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Structures of the Cell Wall Peptidoglycans of *Staphylococcus epidermidis* Texas 26 and *Staphylococcus aureus* Copenhagen. II. Structure of Neutral and Basic Peptides from Hydrolysis with the *Myxobacter* AL-1 Peptidase*

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ABSTRACT: The *Myxobacter* AL-1 enzyme hydrolyzes most of the D-lactyl-L-alanine and all of the D-alanyl-glycine linkages in the cell wall peptidoglycan of *Staphylococcus aureus*. Most of the pentaglycine cross-bridges in this peptidoglycan are also hydrolyzed either between the third and fourth or between the fourth and fifth glycine residues (from their N termini) resulting in the production of equimolar proportions of tri- and tetraglycine. Tetraglycine is subsequently slowly hydrolyzed to diglycine. The major fractions of the remaining basic peptide have been shown to be N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(glycyl)-L-lysyl-D-alanine and the same peptide with 2 moles of glycine on its lysine-ϵ-amino group (Jarvis, D., and Strominger, J. L. (1967), *Biochemistry 6*, 2591). Hydrolysis of the cell wall peptidoglycan of *Staphylococcus epidermidis* strain Texas 26 occurs in identical fashion, liberating polydisperse peptide-free undegraded glycan of average chain length 13 hexosamine residues, two major basic peptides identical with those obtained from *S. aureus* (and containing almost no serine), and neutral di-, tri-, and tetrapeptides of glycine and serine. These results are consistent with the presence of only four types of pentapeptide cross-bridges in *S. epidermidis* cell walls: pentaglycine (20%), and pentapeptides with glycine replaced by serine in the third position (55%), the third and first positions (15%), and the second position (10%) from their N termini. The latter sequence also occurs in about 7% of *S. aureus* bridges.

The mechanisms by which peptides of such constant size and specific sequence might be synthesized are discussed.

Data presented in the preceding paper (Tipper and Berman, 1969) established that the peptide cross-links in the cell wall peptidoglycans of *Staphylococcus aureus* strain Copenhagen and *Staphylococcus epidermidis* strain Texas 26 are pentapeptides. The small amount of serine in *S. aureus* peptidoglycan is localized in the second residue from the N terminus of these pentapeptides, and the much larger amounts of L-serine in *S. epidermidis* pentaglycan account for an average of about 23, 7, 68, 4, and 3% of the first, second, third, fourth, and fifth residues, respectively, a total of about 1 mole/mole of glutamic acid. This nonrandom distribution indicates that individual pentapeptides have a few preferred sequences, and this conclusion is now verified by analyses of the products of digestion of cell walls with the *Myxobacter* AL-1 enzyme. This enzyme has both cell wall lytic and proteolytic activities (Ensign and Wolfe, 1964, 1965) and hydrolyzes D-lactyl-L-alanine,

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D-alanyl-glycine, and glycyl-glycine linkages within the cell wall peptidoglycan of *S. aureus* (Tipper *et al.*, 1967). Since the enzyme is devoid of glycanase activity, its action on cell walls of *S. aureus* resulted in the production of undegraded glycan, a complex of glycan, and teichoic acid and peptides of two types: basic peptides containing the N^{ϵ}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine fragment of the nucleotide precursor, and neutral oligoglycine peptides. Degradative and synthetic studies (Jarvis and Strominger, 1967) established that there were two major components of the basic peptides, carrying 1 or 2 moles of glycine on the ϵ-amino group of lysine. The neutral peptides comprising the remainder of the pentapeptide bridges should therefore be mostly tri- and tetraglycine. This is confirmed by the data presented in this paper, and the same procedure was used for the isolation of large fragments from the pentapeptide cross-bridges of *S. epidermidis*. It has now been demonstrated that the major basic peptides in the AL-1 hydrolysate of *S. epidermidis* are identical with those produced from *S. aureus*, and that the neutral peptides are probably derived from four types of bridges. These data are in accord with recent results on the biosynthesis of the bridges in this organism (Petit *et al.*, 1968).

Materials and Methods

The preparation of cell walls of *S. aureus* and *S. epidermidis*, removal of their ester-linked D-alanine residues, and analyses of the products have been described (Tipper and Berman, 1969). The *Myxobacter* AL-1 enzyme was a partially purified preparation kindly given by Dr. J. C. Ensign, Department of Bacteriology, University of Wisconsin, Madison. Lysostaphin was a gift from Dr. H. Browder, Mead Johnson Co., Evansville, Ind., and the *Chalaropsis* B endo-*N*-acetylmuramidase was a gift from Dr. J. Hash, Vanderbilt University School of Medicine, Nashville, Tenn. Di-, tri-, and tetraglycine, seryl-glycine, glycyl-serine, and seryl-glycyl-glycine were obtained from Cyclo Chemicals Corp., Long Beach, Calif. Determinations of the chain length of fractions of polysaccharide by reduction and periodate oxidation has been previously described (Tipper *et al.*, 1967). The isolation of basic and neutral peptides from the AL-1 enzyme digest of *S. aureus* cell walls has been previously described (Tipper *et al.*, 1967). Edman degradations were performed as described in the preceding paper (Tipper and Berman, 1969). Other analytical procedures have also been previously described (Ghuysen *et al.*, 1966). A Gilson Medical Electronics Model E machine was used for electrophoresis on Whatman No. 3MM paper (Reeve-Angel Co., 110 × 48 cm, 40 V/cm). Buffers were pH 1.9 (20 ml of concentrated formic acid/l.) and pH 4.6 (12 ml of acetic acid and 12 ml of pyridine per l). Peptides were detected by dipping in 0.2% ninhydrin in 95% ethanol containing 4% collidine, followed by heating for 10 min at 70°. Peptides were dinitrophenylated in triethylamine buffer (*cf.* Jarvis and Strominger, 1967) and their DNP derivatives were fractionated by thin-layer chromatography in *t*-amyl alcohol–chloroform–methanol–water–acetic acid (30:30:30:20:3, v/v) on silica gel G.

