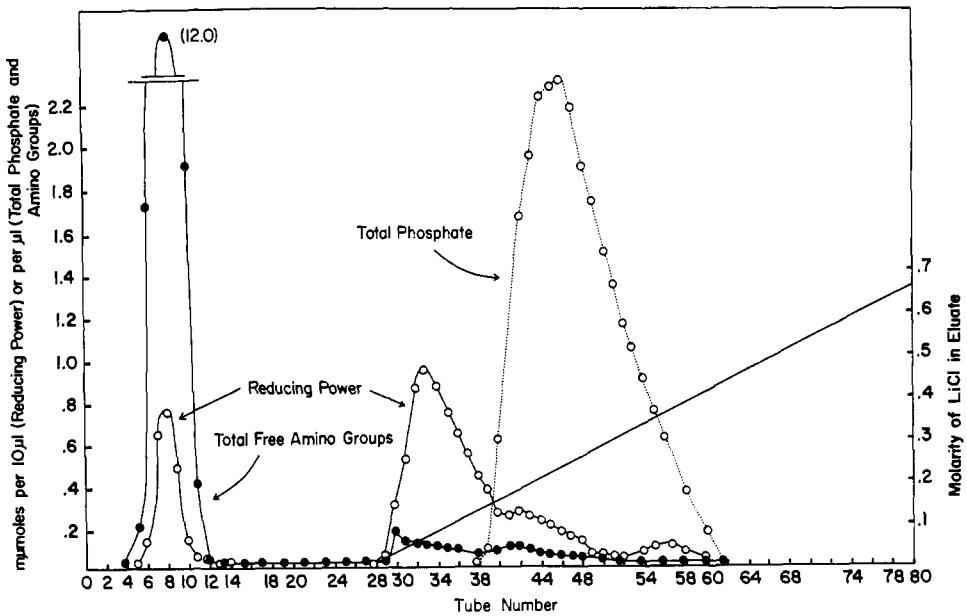


FIGURE 4: Fractionation of *S. aureus* cell wall digest on ECTEOLA-cellulose. The entire digest was applied to a column of ECTEOLA-cellulose (23 × 3 cm), previously washed as described in the text. The column was eluted at 1 ml/min, first with water and then with a gradient of LiCl, as indicated. Fractions (15 ml) were analyzed for reducing power, free amino groups, and total phosphate. LiCl was first detected (by precipitation with $AgNO_3$) in tube 28, and the LiCl concentration in the subsequent fractions was calculated from the volume delivered from the reservoir. The scale for reducing power in this figure and also in Figure 5 is ten times that for phosphate or amino groups. The aliquots for reducing power, total free amino groups, and total phosphate determinations were 26, 3, and 8 μl, respectively. The color yields are not affected by the presence of 0.5 M LiCl in the aliquots. Tubes 4–13 inclusive, 28–38 inclusive, and 39–64 inclusive were pooled, and are referred to in the text as fractions 1, 2, and 3, respectively.

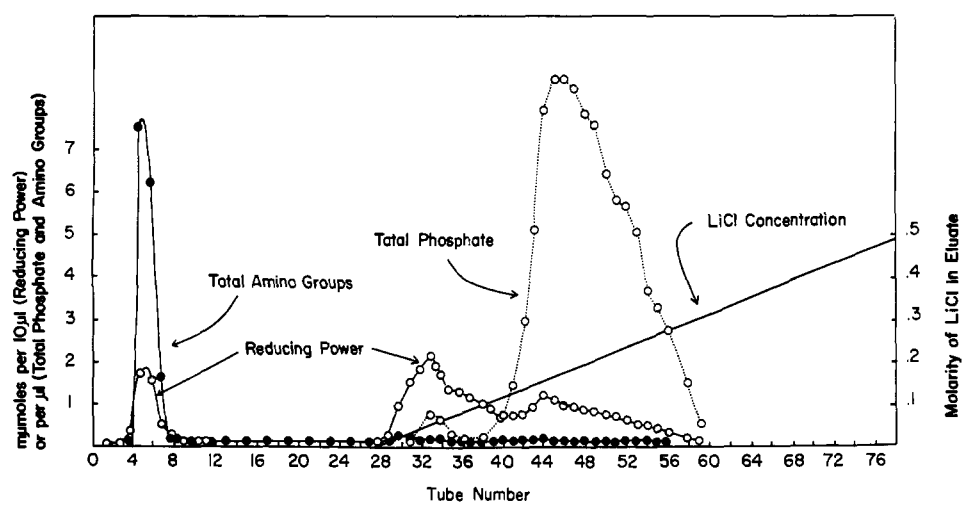


FIGURE 5: Fractionation of *A. crystallopoietes* cell wall digest on ECTEOLA-cellulose. The procedure was identical with that described in Figure 4. The column was first eluted with water, and then with a linear gradient of increasing LiCl concentration, with 350 ml of water initially in the mixing vessel, and 350 ml of 0.5 M LiCl initially in the reservoir. Tubes 3–10 inclusive, 29–39 inclusive, and 40–62 inclusive were pooled, and are called fractions 1, 2, and 3, respectively.

DNP-alanine, while 0.9 mole of D-alanine and 0.15 mole of L-alanine remained undinitrophenylated. The NH_2-terminal alanine is thus L-alanine.

The *Arthrobacter* peptide contained, per mole of glutamic acid, 2.1 moles of total alanine, 1.8 moles of L-alanine, and 0.25 mole of D-alanine. After dinitrophenylation, 1.7 moles of DNP-alanine were produced; 0.3 mole of total alanine remained and no

detectable L-alanine remained undinitrophenylated.

Large-Scale Digestion of Cell Walls of S. aureus and A. crystallopoietes. S. aureus cell walls (345 mg) were treated with *Myxobacter* enzyme (0.46 mg) in 7 ml of 0.02 M sodium barbital buffer, initially at pH 8.9. The pH rapidly dropped as lysis visibly proceeded, and was readjusted over a period of 4 hr by the addition of 1.0 N NaOH (total, 0.5 ml), after which the pH remained virtually constant. The release of amino groups and reducing power was determined on aliquots (Figure 3A). The digestion was terminated after 21 hr.

A. crystallopoietes cell walls (307 mg) were digested with *Myxobacter* enzyme (1.2 mg) in 6 ml of 0.02 M sodium barbital, initially at pH 8.9. As before, the pH dropped rapidly, and was maintained at about 8.9 by additions of 1.0 N NaOH (total, 0.18 ml). The release of reducing groups and amino groups was determined in aliquots (Figure 3B). The digestion was terminated after 21 hr.

Fractionation of the Digests on ECTEOLA-cellulose. Each digest was neutralized with 1.0 N HCl to pH 5.5, diluted to 15 ml to reduce the salt concentration to about 0.04 M, and fractionated on a column of ECTEOLA-cellulose, as described in Figures 4 and 5. Aliquots were removed for determination of reducing power, free amino groups, and total phosphate. The two elution patterns are remarkably similar, indicative of similarity in the proportions and charges of the various types of fragments in the two digests. In each case, almost all of the material with free amino groups was eluted with water; this fraction also contained material with reducing power (peptide and polysaccharide, fraction 1). A second peak of reducing material (polysaccharide, fraction 2) was eluted at low LiCl concentrations, and tailed into the peak of organic phosphate (polysaccharide–teichoic acid complex, fraction 3), eluted at higher LiCl concentrations. There was no contamination of the major part of these polysaccharide peaks by phosphate in either case. The three fractions were obtained in each case by pooling the appropriate tubes. The virtual absence of amino groups in fraction 3 indicated that complete hydrolysis of the ester-linked alanine of the teichoic acid had occurred during the incubation.

