Mechanism of Autolysis of Isolated Cell Walls of 
Staphylococcus aureus

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Autolysis of isolated cell walls of Staphylococcus aureus strain Copenhagen was accompanied by the release of 1 mole of N-terminal alanine per mole of glutamic acid. No other N-terminal amino acids and no C-terminal amino acids were released. These observations indicated that complete hydrolysis of N-acetylmuramyl-L-alanine linkages ("amidase" action) had occurred. This was confirmed by fractionation and analysis of the products. Hydrolysis of 4-O-β-N-acetylglucosaminyl-N-acetylmuramic acid linkages also occurred to a variable extent; on one occasion, complete degradation to disaccharides and hexosamine-free polypeptides (with intact pentaglycine cross-bridges) occurred. In one other instance, hydrolysis within pentaglycine bridges also occurred. Analyses of intact cell walls indicated that, in vivo, glycine endopeptidase activity was negligible and amidase activity was low, but that endo-β-N-acetylglucosaminidase hydrolysed about 8% of the N-acetylglucosaminyl-N-acetylmuramic acid linkages. Autolysis of isolated cell walls was too slow for the enzymes isolated with them to have significant action during this isolation. The possible functions of these autolytic activities are discussed.

Recent investigations have elucidated the basic structure of the peptidoglycan in isolated post-exponential-phase cell walls of Staphylococcus aureus strain Copenhagen [see Tipper et al. (22) and references therein]. It consists of a polysaccharide containing alternating β-1,4-linked residues of N-acetylglicosamine and N-acetylmuramic acid, and a polypeptide consisting of the tetrapeptide units, Nβ(1-lysyl-D-glutaminyl)-L-lysyl-D-alanine, linked to the D-lactate moieties of most of the muramic acid residues, and cross-linked by pentaglycine bridges between the COOH group of D-alanine and the ε-NH2 group of lysine in a neighboring peptide subunit (Fig. 1). Lysis of these cell walls with the Chalaropsis B enzyme, an endo-N-acetylmuramidase which is free from detectable peptidase activity (6, 23), results in the production of soluble glycopeptide in which 4-O-β-N-acetylglucosaminyl-N-acetylmuramic acid disaccharides are interlinked by the polypeptide, which retains its cross links intact (Fig. 1). Fractionation of this glycopeptide by gel filtration into oligomers of increasing chain length has revealed a polydisperse pattern of cross-linking of the peptide, about 7% of which normally exists as the uncrosslinked monomer (19, 21). This monomer, like its uridine diphosphate-N-acetylmuramyl pentapeptide precursor and the COOH-terminal peptide subunits of all the oligomers, contains 2 moles of D-alanine and a total of 3 moles of alanine per mole of glutamic acid (Fig. 1). One mole of D-alanine is lost from each of the other subunits by transpeptidation in formation of D-alanyl-glycine cross-links (19). The average chain length of the peptide is 3.9 subunits (see below). Similarly, lysis of these cell walls with the Myxobacter AL-1 enzyme, a peptidase free from detectable N-acetylmuramidase activity (22), has resulted in the isolation of the intact polysaccharide. Fractionation of this material by gel filtration again has shown it to be polydisperse with an average chain length of 12 disaccharide units (22).

The mode of action of the autolytic enzymes present in isolated cell walls of this strain was studied, first, to see to what extent autolysis might have occurred during isolation, and, second, to see to what extent autolysis might occur in vivo, in the hope of determining the possible functions of these activities during cell growth and division.

MATERIALS AND METHODS

Cell walls. Cultures (1 liter) of S. aureus strain Copenhagen were grown at 37°C in 2-liter conical flasks in gyratory shaker-incubators in a medium containing, per liter: peptone (Difco), 5 g; yeast extract (Difco), 5 g; glucose, 2 g; and K2HPO4, 1 g. Inocula

1 This paper was presented in part at the 68th Annual Meeting of the American Society for Microbiology, Detroit, Mich., May 1968.
were prepared from stock cultures, grown for 16 hr, and diluted 50-fold into fresh media. Cell growth was followed by measurement of absorbance at 700 nm by use of a Zeiss PMQII spectrophotometer and cells of 1-cm path length and 1-ml capacity. At maximal growth, samples (0.2 ml) diluted with sterile medium (0.8 ml) gave readings of about 1.0. (A Beckman DU spectrophotometer used in a similar fashion gave approximately half the absorption at 700 nm.) Cells were harvested either in early log phase (4 hr later, A<sub>760</sub> 0.20 to 0.25 at 1:5 dilution) or in postexponential phase (10 hr later) when growth was approaching the maximum. Cells were broken (except where indicated) in water by shaking with glass beads in a Nossal disintegrator (McDonald Instrument Co., Bay Village, Ohio) in such a way that the temperature never exceeded 15 C. Beads were removed by filtration at 2 C and cell walls were isolated by centrifugation followed by washing once with 0.2 M KH<sub>2</sub>P<sub>O</sub><sub>4</sub>, and twice with water at 0 C.