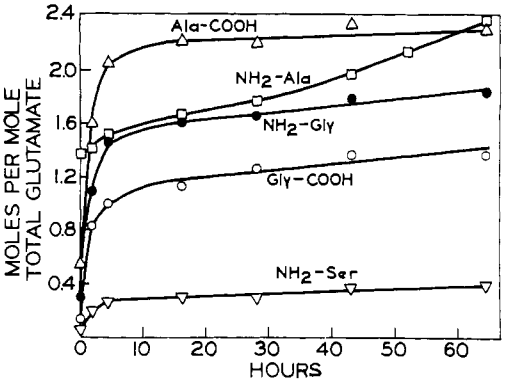

FIGURE 1: Kinetics of hydrolysis of *S. epidermidis* cell walls by AL-1 enzyme. Aliquots of the digest (see text) were immediately acidified to pH 4.5 with acetic acid and frozen. At the end of the incubation, these aliquots were solubilized by incubation with *Chalaropsis* B *N*-acetylmuramidase (5 μg) for 2 hr at 37° to minimize variation due to solubility before removal of aliquots (3 μl) for the determination of N- and C-terminal amino acids. Free amino acids were used as standards except for glycine, for which tetraglycine was used, since the yield of DNP-glycine varies with the chain length of glycine oligopeptides. A separate aliquot was hydrolyzed for the determination of total amino acids, on which the ratios recorded here were based.

Results

Kinetics of Liberation of N- and C-Terminal Amino Acids during Solubilization of S. epidermidis Cell Walls by AL-1 Enzyme. Boiled cell walls (540 mg) were suspended in 0.01 M Veronal buffer (23 ml) and the pH was adjusted to 9.0 with NaOH. AL-1 enzyme (4.1 mg) was added and the suspension was incubated at 37° with a few drops of toluene added. The pH, which drops as ester-alanine and peptide bonds are hydrolyzed, was maintained between 8 and 9 by frequent additions of NaOH. Solubilization was virtually complete in 5 hr, but incubation was continued, since experience with *S. aureus* cell walls (Tipper *et al.*, 1967) had shown that while complete solubilization required fairly complete hydrolysis of criss-bridges, it occurred long before substantial hydrolysis of D-lactyl-L-alanine linkages. After 40 hr, more enzyme (1.5 mg) was added, and the incubation was terminated at 64 hr. Aliquots (20 μl) were removed at intervals for the determination of free C- and N-terminal groups (Figure 1). Since boiled *S. epidermidis* cell walls contain, per mole of glutamic acid, 1.2 moles of D-alanine ester-linked to its teichoic acid (Tipper and Berman, 1969), this accounts for the initial 1.2 moles of N-terminal alanine, for some of the initial C-terminal alanine (since there is some hydrolysis during hydrazinolysis) and for 1.2 moles of the total C-terminal alanine liberated, since it is hydrolyzed to free alanine within a few hours at 37°, pH 9.0 (*cf.* Tipper *et al.*, 1967). Taking this into account, hydrolysis of the cell walls led to the rapid release, per mole of glutamic acid, of about 1 mole of C-terminal alanine and 0.9 mole of C-terminal glycine with parallel release of 1.4 moles of N-terminal glycine and 0.25 mole of N-terminal serine. This corresponds to an average extent of hydrolysis, of about 1.8 bonds/pentapeptide bridge, including almost complete hydrolysis of the linkages between D-alanine