Desalting of the Fractions and Separation of the Peptide and Polysaccharide Components of Fraction 1. All of the fractions were lyophilized. Fractions 2 and 3 were desalted by extraction with 100% ethanol. After vigorous mixing with ethanol, the suspensions were kept at −10° for several hours, and then centrifuged. The remaining traces of salt in the pellets were removed by gel filtration on a column of Sephadex G-25 (medium grade). The fractions were assayed for reducing power, total phosphate, and free amino groups. Chloride ion was detected with silver nitrate solution. The elution pattern for the polysaccharide in the *S. aureus* fraction 2 is illustrated in Figure 6 (top). The polysaccharide in this peak is called SP 2.

Fraction 1, eluted from ECTEOLA-cellulose by water, was expected to contain all of the free alanine removed from the teichoic acid during the incubation at pH 9, as well as all of the free peptide. This material was lyophilized, redissolved in water (2 ml), and applied to a similar column of Sephadex G-25 (Figure 6, bottom). A clear separation of material with reducing power from material with free amino groups was achieved. This latter material includes all the free

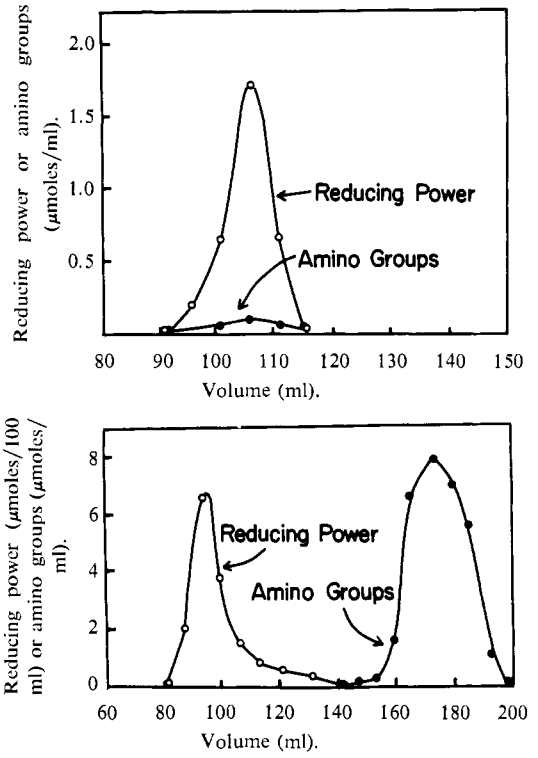


FIGURE 6: Fractionation of fractions 1 and 2 from ECTEOLA chromatography of the *S. aureus* digest on Sephadex G-25. (top) (fraction 2) After extraction of the lyophilized material with ethanol (see text), the residue was dissolved in water (2 ml) and applied to a column (2 × 80 cm) of Sephadex G-25. The column was eluted with water at 0.5 ml/min and fractions of 5 ml were collected. The aliquots for reducing power, free amino groups, and total phosphate determination were 15, 2, and 5 μl, respectively. No phosphate was detectable. This column had a V_0 of 105 ml and a V_i of 115 ml. The reducing power peak eluted from 90 to 115 ml was pooled and lyophilized and is called SP 2. (bottom) (fraction 1) The lyophilized material was dissolved in water (2 ml) and applied to a column (2 × 60 cm) of Sephadex G-25, V_0 85 ml and V_i 95 ml. The aliquots for reducing power, total free amino groups, and total phosphate determinations were 18, 18, and 10 μl, respectively. No phosphate was detectable. The scale for reducing power is 100 times that for free amino groups. The peak of reducing power eluted between 80 and 140 ml was pooled and is referred to as SP 2. The peak of free amino groups eluted between 140 and 200 ml includes the neutral peptides, basic peptides, and free alanine from the digest.

alanine, neutral peptides (molecular weight about 200), and basic peptides (molecular weight about 540, but retarded by ionic interaction with the gel) from the cell wall digests.

The material with reducing power is polysaccharide (SP 1) of shorter chain length and higher peptide content (See below) than that eluted with LiCl. These differences probably account for its lower affinity for ECTEOLA-cellulose.

The total hexosamine content of the polysaccharide fractions SP 1 (20 μmoles) and SP 2 (140 μmoles) was 50% of the theoretical total (324 μmoles) of the glycan which would be contained in 345 mg of *S. aureus* strain Copenhagen cell walls. This theoretical figure is based on a molecular weight of 2080 for the repeating unit of the cell wall, with the formula (Glu), (NH_3), (Lys), $(Ala)_3$, $(Gly)_5$, $(GlcNAc)_3$,[2] (MurNAc), $(acetate)_{0.6}$, $(phosphate)_2$, and $(ribitol)_2$. However, the hexosamine content of the teichoic acid fraction (420 μmoles) was considerably greater than the theoretical figure (324 μmoles). The difference is due to polysaccharide linked to the teichoic acid. The over-all recovery of hexosamines was thus 90%.

Similar results were obtained with fractions 1–3 from *Arthrobacter*. The yield of polysaccharide (AP 2) from fraction 2 was 120 μmoles of total hexosamines. In this case, the acidic polysaccharide and basic peptide in fraction 1 were separated on a column of CM-cellulose (Li form), equilibrated with water. The polysaccharide (AP 1), together with some free alanine, was eluted with water, and then the basic peptides were eluted with 0.3 M LiCl. Using the same column, the neutral and basic components of the peptide fraction from *S. aureus*, eluted after SP 1 from Sephadex G-25 (Figure 6), were separated. The neutral peptides were eluted with water and the basic peptides with 0.3 M LiCl. The basic peptide fractions were lyophilized and then desalted by extraction with 100% ethanol. Their net positive charge at the pH of chromatography on CM-cellulose (5.5) is due to the presence of amide ammonia on the α-carboxyl groups of their glutamic acid residues (Tipper *et al.*, 1967).

Analyses of the Polysaccharide Fractions. Analysis of both fractions from *S. aureus* (SP 1 and SP 2) and of the major fraction from *Arthrobacter* (AP 2) have been carried out (Table III). These fractions did not contain measurable phosphate and, therefore, were not contaminated by teichoic acid. SP 1 contained about 0.3 peptide subunit/disaccharide. SP 2 contained 0.08 peptide subunit and AP 2 about 0.16 peptide subunit/disaccharide. The original walls in both cases contained one peptide subunit per disaccharide. There is one free amino group per peptide residue present, indicating that only one of the two susceptible linkages in each remaining peptide residue had been cleaved. Each fraction contained one-half as much glucosamine (determined enzymatically) as total hexosamine,

TABLE III: Analyses of Polysaccharide Fractions.[a]

	SP 1	SP 2	Redigested SP 2	AP 2
Hexosamines	100	100	100	100
Glucosamine	63	52	–	52
Muramic acid	–	46	–	–
Reducing power	30	6.9	3.7	5.7
O-Acetyl groups	–	8.0	–	0
Glutamic acid	15	3.7	1.7	8.0
Glycine	47.5	6.8	3.4	–
Alanine	25	9.0	2.7	16.4
Lysine	14.2	2.7	1.6	8.3
Free amino groups	7.9	2.2	–	9.2