Turbidity measurements. Turbidity of cell wall suspensions is defined as the absorbance at 535 nm determined by use of a Zeiss PMQII spectrophotometer and cells of 1-cm path length and 0.25-ml capacity. A suspension of walls (1.3 mg) in 0.01 M KPO<sub>4</sub>, pH 7.5 (1 ml), dispersed by brief sonic treatment, had a turbidity of 1.0, and initial turbidity was directly proportional to cell wall concentration.

Enzymes and materials. The *Chalaropsis* B enzyme was a gift from J. Hash, Vanderbilt University School of Medicine, Nashville, Tenn. Authentic muramic acid (0.15%, 113°) was a commercial preparation from Cyclo Chemicals Corp., Los Angeles, Calif. N-acetylglucosamine and glucosamine were obtained from Sigma Chemical Co., St. Louis, Mo.

Ion-exchange chromatography and gel filtration. Columns of ECTEOLA-cellulose (Cellex-E, Bio-Rad Laboratories, Richmond, Calif.) and carboxymethyl cellulose (Cellex-CM, Bio-Rad Laboratories) were operated at room temperature and were washed with 0.4 M NaCl and water before use. Columns of Sephadex (Pharmacia, Inc., Upsala, Sweden) G-25 and G-50, fine bead forms, were operated in series at room temperature in 0.1 M LiCl as described previously (21). Material to be fractionated was applied to a G-50 column (100 X 2.5 cm) whose eluate ran directly into a G-25 column of similar dimensions. Where indicated, the G-25 column was used alone. Individual pooled fractions were concentrated in vacuo and desalted on columns of Biogel P<sub>2</sub> (Bio-Rad Laboratories), 200 to 400 mesh, 2 X 50 cm, eluted with water at room temperature.

Analytical procedures. Analyses for N- and C-terminal amino acids, reducing power, total and inorganic phosphate, and total D- and L-alanine were performed as previously described (5, 22). Addition of 10 N H<sub>2</sub>SO<sub>4</sub>, 10 minutes after color development in the reducing power determination was sometimes necessary to decrease turbidity apparently due to glycan. Analyses for total amino acids and hexosamines (after hydrolysis for 18 hr at 105 C in 6 N HCl and for 8 hr at 100 C in 3 N HCl, respectively) were performed by applying samples of hydrolysates directly to the columns of a Beckman-Spinco amino acid analyzer. A sample of glycopeptide of known muramic acid and glucosamine content, hydrolyzed at the same time, served as standard for these compounds. Chain lengths of oligosaccharide were determined from the release of formaldehyde on periodate oxidation after reduction; samples (0.1 amole of oligosaccharide) were reduced overnight in 0.05 M NaBH<sub>4</sub> (50 amoles; 2.5 amoles) and adjusted to pH 4 by addition of acetic acid. The products had no residual reducing power and were oxidized at pH 4 in 0.001 M IO<sub>4</sub><sup>-</sup> (250 µ-liters); samples (20 amoles) were analyzed at intervals for formaldehyde by a chromotropic acid procedure (22). Plateau values were extrapolated to zero time, and equal samples of unreduced oligosaccharides

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**FIG. 1. Structure of the peptidoglycan of *S. aureus.* In strain Copenhagen, grown as described in Materials and Methods, serine replaces glycine in the bridges to the extent of 0.05 mole per mole of glutamic acid (Tipper and Berman, Biochemistry, in press). The repeating subunit of the cross-linked peptidoglycan is shown in the upper section and the nascent subunit with two D-alanine residues and N-terminal pentaglycine is shown in the lower section.
served as controls. Chain lengths were also determined by the incorporation of tritium on reduction with H\textsubscript{2}-NaBH\textsubscript{4}. Samples containing 15 nmoles of reducing end group were reduced overnight in unbuffered 0.15 M H\textsubscript{2}-NaBH\textsubscript{4}, 43 mc/m mole. After addition of 2 N HCl (7 uliters), samples were dried, hydrolyzed in 4 N HCl (50 uliters) for 4 hr at 105 C, dried, and chromatographed overnight in n-butyl alcohol-acetic acid-water (3:1:1). Spots, detected with a strip scanner, and identified by autoradiography, were cut out and eluted into scintillation fluid. Standard conditions employed throughout these studies were 0.02 m KPO\textsubscript{4} containing 0.001 m MgCl\textsubscript{2} at 37 C with the addition of toluene to retard growth of contaminating organisms.