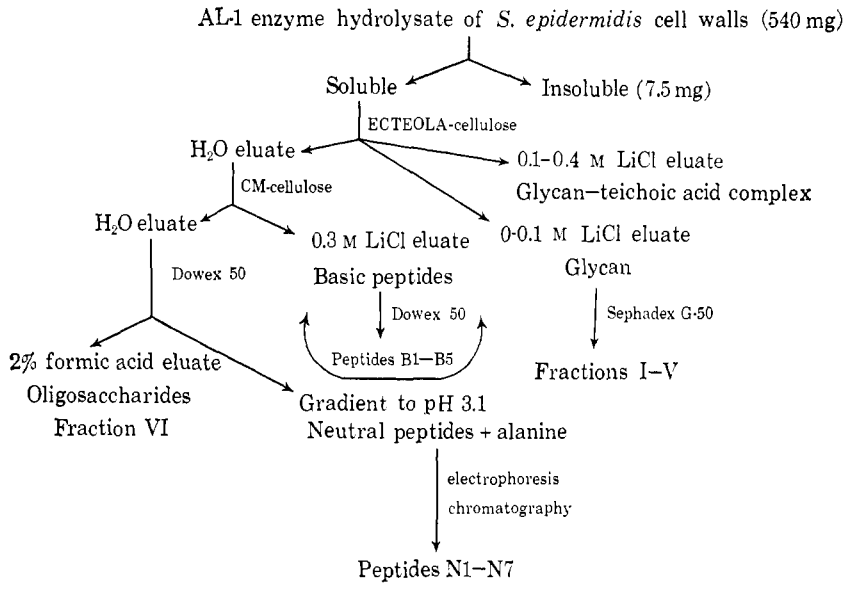

FIGURE 2: Summary of the fractionation of the AL-1 hydrolysate of *S. epidermidis* cell walls. After 64 hr, only 7.5 mg remained insoluble. This was mainly inorganic material and protein.

and the N termini of the pentapeptide cross-bridges. This was followed by slow release of 0.1 mole of N-terminal serine, 0.3 mole of N-terminal glycine, and 0.4 mole of C-terminal glycine, corresponding to further hydrolysis within the pentapeptides. An average of 2.1–2.3 bonds was hydrolyzed per pentapeptide bridge. There was also slow release of 1 mole of N-terminal alanine without parallel release of C-terminal amino acid, which therefore corresponds to hydrolysis of D-lactyl-L-alanine linkages (amidase action) occurring after hydrolysis of linkages within the pentapeptide bridges, as in the case of *S. aureus* (Tipper *et al.*, 1967).

Fractionation of the AL-1-Digest of S. epidermidis Cell Walls. The fractionation sequence was similar to that previously used for fractionation of the AL-1 digest

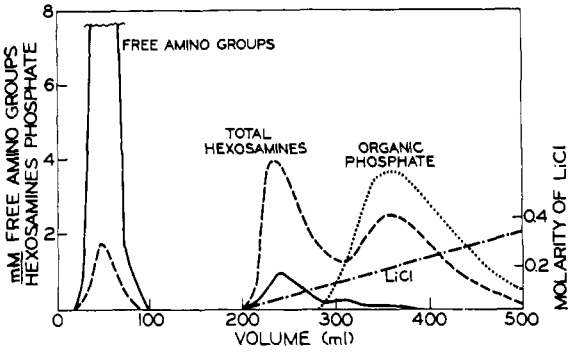

FIGURE 3: Fractionation of the AL-1 digest of *S. epidermidis* cell walls on ECTEOLA-cellulose. The entire digest was applied to a column (2 × 31 cm) of Bio-Rad Cellex E previously washed with 0.7 M LiCl and water. After elution with water (250 ml), a linear gradient of increasing LiCl concentration from 0 to 0.7 M was applied (total volume 900 ml). Aliquots of the fractions (5 ml) were analyzed for reducing power, free amino groups, and organic (total minus inorganic) phosphate with the results shown. LiCl was detected with $AgNO_3$, and its approximate concentration is calculated from the elution volume.

of *S. aureus* cell walls (Tipper *et al.*, 1967; Figure 10) and is summarized in Figure 2. Centrifugation (20 min, 20,000*g*) removed insoluble material (7.5 mg), and the supernatant was fractionated on ECTEOLA-cellulose with the results shown in Figure 3. The pattern was very similar to that produced by fractionation of the AL-1 enzyme digest of *S. aureus* (Tipper *et al.*, 1967). Water eluted an unretarded peak of free amino groups containing some hexosamines, and dilute LiCl eluted a second and major peak of hexosamines (glycan) which contained no phosphate and very few free amino groups and which was followed by a peak containing all the organic phosphate and more hexosamines (teichoic acid plus covalently linked glycan). This fractionation indicates almost complete separation of peptide and glycan by amidase action, as indicated by the data in Figure 1. The water eluate was fractionated at room temperature on a column of CM-cellulose (Bio-Rad Cellex CM, 2 × 60 cm) previously washed with 0.5 M LiCl and water. Water eluted on unretarded peak containing all the reducing power (oligosaccharides) and some of the free amino groups (free alanine and neutral peptides) while 0.3 M LiCl eluted a second peak of free amino groups (basic peptides). These two eluates were separately fractionated on columns of Dowex 50 as described below.

Fractionation on Dowex 50 of the Basic Peptides from AL-1 Enzyme Digestion of S. epidermidis Cell Walls. The 0.3 M LiCl eluate from CM-cellulose was lyophilized and suspended in 95% EtOH. After standing for 2 hr at −15°, the insoluble peptides were precipitated by centrifugation and the LiCl solution was decanted. The residue was dissolved in 2% formic acid and fractionated on Dowex 50 as shown in Figure 4. This pattern is quite similar to that obtained on Dowex 50 chromatography of the basic peptides from AL-1 enzyme digestion of *S. aureus* cell walls (*cf.* Jarvis and Strominger, 1967; Figure 1). Fractions were pooled as shown (B1–B5).