[a] Total hexosamines and glucosamine were determined on samples hydrolyzed for 6 hr at 100° in 4 N HCl and neutralized. Glucosamine was determined enzymatically. Amino acids were determined by quantitative thin layer chromatography of dinitrophenyl derivatives. The hexosamine and amino acid contents of SP 2 were also determined using a Beckman-Spinco amino acid analyzer, which gave results in agreement with the other procedures. *O*-Acetyl groups were determined colorimetrically by hydroxamate formation. Data are expressed as moles/100 moles of total hexosamines.

and the ratios of total hexosamine to reducing power were 3.3, 16.5, and 17.5, respectively. However, experience with the disaccharides *N*-acetylmuramyl-*N*-acetylglucosamine (D3, Tipper and Strominger, 1966) and *N*-acetylglucosaminyl-*N*-acetylmuramic acid (D1, Tipper *et al.*, 1965) indicated that this ratio is not necessarily a reliable guide to chain length, since the reducing powers of the two disaccharides, relative to equimolar amounts of acetylglucosamine, are 0.7 and 1.5, respectively. The higher figure is due to the unusual alkali lability of the glycosidic linkage in the second disaccharide.

Periodate Oxidation of the Reduced and Unreduced Polysaccharides. Reduced and unreduced samples of polysaccharides were oxidized in 0.01 M periodate at pH 4.5 so that periodate consumption could be assessed. Although formaldehyde was produced slowly from unreduced SP 2 (1.4 mole/100 moles of total hexosamine at 18 hr), production from reduced SP 2 (4.3 moles/100 moles of hexosamine) was virtually complete in 10 min, and increased only slowly thereafter. The periodate consumption at 18 hr was 2.7 and 7.1 moles/100 moles of total hexosamine for SP 2 and reduced SP 2, respectively, the difference being equal to the formaldehyde production. The average chain length of SP 2 is, therefore, 23 hexosamines. The periodate consumption by unreduced SP 2 after 18 hr is, therefore, only 0.62 mole/mole of polysaccharide, demonstrating the perio-

[2] Abbreviations used: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; TCA, trichloroacetic acid.

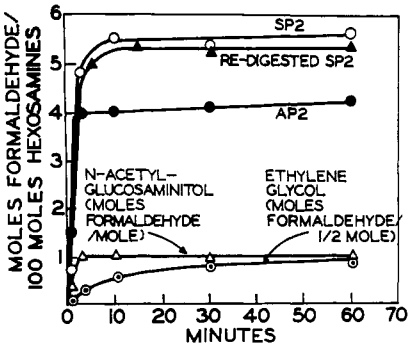


FIGURE 7: Release of formaldehyde during periodate oxidation of reduced samples of SP 2, redigested SP 2, and AP 2 polysaccharides. Aliquots of polysaccharide solutions (*ca.* 0.5 μmole of total hexosamines) were reduced and de-*O*-acetylated as described in the text. Standards were similarly reduced solutions of *N*-acetylglucosamine (25 mμmoles) and ethylene glycol (12 mμmoles). These are difference curves for reduced and unreduced samples of the polysaccharides. The curves for formaldehyde production from *N*-acetylglucosaminitol and ethylene glycol are plotted on a scale such that 1 mole represents 100% oxidation.

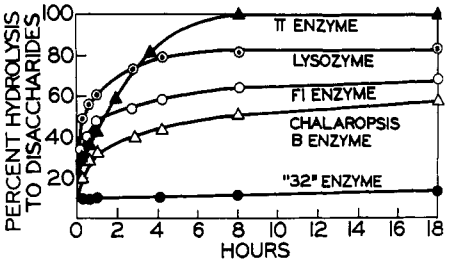


FIGURE 8: Kinetics of hydrolysis of *S. aureus* polysaccharide by endo-*N*-acetylhexosaminidases. SP 2 (41 μg) was incubated at 37° with lysozyme (19 μg) in 38.4 μl of ammonium acetate buffer, pH 6.8, and with F1 enzyme (19 μg), *Chalaropsis* B enzyme (19 μg), or 32 enzyme (15 μg) at pH 4.5 in 38.4 μl of 0.01 M sodium acetate buffer. Aliquots (1.9 μl) were removed at intervals for determination of reducing power, and the extent of hydrolysis to disaccharides was estimated from the ratio of reducing power to total hexosamine, which is 0.75 for the *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides. SP 2 (71.3 μg) was incubated with 0.46 μg of the π enzyme in 61.5 μl of 0.01 M phosphate, pH 7.5. Aliquots (6.3 μl) were removed for reducing power determination and the extent of hydrolysis to disaccharides was again calculated from the ratio of reducing power to total hexosamine, which is 0.36 for the *N*-acetylmuramyl-*N*-acetylglucosamine disaccharides. Note that the amount of π enzyme employed was only 2–3% of that of the *N*-acetylmuramidoses.

date insensitivity of its nonterminal hexosamine residues.

Similarly, the formaldehyde production (5 moles/100 hexosamines, complete in 10 min) and periodate consumption (12.5 moles/100 moles of hexosamines, at 18 hr) of reduced AP 2 indicate an average chain length of 20 hexosamines and demonstrate the periodate insensitivity of its internal hexosamine residues.

Oxidation of reduced polysaccharides in 0.001 M periodate at pH 4.5 and room temperature was employed to obtain data on the rate of production of formaldehyde from their *N*-acetylhexosaminitol termini. These data were compared with the kinetics of formaldehyde production from model compounds (Figure 7). The curves of formaldehyde production from the reduced polysaccharides and from *N*-acetylglucosaminitol were parallel and reached a plateau long before the oxidation of ethylene glycol was complete. Thus the plateau levels could be extrapolated to give estimates of formaldehyde production from reduced terminal groups. These figures should be considerably more accurate than those derived from the initial experiments described above. At this low concentration of periodate, insignificant oxidation of *N*-acetyl-β-glucosaminide residues would occur during the 2-hr duration of the experiments, so kinetic measurements of periodate consumption were not made. Residual periodate was determined at the end of the experiments by measurement of the change in optical density at 224 mμ after the addition of an excess of ethylene glycol and incubating for a further hour at room temperature. In all cases at least 50% of the initial periodate remained after 2 hr, so that the cessation of formaldehyde production was not due to exhaustion of the oxidant. The production of formaldehyde from the unreduced samples was less than 12% of that from the reduced samples in all cases, and showed similar kinetics, being complete within 10 min. This was assumed to be derived from impurities, and was subtracted from the data, which are presented in Figure 7. Extrapolation of the plateaus to zero time gives values for the chain lengths of SP 2 and AP 2 of 18 and 25 hexosamines, respectively.

Measurements of Specific Rotation. The specific rotation at equilibrium of SP 2 was −41.5° (*c* 0.6, water) and that of AP 2 was −52.3° (*c* 0.6, water). The specific rotation of the *S. aureus* peptide was −1.0° and hence the small amount of residual peptide on the polysaccharides could not contribute to the observed rotations. The reason for the difference in these specific rotations is unknown.