**RESULTS**

**Properties of cell walls and condition of autolysis.** Cell walls of *S. aureus* Copenhagen from early exponential- or postexponential-phase\textsuperscript{c} cells, isolated as described above, gave essentially identical analyses (Table 1), showing the presence of only small amounts of nonpeptidoglycan amino acids (with the exception of serine and aspartate). These walls apparently do not contain much enzyme-end group protein (cf. 11). Elluates were suspended in 0.02 M buffers of pH 4.5 to 9.2, autolysis of postexponential-phase cell walls occurred, even though they had been lyophilized and stored for months at \(-20\) C. The optimal pH was 7.5 to 8 (Fig. 2). At the higher pH values, autolysis, though initially fairly rapid, was incomplete, presumably owing to denaturation of the enzymes. In the presence of 0.3 M NaCl, the rate of autolysis in 0.02 m KPO\textsubscript{4} (pH 7.5) was decreased by 25\%, and, in the presence of 0.01 m sodium ethylenediaminetetraacetate (EDTA) and 0.02 m KPO\textsubscript{4} (pH 7.5), autolysis was retarded about 75\%.

The addition of 0.015 M MgCl\textsubscript{2} reversed the inhibition, but neither higher concentrations of Mg\textsuperscript{2+} nor addition of 10\textsuperscript{-3} M Co\textsuperscript{2+}, Ca\textsuperscript{2+}, or Mn\textsuperscript{2+} enhanced the rate of autolysis appreciably. Standard conditions employed throughout these studies were 0.02 m KPO\textsubscript{4} containing 0.001 M MgCl\textsubscript{2} at 37 C with the addition of toluene to retard growth of contaminating organisms.

**Kinetics of hydrolysis during autolysis of postexponential-phase cell walls.** Cell walls (200 mg, prepared from unfrozen cells and lyophilized and stored at \(-20\) C) were suspended in standard buffer (7 ml) and incubated at 37 C. Samples were removed at intervals for the determination of residual turbidity, reducing power, and N- and C-terminal amino acids (Fig. 3). Autolysis of this preparation of walls was slow, 24 hr being required for a 50\% drop in turbidity. Release of 1 mole of N-terminal alanine and 0.3 mole of reducing power per mole of total glutamic acid occurred without release of any C-terminal amino acids or other N-terminal amino acids, indicating hydrolysis of *N*-acytymuramyl-L-alanine linkages (amidase action) and incomplete hydrolysis of the glycan.

**Fractionation of the autolysate.** Insoluble material (5 mg) was removed by centrifugation (30 min at 12,000 \(\times\) g) and on hydrolysis was found to have an amino acid composition typical of proteins. The supernatant liquid was fractionated on a column (2 \(\times\) 50 cm) of ECTEOLA cellulose. A peak of peptide and free D-alanine (detected by determination of free amino groups) and oligosaccharides (detected by determination of reducing power) was eluted with water. A second peak of oligosaccharides (reducing power) was eluted at the start of a gradient of increasing LiCl.

**Table 1. Analyses of cell walls of *S. aureus* strain Copenhagen**

<table>
<thead>
<tr>
<th>Culture age</th>
<th>Glutamine</th>
<th>Alanine</th>
<th>Glycine</th>
<th>Serine</th>
<th>Aspartate</th>
<th>Glucoman</th>
<th>Muramic acid</th>
<th>NH\textsubscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>Glutamine</td>
<td>Alanine</td>
<td>Glycine</td>
<td>Serine</td>
<td>Aspartate</td>
<td>Glucoman</td>
<td>Muramic acid</td>
<td>NH\textsubscript{3}</td>
</tr>
<tr>
<td>4</td>
<td>92</td>
<td>333</td>
<td>229</td>
<td>104</td>
<td>5</td>
<td>12</td>
<td>202</td>
<td>86</td>
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<td>294</td>
<td>199</td>
<td>95</td>
<td>6</td>
<td>12</td>
<td>190</td>
<td>95</td>
</tr>
</tbody>
</table>

* Early exponential- and postexponential-phase cell walls were obtained from 4 and 9 hr cultures. Data are expressed as moles per 100 moles of total lysine. Both preparations of cell walls contained about 0.47 nmoles total glutamic acid per mg. Total amino acids and hexosamines were determined with a Beckman amino acid analyzer as described in the text. D-Alanine and L-alanine were determined on hydrolysates. All other amino acids were present in amounts of less than 3 moles per 100 moles of lysine. These walls contained about 0.06 mole of peptide-linked N-terminal alanine per mole of total glutamic acid (determined by dinitrophenylation after hydrolysis for 3 hr at 38 C, pH 9.2, to remove ester-linked alanine, and solubilization with the Chalaropsis B enzyme).
decrease in S. borate, pH All buffers NaOAc, M 0.02 Samples (15 uliters) AcOH and aureus. Cell the text.

FIG. 2. Effect of pH on initial rate of autolysis of S. aureus cell walls. Rate is defined as the percentage decrease in turbidity (A660) per hour. Buffers were NaOAc, pH 4.5, KPO4, pH 6, 7, 7.5, 8, and 8.5, and borate, pH 9.2. At pH 8.5, and especially at pH 9.2, autolysis stopped before clarification was complete. All buffers were 0.02 M, and incubations were at 37°C.