TABLE I: Analyses of Major Fractions of Basic Peptides from *S. epidermidis* Cell Walls.[a]

Peptide	% of Total Peptide[b]	Lys	NH_3	Gly	Ser	Alanine			N Terminal			C Terminal	
						Total	D	L	Ala	Gly	Ser	Gly	Ala
B2	9	0.9	0.9	1.3	0.2	2.8	1.9	1.1	0.9	0.8	0.1	0	0.9
B3	24	0.9	1.1	1.7	0.1	2.0	1.2	1.0	1.1	0.9	0.1	0.1	1.1
B4	56	1.0	0.9	0.9	<0.1	1.7	1.2	1.2	1.1	0.6	0	<0.1	0.9

[a] Data are expressed as moles per mole of total glutamic acid. Controls for N-terminal glycine, alanine, and serine were diglycine, alanyl-glycine, and seryl-glycine, respectively. Controls for C-terminal alanine and glycine were glycyl-alanine and alanyl-glycine, respectively. Only traces of other C-terminal amino acids were found. Fraction B1 and B5 contained many minor peptides accounting for only 11% of the total glutamic acid. The total serine + glycine content of peptides B1–B7 was 290 μmoles, and their total glutamic acid content was 205 μmoles. [b] Based on glutamic acid content.

Electrophoresis of samples at pH 1.9 and 4.6 showed B1 and B5 to contain several minor ninhydrin-positive components, while B2 and B3 apparently each contained a single component, even though B2 obviously is a mixture (Figure 2). B4 contained a single major ninhydrin-positive component, and two minor components. Essentially the same results were obtained following thin-layer chromatography of the DNP derivatives of these fractions (see Methods). Further fractionation was not attempted. Analyses of pooled fractions B2, B3, and B4 are given in Table I and indicate that peptides B3 and B4, except for the presence of small amounts of serine, have approximately the compositions of the major peptides (2 and 1, respectively, Jarvis and Strominger, 1967) produced on digestion of *S. aureus* cell walls with the AL-1 enzyme. These are N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine with, respectively, diglycine and glycine on the ε-amino group of lysine. Analyses of peptide B2 are consistent with a mixture of approximately equal parts of N^{α}-(L-alanyl-D-isoglutamyl)-L-lysyl-D-alanyl-D-alanine, substituted by either glycine or diglycine. These are presumably derived from the C-terminal peptide subunits of the peptidoglycan (*cf.* Tipper and Berman, 1969). All three peptides contain the expected 1 mole of N- and C-terminal alanine and N-terminal glycine per mole of glutamic acid, and all three are therefore monomers. After one cycle of Edman degradation, N-terminal alanine in peptides B2, B3, and B4 was replaced by N-terminal glutamic acid. No L-alanine remained in the products, indicating (NH_2)-L-alanyl-D-glutamic acid. The C-terminal alanine therefore has the D configuration in all three peptides and lysine must be acylated by glutamic acid in B4, and presumably also in B2 and B3. N-Terminal glycine in B4 was replaced by N^{ϵ}-terminal lysine indicating N^{ϵ}-glycyl-lysine (and N^{α}-glutamyl-lysine by elimination) and this was partially true also for B2, while B3 retained N-terminal glycine. After a second cycle of degradation, N-terminal glutamic acid disappeared in all three peptides without appearance of α-N-terminal lysine, indicating γ-D-glutamyl-lysine. The mole of ammonia in each peptide is therefore on the α-COOH group of glutamic acid. N-Terminal glycine in B3 and residual N-terminal glycine in B2 were replaced by N^{ϵ}-terminal lysine indicating N^{ϵ}-(glycyl-glycyl)-L-lysine in these two peptides. *S. epidermidis* cell walls (1 mg) contained 0.48 μmole of peptidoglycan subunit (based on glutamic acid; Tipper and Berman, 1969), so recovery of basic peptides was 81%.

Fractionation of Neutral Peptides from AL-1 Enzyme Digestion of S. aureus Cell Walls. Peptides in the AL-1 enzyme digest of *S. aureus* cell walls (1.0 g) were fractionated from glycan and teichoic acid by chromatography on ECTEOLA-cellulose followed by chromatography of the water eluate on Sephadex G-25 as previously de-

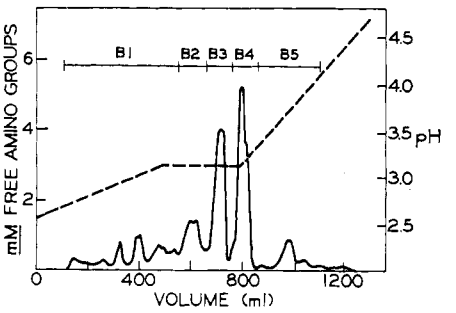

FIGURE 4: Fractionation of the basic peptides from AL-1 enzyme digestion of *S. epidermidis* cell walls on Dowex 50. Dowex 50-X8 (Beckman 15A resin) was washed with 2 N NaOH, water, 2 N HCl, water, and 1% pyridine, in that order. The washed resin, suspended in buffer A (20 ml of 90% formic acid/l., pH 1.9), was packed at 50° in a jacketed column (2 × 15 cm) and equilibrated with buffer A. The basic peptides were applied to the column in buffer A and eluted at 0.5 ml/min first with a linear gradient (500 ml) from pH 2.6 (equal volumes of buffer A and buffer B, the pH 3.1 pyridine acetate buffer of Jones, 1964) to pH 3.1 (buffer B), then with buffer B (300 ml), and finally with a second linear gradient (500 ml) from pH 3.1 to 5.2 (buffer C, pyridine acetate; Jones, 1964). Fractions of 7 ml were collected, and the interpolated variation in pH is shown. Aliquots (40 μl) of the fractions were dried *in vacuo* then analyzed for free amino groups. Fractions B1–B5 were pooled as shown.