Enzymatic Susceptibility of the S. aureus Polysaccharide. The isolated *S. aureus* polysaccharide is an extremely poor substrate for the endoacetylmuramidases which hydrolyze it when it is part of the cell wall peptidoglycan. It can be extensively hydrolyzed by lysozyme in high concentrations, but the action of concentrations of *Streptomyces* F-1 enzyme and *Chalaropsis* B enzyme sufficient to lyse suspensions of *S. aureus* cell walls in a few minutes remains incomplete even after 12 hr at 37°. *Streptomyces* "32"

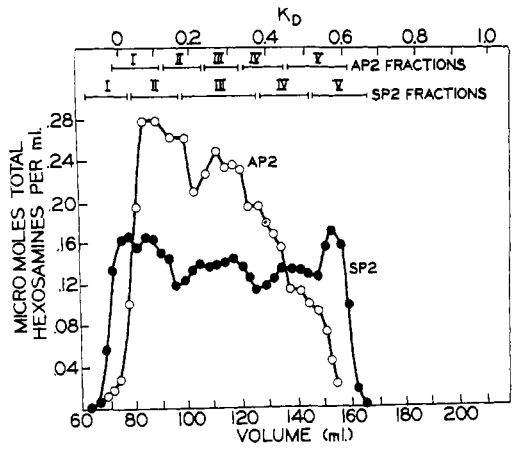


FIGURE 9: Fractionation of SP 2 and AP 2 on Sephadex G-50. SP 2 (65 μmoles of total hexosamine) or AP 2 (15 μmoles of total hexosamine) in water solution (2 ml) was applied to a column of Sephadex G-50 (fine grade, bead form). V_0 (75 ml.) was defined as the elution volume of Blue Dextran 2000 (mol wt 10^6, Pharmacia) and V_i (140 ml) was defined as the additional volume of eluent required to elute DNP-glutamic acid. $K_d = (V_e - V_0)/V_i$, where V_e is the elution volume. The column was eluted with water at 0.3 ml/min and 3-ml fractions were collected. Aliquots (60 μl) were hydrolyzed for determination of total hexosamines in each fraction. The scale for SP 2 has been reduced by a factor of 5.

acetylmuramidase is almost without action on the isolated polysaccharide. The cell wall polysaccharide has also been isolated from autolysates of *Bacillus subtilis* and shown to be relatively insusceptible to lysozyme (Young *et al.*, 1964). In contrast, the isolated polysaccharide is completely and rapidly hydrolyzed to disaccharides by very small amounts of the *Micrococcus* endoacetylglucosaminidase (π enzyme) (Figure 8)

The course of the hydrolysis by lysozyme and the *Micrococcus* enzyme was also followed by thin layer chromatography on boric acid buffered silica gel G in methyl ethyl ketone–acetic acid–water (28:10:12), using *p*-anisaldehyde–sulfuric acid as a detection reagent. *N*-Acetylhexosamine derivatives are much less reactive with this reagent than hexoses, but give colored spots on heating at 150° for 10–15 min. SP 2 (240 μg) was incubated with 41 μg of lysozyme at 37° in 31.3 μl of 0.01 M acetate (pH 6.8), or with 4.1 μg of *Micrococcus* π enzyme at 37° in 31.3 μl of 0.01 M phosphate (pH 7.5). Aliquots (6 μl) were removed, boiled for 3 min, and applied to the thin layer. After 60 min, the *Micrococcus* digest contained only material with the mobility of acetylmuramylacetylglucosamine. The amount of this material increased with time, while that of the other visible component, with a mobility of 0.81 relative to the disaccharide, first increased and then decreased. Both components increased with time in the lysozyme digest, and it is possible that the slower moving component is a tetrasaccharide, similar to the tetrasaccharide derived from lysozyme digests of *Micrococcus lysodeikticus* cell walls (Leyh-Bouille *et al.*, 1966). Thus it is possible that the π enzyme can remove both terminal disaccharides and tetrasaccharides from the polysaccharide, and subsequently degrade the latter to disaccharides. This indicates that the enzyme is not exclusively an exoenzyme. Oligosaccharides with mobility slower than the tetrasaccharide react too weakly with the anisaldehyde reagent to be detected. The ability of these high concentrations of lysozyme to produce tetrasaccharide by transglycosylation (Sharon and Seifter, 1964) prevents any similar deduction on its mode of action.

Redigestion of SP 2 with the Myxobacter Enzyme. Polysaccharide fraction SP 2 still contained about 8% of the peptide subunits to which it was initially linked. SP 2 (40 μmoles of total hexosamines) was incubated at 37° in 2.2 ml of 0.01 M sodium barbital, pH 9.0, containing 0.2 mg of *Myxobacter* enzyme. Additional *Myxobacter* enzyme (0.4 mg) was added at 4 hr. Free amino groups in the digest doubled in the first 4 min of incubation, and then remained constant. After 24 hr, the digest was neutralized to pH 6 with 2 N HCl (15 μl) and fractionated on a column (2.6 × 25 cm) of CM-cellulose. Polysaccharide (36 μmoles of total hexosamines) was eluted by water in a single peak.

The analyses of the polysaccharide eluted by water are presented in Table III. It now contained 0.034 peptide subunit/disaccharide. Thus, a small portion of the peptide subunits appears to be extremely resistant to removal by the *Myxobacter* enzyme. This redigested SP 2 fraction was reduced and oxidized with 0.001 M periodate for the determination of its average chain length, as described above for SP 2. The kinetics of formaldehyde production yields a number-average chain length of 19 hexosamine residues, virtually identical with that obtained for SP 2.

Sephadex G-50 Fractionation of SP 2 and AP 2. Samples of SP 2 and AP 2 (65 and 14 μmoles of total hexosamines respectively) were fractionated on Sephadex G-50 (Figure 9). The tubes were grouped into five fractions, 1–5 (Figure 9). The solutions were lyophilized and the materials were redissolved in water (1 ml). The mean elution volume (V_e), calculated K_d value, and total hexosamine content of each fraction are presented in Table IV. The total recovery of hexosamine from SP 2 and AP 2 was 63.8 and 12.2 μmoles, respectively (98 and 87% yield). The chain lengths of the fractions were determined by periodate oxidation (Table IV). Determination of periodate uptake showed that at least 70% of the periodate was unused in all cases by the termination of the experiment (2 hr). The number-average chain length of each fraction was calculated from the ratio of total hexosamines to formaldehyde produced after reduction (Table IV). As can be seen, the polysaccharide fractions SP 2 and AP 2 are polydisperse and include materials with number-average chain lengths from 12 to 80. Calculation of the chain lengths of the original mixtures from the data in Table IV gives 25 for SP 2 and 33 for AP 2.