Fig. 3. Kinetics of autolysis of cell walls of S. aureus. Cell walls (200 mg) were incubated in 7 ml of 0.02 M KPO4-0.001 M MgCl2, pH 7.5, as described in the text. Samples (10 uliters) were diluted with water (150 uliters) for measurement of turbidity at 535 nm. Samples (15 uliters) were adjusted to pH 4.5 with AcOH and solubilized by incubation for 3 hr at 37°C with Chalaropsis B enzymes (3 µg) before determination of N- and C-terminal amino acids.

concentration, and overlapped with a third peak of reducing power which was eluted at higher LiCl concentrations (0.1 to 0.3 M) and which contained all of the organic phosphate (teichoic acid-oligosaccharide complex; cf. 21). The water eluate from the ECTEOLA cellulose column was fractionated on a column of carboxymethyl (CM) cellulose (30 x 1.6 cm). Water eluted the oligosaccharides (reducing power) and free alanine (free amino groups). A linear gradient of increasing LiCl concentration eluted the oligopeptides in a series of poorly resolved peaks between 0.1 and 0.4 M. The compositions of the various fractions are summarized in Fig. 4 and Table 2.

The LiCl eluate from CM cellulose contained 82% of the total glutamic acid of the original cell walls together with the other amino acid components of the peptidoglycan, but very little of the hexosamines. This and the isolation of oligosaccharide fractions containing relatively little peptide is further evidence of almost complete amidase action. The peptide fraction was further fractionated on columns of Sephadex G-50 and G-25 connected in series, with the results shown in Fig. 5. Fractions were pooled as indicated, and analyses are presented in Table 3. Each fraction contained approximately 1 mole of N-terminal alanine per mole of glutamic acid and alanine as the only C-terminal amino acid. Amounts of N-terminal glycine and total alanine were consistent with the nominal chain lengths given in Table 3; thus, if no hydrolysis within pentaglycine cross-bridges had occurred, a polymer of chain length n subunits should contain 2 + 1/n moles of total alanine and 1/n moles of N-terminal glycine per mole of subunit (glutamic acid), as was found for the glycopeptide oligomers produced by digestion of unautolyzed cell walls with Chalaropsis B enzyme (21). Fractionation of such a digest on Sephadex G-50/ G-25 is shown in Fig. 6. Fractions 1, 2, 3, 4, and 5 comprised 9, 10, 13, 11, and 57%, respectively, of the total glycopeptide fractionated and had average chain lengths of 1, 2, 3, 4.5, and about 10 subunits, giving an overall average chain length of 3.9. This is considerably more accurate than figures derived from overall alanine content, N-terminal glycine content, or C-terminal alanine content.

Similarly, if the fractions of the oligopeptides from autolysis are assumed to have the nominal chain lengths given in Table 3, then an overall chain length of 5.6 can be calculated from the proportion of the total glutamic acid in each fraction. Previous experience with Cellex CM indicated that it retarded efficiently only peptide oligomers, so that the peptide fractionated in Fig. 5 is probably deficient in monomers and
AUTOLYSIS OF STAPHYLOCOCCUS AUREUS CELL WALLS

S. aureus Cell Wall Autolysate

Supernatant liquid

ECTEOLA

Water eluate

Pellet (1.5% of total)

LiCl eluate

CM

0 to 0.1 M LiCl eluate

CM Teichoic acid complex

G-50/G-25

G-25

Excluded peak of oligosaccharide (D)

Table 2. Recoveries from fractionation of first autolysate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oligopeptide fractions</th>
<th>Oligosaccharide fractions</th>
<th>Teichoic acid fractions</th>
<th>All fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muramic acid</td>
<td>4</td>
<td>52</td>
<td>16</td>
<td>72</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>62</td>
<td>6</td>
<td>8</td>
<td>76</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0</td>
<td>0</td>
<td>140</td>
<td>140</td>
</tr>
</tbody>
</table>

* Details of the fractionation are given in Fig. 4 and in the text. Total muramic and glutamic acid contents were determined on hydrolysates of samples by use of an amino acid analyzer. Organic phosphate is total phosphate minus inorganic phosphate. A 200-mg amount of S. aureus cell walls (0.47 μmole of glutamic acid per mg, Table 1) contains about 94 μmole of peptidoglycan and 188 μmole of total phosphate. Data are not corrected for analytical losses.

dimers, thus accounting for its high average chain length. It is concluded that no hydrolysis within pentaglycine cross-bidges occurred during this autolysis.