TABLE II: Chromatographic and Electrophoretic Mobilities of Glycine and Serine Peptides.[a]

Peptide	Ser	Ala	Gly	Di-Gly	Tri-Gly	Tetra-Gly	Penta-Gly	SG	GS	SGG
Paper chromatography	100	190	106	100	78	60	50	96	81	78
Thin-layer chromatography		120	104	100	77	62	52	98	85	83
Electrophoresis, pH 1.9	68	75	91	100	87	76	69	88	89	79
Electrophoresis, pH 9.2		18		100	92	85		87	94	
Thin-layer chromatography of dinitrophenyl derivatives										
Solvent C			190	100	63	42	30			
Solvent D		123		100	81	66	55			

[a] All mobilities are given relative to diglycine. The solvent for thin-layer chromatography on silica gel G was the same as used for paper chromatography (butanol–acetic acid–water, 3:1:1, v/v), two sequential elutions being made in each case. pH 1.9 buffer contained 20 ml of 90% formic acid/l., and pH 9.2 buffer was 0.05 M $Na_2B_4O_7$. Solvent C for dinitrophenyl derivatives was butanol–water–contentrated NH_3 (100:100:2, v/v), upper layer, and solvent D was *t*-amyl alcohol–chloroform–methanol–water–concentrated NH_3 (30:30:30:6:2, v/v). Peptides were detected with 0.2% ninhydrin in 100% ethanol containing 2% trimethylpyridine. Papers were dipped and thin layers were sprayed and heated for 5 min at 70°. After electrophoresis in borate, the paper was first sprayed with 5% ethanolic acetic acid and thoroughly dried at 80° before detection with ninhydrin.

scribed (Tipper *et al.*, 1967). This peptide fraction was further fractionated into neutral and basic peptides by chromatography on CM-cellulose as described above, water eluting the unretarded neutral peptides and free alanine. Amino acid analysis showed this mixture to consist almost entirely of glycine and alanine, and paper and thin-layer chromatography and paper electrophoresis and thin-layer chromatography of dinitrophenyl derivatives indicated the presence of free alanine and of di-, tri-, and tetraglycine (Table II). Peptides with N-terminal glycine initially give an easily identifiable yellow color with ninhydrin reagent, whereas seryl glycine, for example, gives an initial blue color. It was anticipated from the pK_a values of di-, tri-, and tetraglycine (3.0–3.3 at 50°) that they would be rapidly eluted from Dowex 50 by buffer B (pH 3.1, the lowest pH for elution by Jones (1964)). A trial fractionation of di-, tri-, and tetraglycine (10 μmoles each) was made using the column described in Figure 4, previously equilibrated with buffer A. A linear gradient from pH 1.9 (buffer A) to pH 3.1 (buffer B, total volume 500 ml) was applied and followed by elution with buffer B (300 ml). Analysis of the fractions by dinitrophenylation and thin-

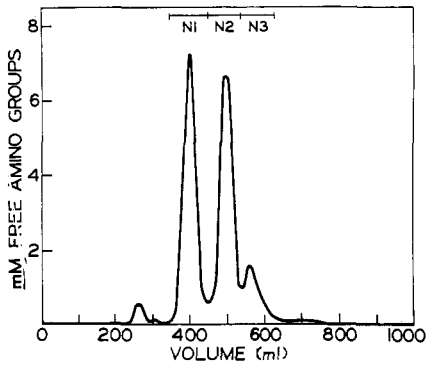

FIGURE 5: Fractionation of the neutral peptides from AL-1 enzyme digestion of *S. aureus* cell walls on Dowex 50. The column described in Figure 4 was reequilibrated with buffer A and the mixture of peptides was applied in this buffer. The column was eluted with a linear gradient from a mixture of buffer A (300 ml) with buffer B (200 ml) to buffer B (total volume 1000 ml) and aliquots (50 μl) of the fractions (7.5 ml) were lyophilized and analyzed for free amino groups by dinitrophenylation. Peaks were pooled as shown.

TABLE III: Analyses of the Neutral Peptides from AL-1 Enzyme Digestion of *S. aureus* Cell Walls.[a]

Peptide	Moles/Mole of Total Glycine: N-Terminal Glycine	Moles/Mole of Total Glycine: C-Terminal Glycine	Identity	Peptide (μmoles)
N1	0.23	0.27	Tetraglycine	214
N2	0.31	0.35	Triglycine	242
N3	0.46	0.49	Diglycine	40

[a] Glycine was the only amino acid present in each peptide, except for traces of serine. The micromoles of peptide were calculated from the total glycine contents assuming the identities indicated, and allowing for aliquots removed during fractionation. The controls for N- and C-terminal determinations were di-, tri-, and tetraglycine, and the yield of N-terminal glycine was improved by dinitrophenylation for 60 min at 60° in 0.1 M triethylamine–0.05 M fluorodinitrobenzene in 60% ethanol (60 μl).