TABLE IV: Fractions of SP 2 and AP 2 from Sephadex G-50 Fractionation.[a]

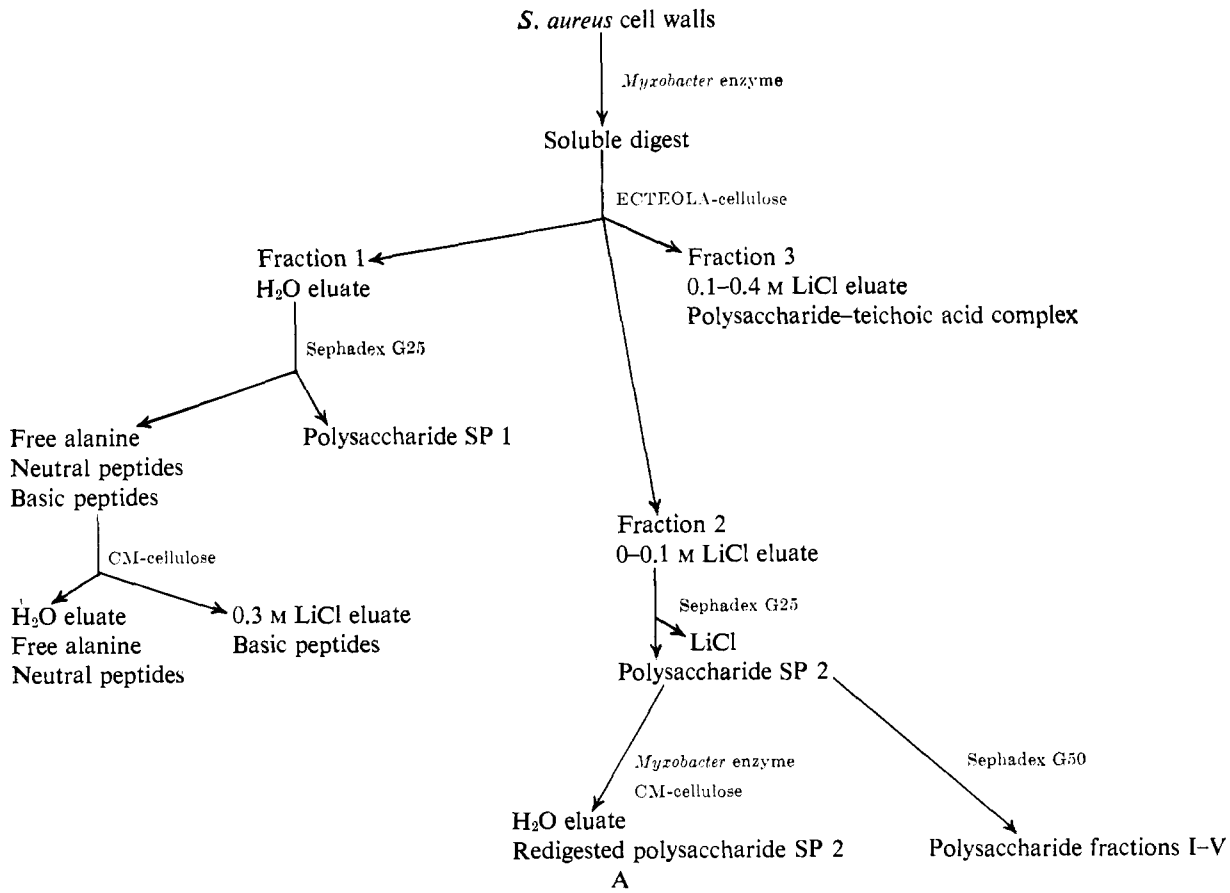
Fraction	Mean V_e (ml)	Mean K_d	Hexosamine Content (μmoles)	% of Total Hexosamines	Moles of Formaldehyde/100 Moles of Hexosamine	No.-Av Chain Length
			SP 2			
I	75	0	8	12.3	1.1-1.3	75-90
II	90	0.11	13.6	20.7	2.2-2.4	40-45
III	114	0.28	21.9	33.3	3.3-3.5	30
IV	137	0.66	12.0	18.3	5.6-5.9	18
V	155	0.66	10.1	15.4	8.0-8.5	12
			AP 2			
I	83	0.06	2.96	24.4	1.5-1.8	55-65
II	100	0.17	3.25	26.7	2.0-2.5	40-50
III	115	0.29	2.64	21.7	2.6-3.0	33-38
IV	129	0.39	2.08	17.1	3.6-4.0	25-27
V	145	0.50	1.24	10.1	7.8-8.4	12

[a] The number-average chain lengths (final column) are the reciprocals of the moles of formaldehyde/100 moles of hexosamine, produced during oxidation of samples of the fractions, with and without prior reduction, in 0.001 M periodate at pH 4.5. These data are given with and without subtraction of the values for the unreduced controls (penultimate column). In each case, the kinetics of formaldehyde production from the samples were identical with those in Figure 7, with maximum production within 10 min.

Discussion

The Mode of Action of the Myxobacter Enzyme on S. aureus Cell Walls. Kinetic analysis of the peptide terminal groups released during digestion of ester alanine-free *S. aureus* cell walls with *Myxobacter* enzyme is presented in Figure 1. Only traces of free alanine were detected at the termination of the experiment, demonstrating the efficiency of the prior removal of ester-linked D-alanine. Digestion resulted in the rapid appearance of NH_2-terminal glycine, COOH-terminal glycine, and COOH-terminal alanine. This COOH-terminal alanine was almost exclusively of the D configuration, and so all these end groups resulted from hydrolysis within the interpeptide bridges. The release of NH_2-terminal alanine was slower, and mostly occurred subsequent to the release of COOH-terminal amino acids. This NH_2-terminal alanine must, therefore, be derived from hydrolysis of the *N*-acetylmuramyl-L-alanine linkages (amidase action), since the COOH-terminal *N*-acetylmuramic acid liberated by this amidase action is not detected by the hydrazinolysis procedure. This was confirmed by analysis of the peptides fractionated from the large-scale digestion of *S. aureus* cell walls with the *Myxobacter* enzyme (see below), since these peptides were free of hexosamines, and their NH_2-terminal alanine was almost exclusively of the L configuration. These data show that bridge-splitting precedes amidase action, which was still incomplete at the termination of this experiment. The fractionation of the large-scale *Myxobacter* enzyme digest of *S. aureus* cell walls is summarized in Figure 10A. The polysaccharide–teichoic acid complex, like the polysaccharide fractions, contained very little peptide and thus the *Myxobacter* enzyme liberates virtually all of the peptide from the polysaccharide moiety of the peptidoglycan by cleavage of the *N*-acetylmuramyl-L-alanine linkages. Examination of the peptide fraction has confirmed the fact that the *Myxobacter* enzyme had also catalyzed the hydrolysis of an average of two peptide bonds in every interpeptide bridge, as was indicated by the kinetic analysis (Figure 1) which showed the appearance of one mole each of COOH-terminal glycine and alanine per peptide subunit Fractionation of these peptides[3] has revealed that they contain triglycine, a hexapeptide (containing the basal tetrapeptide, L-Ala-D-γ-Glu-(α-$CONH_2$)-L-Lys-D-Ala, plus two glycine residues), tetraglycine and a pentapeptide (containing the basal tetrapeptide, plus one glycine residue). The pentapeptide and hexapeptide were positively charged at pH 8.0, and each contained 1 mole of NH_2-terminal alanine 1 mole of NH_2-terminal glycine, and no free ϵ-amino groups of lysine.[3] Thus, both the kinetic data and the analysis of these peptides indicate that

[3] The neutral peptides and basic peptides were separated as described in Methods. Further fractionation and characterization of the basic peptides was carried out by D. Jarvis (manuscript in preparation). The neutral peptides were separated from free D-alanine and fractionated into di-, tri-, and tetraglycine (obtained in amounts of 0.1, 0.5, and 0.45 mole/mole of total glutamic acid in the cell walls, respectively) by chromatography on Dowex 50. The glycyl peptides were characterized by paper and thin layer chromatography, paper electrophoresis at pH 1.9, and analyses of total and terminal amino acids. Details will be described in a future publication.