Second autolysis and Sephadex fractionation of the products without prior ion-exchange chromatography. With the object of obtaining a complete pattern of oligopeptides unaffected by preferential losses during ion-exchange chromatography, a new preparation of walls was autolyzed, and the products were fractionated immediately on Sephadex G-50/G-25. In this instance, cells from early exponential-growth phase which had been frozen for several months (6 g, wet weight) were suspended in water (total volume, 30 ml)
TABLE 3. First autolysis: analyses of peptide oligomers from Sephadex G-50/G-25 fractionation (Fig. 5)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total glutamic acid (moles)</th>
<th>Lysine</th>
<th>Glycine</th>
<th>Alanine</th>
<th>N-terminal Glutamic Acid</th>
<th>Glutamic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>103</td>
<td>450</td>
<td>280</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>98</td>
<td>490</td>
<td>245</td>
<td>40</td>
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<td>3</td>
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<td>460</td>
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<td>34</td>
<td>90</td>
<td>440</td>
<td>210</td>
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<td>100</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
<td>10</td>
<td>86</td>
<td>455</td>
<td>195</td>
<td>9</td>
</tr>
</tbody>
</table>

* Data are expressed as moles per 100 moles of total glutamic acid. The correspondence between peak and fraction number is as indicated in Fig. 5. The only C-terminal amino acid present in significant amounts was alanine, and no fraction contained more than traces of hexosamines.

and treated for 20 min with a Branson J17 sonic oscillator, with the use of a sealed atmosphere cell and 10 ml of 5-μm glass beads (Heat Systems, Inc., Melville, N.Y.). The walls (about 200 mg) were washed twice with 0.01 M KPO₄, pH 7.5, and were finally suspended in 14 ml of 0.01 M KPO₄, 0.001 M MgCl₂, pH 8, and incubated at 37 C. The pH was adjusted at intervals, and remained between 8 and 8.5. Clarification was fairly rapid, being complete in 30 hr, by which time release of reducing power had ceased, although the final amount was again only about 0.3 mole per mole of total glutamic acid. Release of free amino groups continued, and the autolysis was terminated after 66 hr. The residual insoluble material (mostly metal fragments from the sonic treatment, 20 mg) was removed by centrifugation, and the supernatant liquid was concentrated to 3 ml and fractionated on Sephadex G-50/G-25 in 0.1 M LiCl as before (Fig. 7).

All of the organic phosphate (not shown, teichoic acid) was eluted in the excluded peak (Kₒ = 0) together with a small proportion of the reducing power (oligosaccharide-teichoic acid complex) and oligopeptides (free amino groups). Free alanine, produced by hydrolysis (under the conditions of autolysis) of the ester-linked D-alanyl residue of the teichoic acid, gave rise to the peak of free amino groups of Kₒ = 1. The bulk of the reducing power eluted in a series of poorly resolved peaks centering about a peak at Kₒ 0.5, which is probably hexa- or tetrasaccharide (cf. Fig. 9, below). The bulk of the peptide eluted in peaks of Kₒ 0.65 to 0.8 (monomer), 0.55 to 0.65 (dimer), and 0.45 to 0.55 (trimer), about 53% of the total being in the monomer peak alone. Analyses of the monomer and dimer peaks are given in Table 4. In contrast to the data on the products of the first autolysis (Table 3), these fractions contained only 2 moles of total alanine per mole of glutamic acid, and contained considerable amounts of C-terminal glycine, showing them to be products of hydrolysis within pentaglycine cross-bridges.

Autolysis of cell walls from early exponential- and postexponential-phase cells from the same culture. Twenty-four flasks, each containing 1 liter of medium, were inoculated from the same S. aureus Copenhagen culture. Half were harvested after 4 hr when growth was 20% of maximum, and the rest were harvested after 7 hr of...