TABLE IV: Chromatographic and Electrophoretic Mobilities of Neutral Peptides from AL-1 Digestion of *S. epidermidis* Cell Walls.[a]

Peptide:	N1	N2	N3	N4	N5	N6	N7
Identity:	SGSG	GGSG	GGG	GSG	GGG	SG	GG
Paper chromatography	55	55	60	70	78	96	100
Thin-layer chromatography	60	60	62	75	77	98	100
Paper electrophoresis	65	71	76	79	87	88	100

[a] Mobilities are expressed relative to diglycine. Solvent A (Table II) was used for paper and thin-layer chromatography, and pH 1.9 buffer (Table II) for electrophoresis. Identities given were deduced from subsequent analyses (see text): SG = seryl-glycine, etc.

layer chromatography indicated elution of tetraglycine at 400 ml, completely separated from triglycine at 450 ml and diglycine at 475 ml.

The column was reequilibrated with buffer A, and the mixture of neutral peptides was applied in this buffer and eluted as shown in Figure 5. Peak N1 behaved chromatographically and electrophoretically as tetraglycine, peak N2 as triglycine, and peak N3 as diglycine. Their analyses are given in Table III, and confirm these identities. Since 1 g of *S. aureus* cell walls contained 480 μmoles of peptidoglycan subunit (Tipper and Berman, 1969), the yields of di-, tri-, and tetraglycine, per mole of total glutamic acid, were 0.1, 0.5, and 0.45 mole, respectively.

Hydrolysis of Glycine Oligopeptides by the AL-1 Enzyme. Samples of di-, tri-, tetra-, and pentaglycine (0.1 μmole) were incubated at 37° in 0.01 M Veronal buffer (pH 9, 50 μl) containing AL-1 enzyme (5 μg). After 18 hr, aliquots (10 μl) were dinitrophenylated and subjected to thin-layer chromatography in solvent C (see Table II). Spots corresponding to the different DNP-oligoglycine peptides were extracted and quantitated. While di- and triglycine were unaffected, 15% of the tetraglycine was converted into diglycine, and 75% of the pentaglycine was converted into equimolar amounts of di- and triglycine. No peptide gave rise to free glycine. It is therefore concluded that the AL-1 enzyme does not further hydrolyze di- and triglycine, while it very slowly cleaves tetraglycine to 2 moles of diglycine, and more rapidly cleaves pentaglycine to di- and triglycine. Similar results have recently been reported by Jackson and Wolfe (1968). Assuming that all the diglycine in the digest of *S. aureus* was derived from tetraglycine, the digestion resulted in hydrolysis of half of the bridges between the third and fourth glycine residues (producing triglycine), and half between the fourth and fifth residues (producing tetraglycine, 10% of which was subsequently hydrolyzed to diglycine).

Fractionation of the Neutral Peptides from AL-1 Enzyme Digestion of S. epidermidis Cell Walls. The water eluate from ECTEOLA chromatography of the digest contained peptides, free alanine, and unretarded oligosaccharides of low molecular weight. All but the basic peptides were also eluted with water from the CM-cellulose column. This eluate was fractionated on Dowex 50 with the results shown in Figure 6. Initial elution with buffer A alone eluted the unretarded oligosaccharide fraction VI (not shown, detected by determination of total hexosamines) while the gradient of increasing pH eluted a series of peaks containing free amino groups. Individual fractions were analyzed by paper and thin-layer chromatography and by paper electrophoresis (Table IV). The largest peak (fractions 22–24) corresponded to the elution of free alanine. Fractions 19 and 20 contained several minor unidentified peptides of low mobility while fractions 20 and 21 contained two major peptides, N1 and N2. These peptides gave a single band moving more slowly than tetraglycine on chromatography, but were narrowly separated by paper electrophoresis at pH 1.9 where they again moved slower than tetraglycine. The initial colors given on paper with ninhydrin by peptides N1 and N2 were blue and yellow, respectively, so peptide N2 (but not peptide N1) apparently had N-terminal glycine. Peptide N2 did not

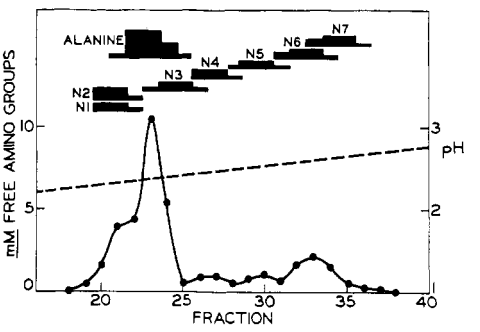

FIGURE 6: Fractionation of the neutral peptides from AL-1 enzyme digestion of *S. epidermidis* cell walls on Dowex 50. The mixture of neutral peptides, free alanine, and oligosaccharides, eluted with water from the CM-cellulose column, was concentrated to 5 ml, adjusted to pH 1.9 with formic acid, and applied to the column used in Figure 5 which had been previously equilibrated with buffer A (2% formic acid, pH 1.9) at 50°. The column was eluted at 1 ml/min first with buffer A (100 ml) then with a gradient from buffer A plus buffer B (200 ml plus 300 ml) to buffer B (500 ml). Fractions of 8 ml were collected and analyzed for free amino groups as shown. All fractions were also analyzed by paper electrophoresis and chromatography. The presence of free glycine, alanine, and peptides NI–N7 in the fractions was as indicated (see text).