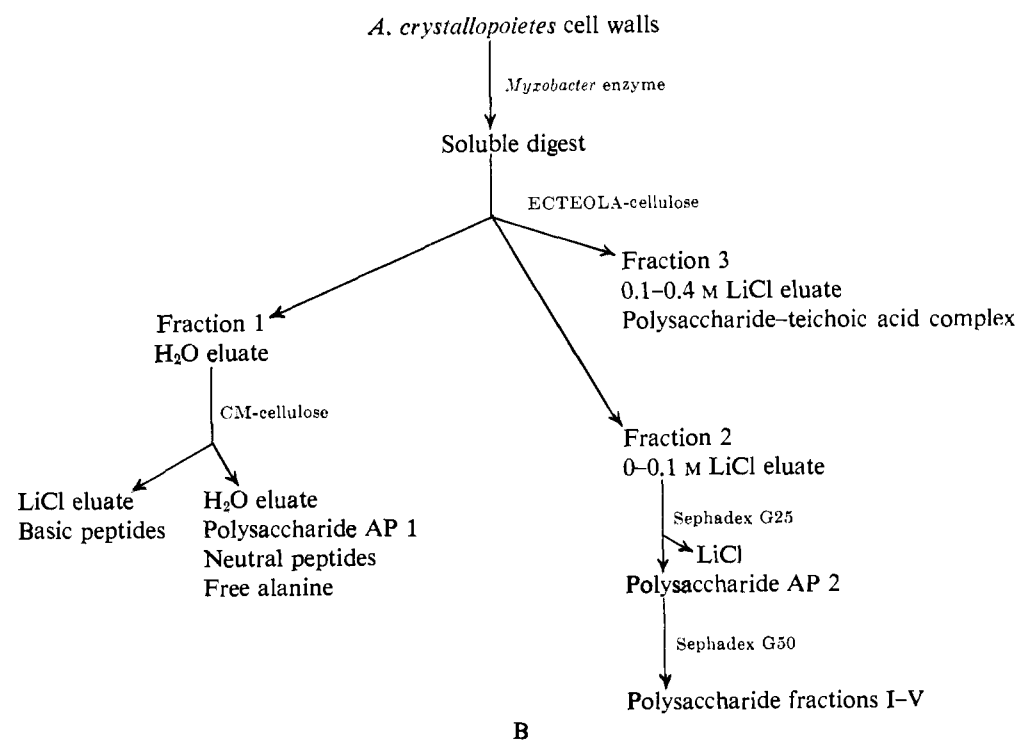


 FIGURE 10: Fractionation scheme for the *Myxobacter* digest of (A) *S. aureus* and (B) *A. crystallopoietes* cell walls.

the *Myxobacter* enzyme catalyzes the cleavage of the peptidoglycan of *S. aureus* at the three positions indicated in Figure 11A.

The kinetic data (Figure 1) show, moreover that the *Myxobacter* enzyme catalyzes the release of both COOH-terminal alanine and glycine at a rapid rate until about one bond per glutamic acid residue has been hydrolyzed, at which time the cell walls are completely solubilized. Thereafter, COOH-terminal alanine continues to be released rapidly. The release of COOH-terminal glycine continues more slowly, however, and is only complete at 21 hr. It is concluded that the *Myxobacter* enzyme had catalyzed the rapid hydrolysis of D-alanylglycine (arrow 1, Figure 11A) and glycylglycine (arrow 2, Figure 11A) linkages in the interpeptide bridges and that the former reaction continued at a relatively rapid rate after the production of one break per polyglycine bridge, while the latter reaction continued at a much slower rate.

The demonstration of the presence of ammonia on the α-carboxyl groups of glutamic acid in the cell walls of *S. aureus* (Tipper and Strominger, 1965; Tipper *et al.*, 1967; Muñoz *et al.*, 1966b) has precluded this position for the NH_2-terminal end of the pentaglycine bridges. The presence of D-alanylglycine linkages in *S. aureus* cell walls is now directly demonstrated by the production of COOH-terminal D-alanine and NH_2-glycine on lysis of these walls with the *Myxobacter* enzyme. The same result has also been obtained with the SA endopeptidase from *S. albus* G (Petit *et al*, 1966; Muñoz *et al.*, 1966b) and with the staphylolytic L_{11} enzyme from *Flavobacterium* (Kato *et al.*, 1962; K. Kato and J. L. Strominger, in preparation, 1967)

The kinetics of NH_2-terminal L-alanine release (Figure 1) demonstrate that amidase action (arrow 3, Figure 11A) is slow relative to bridge splitting, and largely occurs after solubilization. It may be that the *Myxobacter* enzyme can only attack the *N*-acetylmuramyl-L-alanine linkage after the wall structure has been opened up by breakage of the peptide cross-links. If so, then the amidase action of the enzyme is nonlytic, like the amidase from *S. albus*, which only acts after lysis of cell walls with an endoacetylmuramidase (Ghuysen *et al.*, 1962). By contrast with the *S. albus* amidase, the *Myxobacter* enzyme is almost without effect on these amide linkages after lysis of the walls with an endoacetylmuramidase[4] (Table I). The autolytic enzyme of *B. subtilis* (168 I^-C^+) is an example of a lytic amidase (Young *et al.*, 1964) and a similar enzyme causes the autolysis of cell walls of *S. aureus* strain Copenhagen (D. J. Tipper, unpublished observations).

Structure of the Peptidoglycan in the Cell Wall of A. crystallopoietes and the Mode of Its Degradation by the Myxobacter Enzyme. Arthrobacter are Gram-positive organisms which characteristically undergo a change in morphology from sphere to rod in response to certain nutrients (Ensign and Wolfe, 1964). The cell wall peptidoglycan of members of this species can contain either lysine or diaminopimelic acid. The particular strain used in these studies, *A. crystallopoietes*, is one of the lysine-containing group (Table II). The cell walls were prepared from organisms growing exclusively as spheres, as previously described (Ensign and Wolfe, 1965). The data reported here on the chemistry of these walls are still provisional, and are included to illustrate the mode of action of the *Myxobacter* enzyme and to provide a second example of the isolation of an intact cell wall polysaccharide. The structures of the peptidoglycans in both rod and spherical form organisms are being extensively studied in our laboratories by Krulwich (1966). The spherical form cell walls had 1 mole of peptide subunit/mole of acetylmuramic acid (Table II).

Kinetic analysis of the peptide terminal groups released during digestion of *A. crystallopoietes* cell walls with *Myxobacter* enzyme are presented in Figure 2. Digestion resulted in the parallel liberation of both COOH- and NH_2-terminal alanine, and occurred rapidly until both had increased by about 1 mole/mole of glutamic acid. This COOH-terminal alanine was mostly of the D configuration,[5] and so resulted from hydrolysis within the interpeptide bridges. The walls were completely solubilized after 1 hr of digestion. The release of NH_2-terminal alanine then continued, without further appearance of COOH-terminal amino acids and as in the *S. aureus* digest, presumably arose from splitting of *N*-acetylmuramyl-L-alanine linkages. The fractionation of the large-scale *Myxobacter* enzyme digest of these walls is summarized in Figure 10B. The nature of the phosphate-containing polymer has not been investigated, but it has the same chromatographic behavior on ECTEOLA-cellulose as the polysaccharide–teichoic acid complex from *S. aureus* (see Figures 4 and 5) and has been shown to contain sugars with the mobilities of glucose and glucosamine by acid hydrolysis and paper chromatography. It is presumed to be a teichoic acid–polysaccharide complex. This fraction and the polysaccharide fractions, AP 1 and AP 2, contain more peptide (0.16 subunit/disaccharide) than the corresponding *S. aureus* fractions. The basic peptide fraction is free of hexosamines, and the majority of the *N*-acetylmuramyl-L-alanine linkages in the cell walls have, therefore, been hydrolyzed. The hexosamine-free peptides contained approximately 2 moles of NH_2-terminal L-alanine/mole of glutamic acid. Edman degradation of these peptides yielded, after one cycle,

[4] The amidase action of lysostaphin is similarly inhibited by predigestion of *S. aureus* cell walls with an endoacetylmuramidase.