![Fig. 6. Sephadex G-50/G-25 fractionation of glycopeptides solubilized by the action of Chalaropsis B enzyme on S. aureus Copenhagen cell walls. Cell walls (from postexponential-phase cells, 1.53 g) were hydrolyzed with enzyme (5 mg) in 0.01 M NaOAc, pH 4.5 (30 ml), at 37 C for 24 hr. Release of reducing power was maximal at 7 hr. The complex of teichoic acid with glycopeptide (about 20% of the total glycopeptide) was adsorbed on ECTEOLA cellulose and the teichoic acid-free glycopeptide, eluted with water, was fractionated on G-50/G-25 as described in Materials and Methods. All of the muramic acid in the glycopeptide was susceptible to reduction with NaBH₄, demonstrating that N-acetylmuramidase action had been complete. Therefore, as confirmed by analyses, each peptide subunit carried a 4-O-β-N-acetylmuramyl-N-acetylmuramic acid disaccharide unit on its l-alanine N-terminus, and reducing power is proportional to total peptide subunit concentration. Fractions were pooled as follows: 5, Kₒ 0 to 0.15; 4, 0.16 to 0.20; 3, 0.20 to 0.30; 2, 0.30 to 0.45; 1, 0.45 to 0.7. Kₒ is defined in the legend to Fig. 5.](http://jb.asm.org)
growth (90% of maximum). Cell walls were prepared from all of the first and about one-third of the second cell pellets, as described in Materials and Methods, giving E and P cell walls, respectively. These walls were not lyophilized or frozen. Autolysis at 37 C in 0.02 m KPO4, 0.001 m MgCl2 at pH 7.5 to 8 was followed by release of reducing power which was almost complete in 10 hr with E walls; the suspension of E walls was completely clarified by 15 hr. Release of reducing power from P walls ceased at about 36 hr, and clarification was only complete by 48 hr. Determination of N-terminal amino acids at intervals on samples solubilized with Chalaropsis B enzyme showed no increase of N-terminal glycine in either case, but release of N-terminal alanine occurred in both, terminating at about 24 hr in E and at about 76 hr in P. Both autolyses were centrifuged after 84 hr, and the supernatants were fractionated on G-50/G-25 as before (Fig. 8). Both patterns are similar and, unlike that shown in Fig. 7, resemble that found in Chalaropsis B enzyme digests of unautolyzed cell walls (Fig. 6). Displacement of the peaks to higher KD values occurred, since almost half of the molecular weight of the glycopeptide subunit resides in its disaccharide moiety. As in Fig. 7, the totally excluded peak coincided with the peak containing all of the organic phosphate and about half of the hexosamines (oligosaccharide-teichoic acid complex), and the peak at KD = 1 is free alanine. The monomer and dimer peaks were pooled and analyzed with the results shown in Table 4. As in the products of the first autolysis, all contained about 1 mole of N-terminal alanine, and amounts of N-terminal glycine, total alanine, and C-terminal alanine consistent with their chain lengths and the presence of exclusively D-alanyl-D-alanine C-termini. Thus, in neither case did significant hydrolysis within pentaglycine cross-bridges occur, although a somewhat higher degree of cross-linking in the P

![Image](https://via.placeholder.com/150)

**Fig. 7. Fractionation on Sephadex G-50/G-25 of products of autolysis of early exponential-phase cell walls. See Materials and Methods and legend to Fig. 5 for details. The solid line represents the concentration of free amino groups. The total reducing power (dotted line) gives a measure of the concentration of oligosaccharides, not of total hexosamines, and underestimates the concentration of higher oligosaccharides as these have a somewhat lower molar reducing power (5).**

**Table 4. Second and third autolyses: analyses of peptide dimers and monomers from Sephadex G-50/G-25 fractionations (Fig. 7 and 8)***

<table>
<thead>
<tr>
<th>Figure</th>
<th>Fraction</th>
<th>Lysine</th>
<th>Glycine</th>
<th>Alanine</th>
<th>N-terminal Glycine</th>
<th>Alanine</th>
<th>C-terminal Glycine</th>
<th>Alanine</th>
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</thead>
<tbody>
<tr>
<td>7</td>
<td>Monomer</td>
<td>97</td>
<td>480</td>
<td>208</td>
<td>80</td>
<td>80</td>
<td>60</td>
<td>30</td>
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<tr>
<td></td>
<td>Dimer</td>
<td>98</td>
<td>435</td>
<td>176</td>
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</tbody>
</table>

* Data are expressed as moles per 100 moles of total glutamic acid. The nominal chain lengths of the fractions are derived from N-terminal glycine and total alanine and C-terminal contents. The designations 8E and 8P refer to the Sephadex G-50/G-25 fractionations of 4- and 7-hr cell wall autolyses, respectively (Fig. 8). In each case, monomer is the peak with KD 0.65 to 0.8 and dimer is the peak with KD 0.55 to 0.65.

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cell walls is evident from the patterns of peptide oligomers (Fig. 8).

Fractionation and analysis of the oligosaccharides from the first autolysis. The oligosaccharide fractions (Fig. 4) were fractionated on Sephadex G-25 with the results shown in Fig. 9. Analyses of the pooled fractions are given in Table 5. The material retarded by ECTEOLA was almost totally excluded from the gel, whereas the material eluted with water from both ECTEOLA and CM cellulose was of lower average molecular weight, 40% of the total hexosamine being in fraction A alone. Chain-lengths were consistent with the $K_D$ values and indicated that fraction A was a disaccharide, and that the average chain-length of all fractions was 4. Thus, glycanase action was 50% complete during this autolysis. All fractions contained equimolar amounts of glucosamine and muramic acid. After reduction, fraction A contained equimolar glucosaminol and muramic acid (determined with the amino acid analyzer) and no residual glucosamine, showing it to be N-acetylmuramyl-N-acetylglucosamine. After reduction of all 4 fractions with $^3$H-NaBH$_4$, label was found exclusively in glucosaminol, in amounts corresponding to the chain-lengths given in Table 5, which are in reasonable agreement with the periodate oxidation data. The glycan of the peptidoglycan had previously been isolated from cell walls of the organism, without degradation during isolation, by use of the *Myxobacter* AL-1 enzyme (22). This glycan was separated into fractions of increasing average chain-length on Sephadex G-50. Reduction of these fractions with $^3$H-NaBH$_4$ gave data for their chain lengths (9, 15, 19, 33, and 56 hexosamine residues) in reasonable agreement with those previously obtained (22) by periodate oxidation (11, 17, 28, 42, and about 80), and showed that, for these fractions, $N$-acetylmuramylglucosamine accounted for 94, 95, 95, 92, and 72%, respectively, of the reducing end groups.