TABLE V: Analyses of Neutral Peptides from AL-1 Enzyme Digestion of *S. epidermidis* Cell Walls.[a]

Peptide	Identity	Total μmoles		Peptide (μmoles)	N Terminal		C Terminal	Products of Edman Degradation		
		Glycine	Serine		Gly	Ser	Gly	First	Second	Third
N1	Ser-Gly-Ser-Gly	38.2	36.7	37.5	0.10	0.85	1.05	Gly-Ser-Gly	Ser-Gly	Gly
N2	Gly-Gly-Ser-Gly	24.8	84.6	83.1	0.95	0	0.70	Gly-Ser-Gly	Ser-Gly	Gly
N3	Tetra-Gly	38.6	1.6	10.0	0.95	0	0.90	Tri-Gly	Di-Gly	Gly
N4	Gly-Ser-Gly	34.2	17.6	17.3	1.05	0	1.30	Ser-Gly	Gly	
N5	Tri-Gly	70.1	0.9	23.5	0.85	0	1.10	Di-Gly	Gly	
N6	Ser-Gly	32.3	30.3	31.3	0.15	0.80	0.90	Gly		
N7	Gly-Gly	34.3	0.2	17.2	0.90	0	0.95	Gly		
Glycine	Gly	4.5	0	4.5	1.0	0	1.0			

[a] Total amino acids were determined with the amino acid analyzer. The total micromoles present in each peptide were corrected for losses during fractionation (*e.g.*, on guide strips removed during preparative chromatography) and for aliquots removed for analyses. N- and C-terminal amino acids are expressed as moles per mole of peptide of the assumed structure. No other amino acids or C-terminal amino acids were present. Unique products, as indicated, were formed at each stage of Edman degradation of all peptides (analyzed by chromatography and electrophoresis).

have the same mobilities as pentaglycine. Fractions 23–25 contained a peptide (N3), indistinguishable from tetraglycine (Tables II and IV), and fraction 24 also contained a trace of free glycine. Fractions 26–29 contained a peptide (N4) giving a yellow color with ninhydrin and moving between tri- and tetraglycine on chromatography and electrophoresis, while fractions 28–31 contained a peptide (N5) indistinguishable from triglycine (Tables II and IV). Fractions 31–34 contained a peptide (N6) indistinguishable from serylglycine (blue with ninhydrin), and 33–36 a peptide (N7) (yellow with ninhydrin) indistinguishable from glycylglycine, but distinguishable from glycyl-serine by chromatography, and electrophoresis. The approximate distribution of these peptides in the Dowex 50 fractions is indicated in Figure 6.

Peptides N1 and N2 were first fractionated from free alanine and contaminating minor peptides by preparative paper chromatography. Fractions 20–22 were pooled, concentrated to dryness *in vacuo*, redissolved in water, streaked along the base line of a sheet of Whatman No. 3MM paper, and eluted with solvent A overnight. Peptides N1 and N2 were detected on guide strips with ninhydrin and eluted from the rest of the paper with 0.1 M acetic acid. The eluate was concentrated to dryness and shown by chromatography of an aliquot to be free of alanine and other peptides. This material was then applied across the width of three sheets (48 cm wide) of Whatman No. 3MM paper for preparative electrophoresis at pH 1.9. Again peptides bands were located by treating guide strips with ninhydrin, and the areas corresponding to the blue (peptide N1) and yellow (peptide N2) bands were eluted as before. The eluates were dried, and electrophoresis of aliquots showed peptide N2 to be free of peptide N1 while peptide N1 was still contaminated by peptide N2. These were completely separated by a second preparative electrophoresis.

Peptide N3 was isolated free of alanine and glycine after preparative paper chromatography as described above. Peptides N4 and N5 were substantially pure in fractions 26–27 and 29–30, respectively, and peptides N5 and N6 in fraction 31 were fractionated by preparative paper chromatography as described above. Fractions 32–35 were pooled, dried, and fractionated by preparative electrophoresis at pH 1.9 as described above giving complete separation of peptides N6 and N7. The fractionated peptides, N1–N7, were finally separated from contaminants (derived from the Dowex 50 column buffers and the elutions from paper) by fractionation, in water, on columns (40 × 1 cm) of Bio-Gel P2. Peptide peaks were detected by determination of free amino groups and pooled.