[5] Some free D-alanine was also liberated during this digestion, which was also accompanied by an increase in COOH-terminal lysine (Figure 2), indicative of some carboxypeptidase action. This may explain the low content of D-alanine in the isolated peptides, but a discrepancy remains between the sum of free alanine and the D-alanine in these peptides, and the total COOH-terminal alanine measured in the digest. This discrepancy is being further investigated.

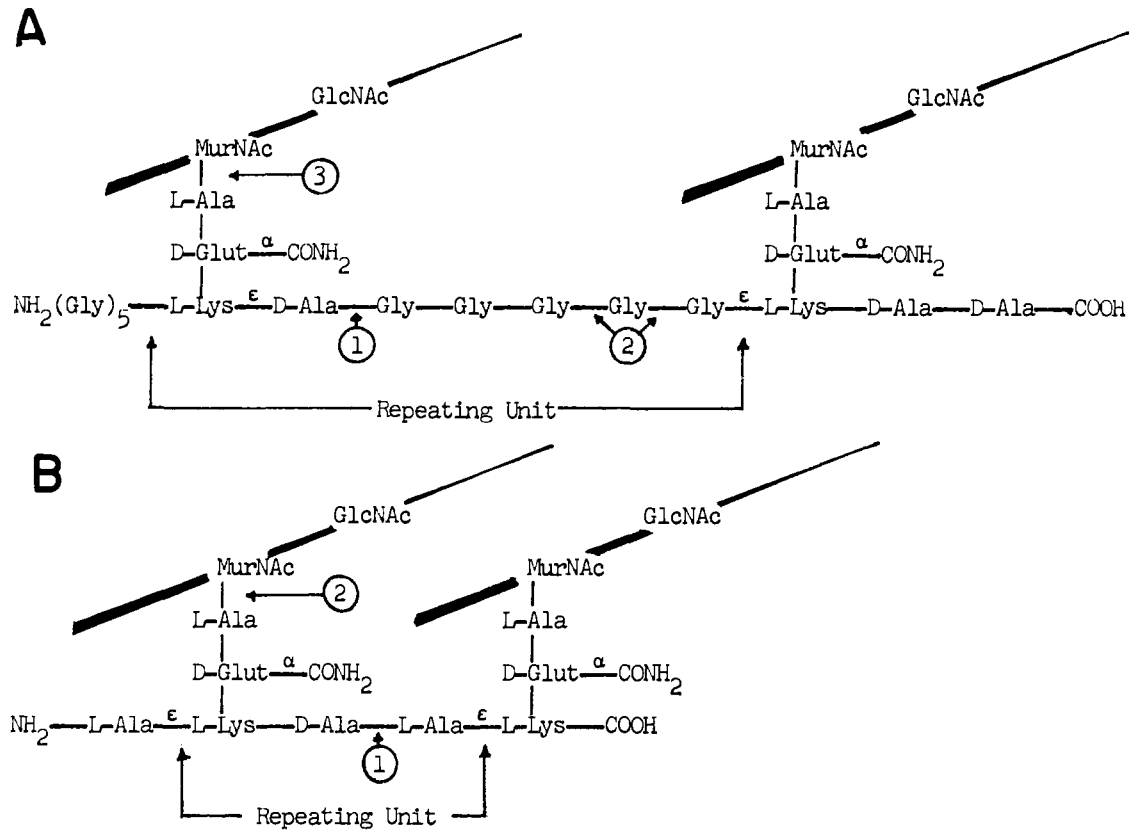


FIGURE 11: Structures of the peptidoglycans in the cell walls of (A) *S. aureus* and (B) *A. crystallopoietes*. The interpeptide bridge peptides are pentaglycine in the former and L-alanine in the latter structure. The most common NH_2- and COOH-terminal groups are represented. The γ-carboxyl group of glutamic acid is linked to the α-amino group of lysine in both structures.

approximately 0.8 mole each of NH_2-terminal glutamic acid and ϵ-NH_2-terminal lysine, with disappearance of both of the NH_2-terminal L-alanine residues (Tipper *et al.*, 1967). These data indicate that one of the L-alanine residues is attached to the ϵ-NH_2 group of lysine in the isolated peptides, and presumably formed an interpeptide bridge in the peptidoglycan consisting of a single L-alanine residue. Solubilization of the cell walls by the *Myxobacter* enzyme probably involves an initial attack on these bridges as indicated in Figure 11B (arrow 1), followed by amidase action (arrow 2).

While this paper was in preparation, a series of papers dealing with the isolation and mode of action of lytic enzymes from *Sorangium* sp. appeared (Whitaker, 1965; Whitaker *et al.*, 1965; Tsai *et al.*, 1965). These workers isolated two peptidases from the culture supernatant which were lytic for cells and peptidoglycan preparations of *Arthrobacter globiformis* and *M. lysodeikticus*. Analysis of the products of degradation of *M. lysodeikticus* peptidoglycan led to the conclusion that both enzymes cleave *N*-acetylmuramyl-L-alanine linkages and peptide cross-linkages. By consideration also of the nature of the action of these enzymes on insulin, it was concluded that they have a specificity for linkages in which the carbonyl donor is a neutral amino acid of D or L configuration, or glycine. The carbonyl donors of cell wall peptide bonds known to be split by the *Myxobacter* enzyme are glycyl, D-alanyl, and D-lactyl. The amino donors are glycine and L-alanine. Thus, the specificity of the *Myxobacter* enzyme is in many respects similar to that of the *Sorangium* enzyme, but the *Myxobacter* enzyme clearly differs from the *Sorangium* enzyme in that, unlike the latter, it does not hydrolyze the D-alanyl-N^{ϵ}-lysine linkages in the cell wall of *M. lysodeikticus* (Katz and Strominger, 1967).

Structure of the Polysaccharides. Digestion of cell walls of *S. aureus* and *A. crystallopoietes* with the *Myxobacter* enzyme failed to release significant amounts of reducing material (Figure3A, B). Redigestion of SP 2 with large amounts of the enzyme caused no change in its chain length (Figure 7). It is concluded that the *Myxobacter* enzyme is devoid of *N*-acetylhexosaminidase activity. The isolated polysaccharide chains are, therefore, undegraded. Many of the *O*-acetyl groups in the *S. aureus* polysaccharide had been lost by chemical hydrolysis at the pH of incubation with the *Myxobacter* enzyme. About 60% of the *N*-acetylmuramic acid residues in *S. aureus* cell walls contain such substituents (Ghuysen and Strominger,

1963). SP 2, however, retains only about 13% of its *O*-acetyl groups (Table III). The total absence of such groups in AP 2 makes it unlikely that they exist at all in the *Arthrobacter* cell walls.