![Figure 8](http://jb.asm.org/) Fractionation on Sephadex G-50/G-25 of products of autolysis of early exponential-phase (E) and postexponential-phase (P) cell walls. Preparation of the autolysates and details of their fractionation are described in the text. Insoluble material was 4 and 9 mg for E and P cell walls, respectively. Monomer and dimer fractions were pooled as shown in Table 4. The solid and dotted lines represent free amino groups in the fractions from E and P cell walls, respectively. In both autolysates, about 50% of the hexosamines occurred with all of the organic phosphate in the totally excluded peak of teichoic acid complex (not shown). In the fractions from the E cell wall autolysate, the only other peak of hexosamines occurred at $K_D$ 0.75, as shown by the dashed line. This indicates a molecular size slightly smaller than peptide monomer, and therefore corresponds to disaccharide. The P cell wall autolysate contained this peak (disaccharide) and also a peak at $K_D$ 0.55 (probably tetrasaccharide).

![Figure 9](http://jb.asm.org/) Fractionation on Sephadex G-25 of the oligosaccharide fractions from the first autolysis. Left-hand side: fractionation of the water eluate from CM cellulose (Fig. 4). This eluate was concentrated to 2 ml, applied to a column (1.5 × 100 cm) of Sephadex G-25, and eluted with water. Samples (8 ml) were analyzed for reducing power (dotted lines), and samples (24 ml) were analyzed for total hexosamines (solid lines). Right-hand side: fractionation on the 0 to 0.1 M LiCl eluted from ECTEOLA-cellulose (Fig. 4). This eluate was concentrated, fractionated, and analyzed as above. Fractions were pooled as indicated.
**DISCUSSION**

Autolysis of isolated cell walls of *S. aureus* (strain Duncan) was first reported by Mitchell and Moyle (12), although lysis of heat-killed *S. aureus* cells by products of other *S. aureus* strains, possibly virus-induced lysins (cf. 14), was first reported by Welch and Salmon (25). *S. aureus* "lysozyme," an extracellular activity defined by its ability to lyse heat-killed cells of *Micrococcus lysodeikticus*, was first named by Welch (24) and has been reported to be strongly correlated with pathogenicity (10). Richmond (15) reported that this activity solubilized cell walls of *S. aureus* and *M. lysodeikticus* with the release of reducing power and material reacting weakly as *N*-acetylamin sugar. Autolysis of cell walls of strain Copenhagen was also accompanied by release of reducing power, and was inhibited by EDTA, as reported by Mitchell and Moyle (12), though their reported pH optimum was 6.

Huff and Silverman (9) have recently reported the presence of enzymes able to solubilize isolated cell walls of the parent organism in various fractions from *S. aureus* Oeding 8507. Activity was found in the growth medium, the soluble portion of cell extracts, and in the isolated cell walls. Freezing and thawing released 2% of the cell protein and 75% of the activity, which was active on cell walls but not on intact cells. It was deduced that, in intact cells, the soluble enzyme probably resides close to its substrate, the inner surface of the cell walls. Shockman et al. (18) have extensively studied the autolytic *N*-acetylmuramidase of *Streptococcus faecalis* 9790 which is isolated largely in the cell wall fraction, and found that the soluble enzyme is irreversibly bound by its substrate. They also found preferential release of newly synthesized peptidoglycan during autolysis (but not when heat-treated walls were digested with soluble autolysin), indicating localization of the activity at the site of synthesis. Mitchell and Moyle (12) earlier observed that autolysis of intact cells of *S. aureus* in 1.2 M sucrose followed by osmotic shock resulted in the formation of hemispheres, owing to preferential lysis at points of separation. More recently, this has been graphically demonstrated in sections of nondividing cells of *S. aureus* JHM in 4% NaCl (2). Thus, the isolation of totally autolyzable cell walls may be artifactual, and breakage of cells may disrupt cell wall synthesis but activate cell wall hydrolysis, although these two types of activity may be under integrated control in vivo.