Precautions had been taken to minimize degradation of the polysaccharide by autolytic enzymes during preparation of the *S. aureus* cell walls, and these walls failed to autolyze during prolonged incubation at 37° and pH 9. Walls have also been produced from cells at the same stage of growth without heat treatment and trypsin digestion and have been found to autolyze slowly. The pH optimum for this autolysis is about 8, and is accompanied by eventual complete hydrolysis of the *N*-acetylmuramyl-L-alanine bonds and partial hydrolysis of *N*-acetylglucosaminidic linkages (D. J. Tipper, unpublished data). These activities are absent from the wall preparation described here, and the polysaccharide in the isolated cell walls is, therefore, probably unaltered from its state in the original cells. The same arguments apply to the *A. crystallopoietes* polysaccharide fractions, since similar precautions were taken during cell wall preparation (from log-phase cells). These cell walls failed to autolyze at 37° and pH 9, even though they had not been heat treated or trypsin digested.

The major polysaccharide fractions, SP 2 from *S. aureus* and AP 2 from *A. crystallopoietes*, contain at least 40% of the total glycan in the original cell walls, most of the rest being bound to teichoic acid in the complexes that were separated from AP 2 and SP 2 by ECTEOLA-cellulose chromatography (Figures 4 and 5). Small amounts of polysaccharide (SP 1 and AP 1) in both cases failed to be absorbed by ECTEOLA-cellulose and were eluted with the peptide fractions. Only SP 1 has been analyzed. Relative to SP 2 it has a high peptide content and reducing power, and may represent recently synthesized peptidoglycan, some of whose *N*-acetylmuramyl-L-alanine linkages are relatively insusceptible to the *Myxobacter* enzyme because of their proximity to reducing end groups in short chain length oligosaccharides. The reducing power of SP 1 indicates an average chain length of about four hexosamines, assuming that its reducing termini are *N*-acetylmuramic acid residues. Such a limitation on susceptibility of these linkages to the *Myxobacter* enzyme is indicated by their resistance to the enzyme in *Chalaropsis* glycopeptide (Table II), and may also explain the residue of peptide remaining in SP 2 after exhaustive redigestion with the *Myxobacter* enzyme (Table III). This residual peptide corresponds to 1 mole of peptide subunit/3 moles of polysaccharide with an average chain length of 19 hexosamine residues.

Glycopeptide, isolated from cell walls of *Staphylococcus aureus*, strain Copenhagen after digestion with endoacetylmuramidases, has been shown to contain equimolar amounts of *N*-acetylglucosamine and *N*-acetylmuramic acid, and the *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides (D1 and D2) isolated from this glycopeptide in high yield, have been shown to be β-1,4 linked (Ghuysen and Strominger, 1963; Tipper *et al.*, 1965). The isomeric *N*-acetylmuramyl-*N*-acetylglucosamine disaccharides, isolated in high yield from the same cell walls after lysis with lysostaphin, have also been shown to be β-1,4 linked (Tipper and Strominger, 1966). These data indicate that the polysaccharide in the peptidoglycan is built up of alternating β-1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid. Elucidation of the mechanism of biosynthesis of this polysaccharide, which is formed by polymerization of preformed, lipid-bound *N*-acetylglucosaminyl-*N*-acetylmuramic acid (peptide) subunits (Anderson *et al.*, 1965) demonstrated the mechanism by which this strict alternation is ensured. Branching, if it occurs, cannot be frequent. These predictions are substantiated by the 1:1 ratios of glucosamine:muramic acid in these polysaccharides (Table III) and by their high negative specific rotations, to which the residual peptide makes a negligible contribution.

The terminal reducing *N*-acetylhexosamine of a 1,4-linked polysaccharide (or disaccharide), in its aldehyde form, should be oxidized by periodate between C_5 and C_6 with the production of 1 mole of formaldehyde. The ratio of formaldehyde produced to total hexosamine content should be equal to the chain length of the polysaccharide. After reduction, the isomeric disaccharides derived from this polysaccharide are rapidly oxidized at pH 4.5 by 0.001 M periodate with production of 1 mole of formaldehyde (Tipper *et al.*, 1965; Tipper and Strominger, 1966). Under these conditions, no detectable formaldehyde is produced from the unreduced disaccharides, whose reducing termini are unusually resistant to oxidation. In determination of polysaccharide chain lengths by periodate oxidation under these conditions, therefore, oxidation of unreduced polysaccharide served as a control for nonspecific formaldehyde production.

The nonreducing residue of 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid consumes 1 mole of periodate at pH 4.5 over a period of 24 hr in 0.01 M periodate (Tipper *et al.*, 1965). The very low consumption of periodate by unreduced SP 2 and AP 2 under these conditions is probably owing to *N*-acetylglucosaminyl nonreducing termini, and demonstrates that the nonterminal residues of these polysaccharides are largely resistant to periodate oxidation. This is expected for a 1,4-linked structure, and rules out a significant content of linkages to the 6 position of *N*-acetylglucosamine, or of non-*N*-acylated glucosamine residues. On oxidation of reduced SP 2 and AP 2 under these conditions, the consumption of periodate in excess of formaldehyde production is similarly low, while the ratio of total hexosamines to liberated formaldehyde gave average chain lengths of 24 hexosamine residues for SP 2 and 20 hexosamine residues for AP 2. The production of formaldehyde from reduced and de-*O*-acetylated samples of SP 2 and AP 2 was followed kinetically in 0.001 M periodate as shown in Figure 7. From these data, more accurate average chain lengths of 18 for SP 2 and 25 for AP 2 were calculated. After redigestion with a relatively high concentration of the *Myxobacter* enzyme, the chain

length of SP 2, determined by this same procedure, remained 19 hexosamines.

SP 2 and AP 2 were fractionated by filtration on a column of Sephadex G-50 (Figure 9). The tubes were pooled into 5 fractions in each case, which were analyzed for total hexosamine content and chain length. The results, together with approximate K_d values, are presented in Table IV. The K_d value is a measure of the fraction of the internal volume of the gel available to the solute, and is inversely related to the molecular weight of the solute. Both polysaccharides are polydisperse with fractions varying in average chain length between 12 and 70 hexosamines, or between about 3000 and 17,500 in molecular weight.

Although the polysaccharide when substituted by peptide in the cell wall peptidoglycan of *S. aureus* is an excellent substrate for endoacetylmuramidases (*Chalaropsis* B enzyme, F1 enzyme, and 32 enzyme), and the TCA-peptidoglycan is a substrate for lysozyme, none of these enzymes hydrolyze the isolated polysaccharide except in high concentrations (Figure 8). The 32 enzyme is almost without effect, F1 and *Chalaropsis* B enzymes are intermediate, and lysozyme gives the greatest extent of hydrolysis, the final mixture probably containing an equilibrium mixture of di- and tetrasaccharides, as shown by thin layer chromatography. It is probable that these enzymes, to varying degrees, require a substituted *N*-acetylmuramyl carboxyl group if they are to hydrolyze the neighboring glycosidic linkage (*cf.* Muñoz *et al.*, 1966a). On the contrary, the *Micrococcus* endoacetylglucosaminidase (π enzyme) hydrolyzes the polysaccharides to disaccharides with high efficiency. This enzyme may require unsubstituted *N*-acetylmuramyl carboxyl groups in its substrate, since it is nonlytic on *S. aureus* cell walls when separated from its associated staphylolytic peptidase (Browder *et al.*, 1965). Its action on *S. aureus* cell walls probably follows that of the amidase action of the *Micrococcus* peptidase as shown by the relative kinetics of NH_2-terminal L-alanine and reducing power production.

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