**Table 5. Analyses of oligosaccharide fractions from fractionation on Sephadex G-25**

<table>
<thead>
<tr>
<th>Oligosaccharide Fraction</th>
<th>Percentage of total</th>
<th>Molar ratio, MurNAc/GlcNAc</th>
<th>Chain length (hexosamines)</th>
<th>GlcNAc reducing end group (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>26</td>
<td>0.9</td>
<td>2.0 2.2 96</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>14</td>
<td>0.8</td>
<td>3.2 3.0 99</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>0.9</td>
<td>4.7 5.2 97</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>0.8</td>
<td>8 10 96</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>0.8</td>
<td>3.7 4.0 97</td>
<td></td>
</tr>
</tbody>
</table>

* Muramic acid was determined with an amino acid analyzer. Chain lengths were determined by periodate oxidation (10^-1) or by reduction with tritiated borohydride (H-NaBH4) as described in Materials and Methods. The last column shows the ratio of tritium in glucosaminol to total tritium incorporated × 100. MurNAc = *N*-acetylmuramic acid; GlcNAc = *N*-acetylglucosamine.
tion isolated from one strain of *Staphylococcus* that is lytic for others (1). It resembles the autoly-
sin also in having a pH optimum of 7.5 and containing amidase activity. While *N*-acetyl-
muramidases are common, endo-*N*-acetylglu-
cosaminidases are rare (4), and therefore the
resemblance between lysostaphin and *S. aureus*
autolysin is striking and suggests that the lysos-
olphin-producing strain may be producing and
excreting abnormally large amounts of normal
autolytic activities. Lysostaphin and an autolyse of
*S. aureus* Copenhagen walls (and possibly staphyloccocal lysozyme) also lyse *M. lysode-
ikticus* cells walls, probably due to their endo-β-
*N*-acetylglicosaminidase activity, since, in *M.
lysodeikticus* walls, about 60% of the muramic
acid residues have free carboxyl groups and these
areas of the glycan are therefore susceptible to the
enzyme. Lysostaphin, however, also contains a
powerful glycine endopeptidase which is responsible
for its selective activity on staphyloccocal cell
walls. This type of activity was found in only a
single instance of autolysis, a fact which is at
present not understood but possibly due to a
chance phage infection.

If amidase action is primarily responsible for
autolysis of walls of this strain, then they re-
ssemble walls of *B. subtilis* 168 (26) and *Listeria
monocytogenes* (Tinellii and Ghuysen, in prepara-
tion). The same activity is found in the *Escherichia
coli* autolytic complex (13). Hisaizumi et al. (7,
8) have described the isolation, from culture filtrates of "the Wiley viscous wound strain" of
*S. aureus*, of nonhydrolyzable antigenically ac-
tive polypeptide that has all the properties of the
higher molecular weight fraction of the poly-
peptide produced during autolysis of *S. aureus*
Copenhagen. Thus, their strain also must have
an amidase activity and be free from significant
pentaglycine endopeptidase activity.

Autolytic enzymes, as has frequently been pro-
posed, may play a role in determining cell wall
structure at the time of its synthesis. Since the
glycan of the peptidoglycan is synthesized by
polymerization of *N*-acetylglucosaminyl-*N*-acetyl
muramic acid-peptide subunits, the almost ex-
clusive presence of *N*-acetylglucosamine reducing
end groups in the glycan indicates endo-*N*-acetyl-
glicosaminidase hydrolysis of about 8% of the
susceptible linkages in vivo. Indeed, if the non-
reducing terminus of the glycan is the acceptor,
the polymerization would result in a continuous
molecule, whose hydrolysis might be necessary
to maintain wall flexibility. Measurement of the
result of in vivo amidase activity is more difficult,
but it seems to be minimal, since no more than
6% of the l-alanyl residues are N-terminal in
isolated cell walls (Table 1). It is possible, how-
ever, that very extensive local amidase action
occurs, for example, at the sites of cell separation,
where hydrolysis of peptidoglycan may be neces-
sary.

None of the hydrolytic activities found in cell
walls of *S. aureus* produce acceptors for addition
to or cross-linking of peptidoglycan subunits by
the presently known mechanisms. These would
require nonreducing end groups of *N*-acetylglu-
cosamine, N-terminal pentaglycine residues, or
C-terminal d-alanyl-d-alanine residues. Indeed, it
seems probable that a glycan end group pro-
duced, for instance, by *N*-acetylmuramidase action,
would be too constrained by its attachment to a
nonextensible network to enter the active site of a
membrane-bound biosynthetic enzyme. More
likely, a peptidoglycan subunit, newly added to
the glycan, might be cross-linked to an adjacent
nascent subunit and then pass to the acceptor
site and be linked to the next incoming subunit
before it is released from the membrane, so that
glycan polymerization and transpeptidation are
spatially and temporally integrated. Uncross-
linked subunits, introduced into walls of *S. aureus*
growing in the presence of penicillin, do not be-
come cross-linked on subsequent growth in the
absence of penicillin (21). The situation may
differ in *E. coli*, where recent studies (17) have
indicated that peptidoglycan subunits, incorpo-
rated in the presence of penicillin, may subse-
quently become cross-linked during growth in the
absence of penicillin. In some instances, poly-
merization of peptidoglycan may also be coordi-
nated with hydrolytic activity (amidase) to form the "head to tail" linkage of peptide subunits re-
cently demonstrated to occur in *M. lysodeikticus*
(3, 16).

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