Analyses and Identification of the Neutral Peptides from S. epidermidis. Analyses of the fractionated and purified peptides are given in Table V. Glycine and serine were the only amino acids present. Each peptide had a unique N-terminal amino acid that corresponded with the color given with ninhydrin, and each had exclusively C-terminal glycine. Amounts of C- and N-terminal amino acids were consistent with chain lengths of four for peptides N1, N2, and N3, three for peptides

TABLE VI: Glycan Fractions from AL-1 Enzyme Digestion of *S. epidermidis* Cell Walls.[a]

Fraction	Hexosamine Content	% Total Hexosamines	Av Chain Length	Glucosamine	Glutamic Acid
I	27.2	10.3	89	102	14
II	53.0	20.0	46	107	13
III	41.8	15.8	25	108	12
IV	33.6	12.7	16	103	14
V	50.7	19.2	10	106	16
VI	58.1	22.0	6	108	18

[a] Fractions I–V are those obtained from Sephadex G-50 fractionation (Figure 7) of the glycan eluted from ECTEOLA-cellulose with 0–0.1 M LiCl. Fraction VI is glycan unretarded by ECTEOLA and CM columns, and eluted from Dowex 50 with buffer A. This eluate was concentrated *in vacuo* to about 1 ml and deionized by passage through a column of Retardion resin (Bio-Rad AG-11A8, 1 × 30 cm) eluted with water. Figures for total hexosamines are corrected for aliquots removed for analyses, etc. Glucosamine and glutamic acid contents are given per 100 moles of muramic acid. Each fraction contained approximately equimolar glutamic acid, lysine, glycine, and ammonia, 2 moles of alanine, and only traces of serine. Chain lengths were estimated from K_D values on Sephadex G-50 (Figure 7), and aliquots containing about 0.1 μmole of reducing end group were reduced overnight at room temperature in unbuffered 0.04 M $NaBH_4$ (50 μl). After acidification to pH 4.5 with 0.2 M ACOH (20 μl), no reducing power remained. $NaIO_4$ (0.01 M, 8 μl) was added, and aliquots (7 μl) were analyzed for formaldehyde at intervals. A plateau was reached within 10 min and the chain length is the ratio of this plateau level of formaldehyde released to total hexosamine content, after subtraction of the formaldehyde produced by oxidation of the unreduced glycan fraction under identical conditions. The disaccharide subunit of the glycan has a molecular weight of 478, so that fractions I and II have average molecular weights of 21,000 and 11,000, respectively, based on formaldehyde production. Sephadex G-50 excludes dextran of mol wt 10,000 and globular protein of mol wt 70,000.

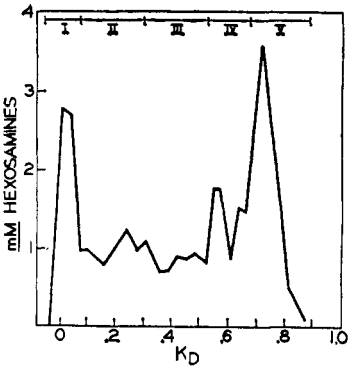

FIGURE 7: Fractionation on Sephadex G-50 of the glycan from AL-1 enzyme digestion of *S. epidermidis* cell walls. The glycan was applied to a column (2 × 60 cm) of Sephadex G-50 (fine bead form) and eluted with water at room temperature at 0.3 ml/min. Aliquots (17 μl) of the fractions (4 ml) were analyzed for total hexosamines. Fractions I–V were pooled as indicated, lyophilized, and redissolved in water (2 ml).

N4 and N5, and two for peptides N6 and N7. These data indicated peptide N1 to be NH_2-Ser-(Ser,Gly)-Gly-COOH, peptide N2 to be NH_2-Gly-(Ser,Gly)-Gly-COOH, peptide N3 to be tetraglycine, peptide N4 to be NH_2-Gly-Ser-Gly-COOH, peptide N5 to be triglycine, peptide N6 to be Ser-Gly, and peptide N7 to be diglycine .The sequences in N1 and N2 were demonstrated to be Ser-Gly-Ser-Gly and Gly-Gly-Ser-Gly, respectively, by two methods: partial acid hydrolysis and Edman degradation. The sequence in peptides N3–N7 was confirmed by the same methods.

Acyl-serine linkages are peculiarly sensitive to acid hydrolysis (Desnuelle and Casal, 1948). Nishimura *et al.* (1965) used 10 N HCl at 30° to achieve partial acid hydrolysis of alternating Ser-Leu polypeptide, but 10 N HCl at room temperature was found to give a greater differential rate of hydrolysis of acyl-glycine and acyl-serine linkages in the present studies: samples (50 mμ-moles) of di-, tri-, and tetraglycine, of seryl-glycyl-glycine, seryl-glycine, and of peptides N1, N2, and N4 were sealed in tubes containing 10 N HCl and incubated at room temperature. Tubes were opened and lyophilized after 0, 2, 4, 8, 14, 28, and 57 hr. All were subsequently analyzed qualitatively by electrophoresis at pH 1.9 and quantitatively by dinitrophenylation and thin-layer chromatography; 95% of diglycine remained unhydrolyzed after 57 hr, while 40% of triglycine (and peptide N5) was converted into roughly equimolar amounts of glycine and diglycine. Tetraglycine (and peptide N3) was almost completely hydrolyzed by 57 hr, mostly to diglycine though some glycine and triglycine were produced. Seryl-glycine (and peptide N6) was totally resistant to hydrolysis and seryl-glycyl-glycine was 20% hydrolyzed to seryl-glycine plus glycine. Peptide N1 was completely hydrolyzed within 12 hr, giving rise to 2 moles of seryl-glycine and traces of free serine and glycine. Peptide N2 was hydrolyzed at a similar rate, to equimolar amounts of seryl-glycine and diglycine while peptide N4 was hydrolyzed somewhat more slowly but completely to free glycine and seryl-glycine.

The products of Edman degradation were qualitatively analyzed by paper electrophoresis and paper chromatography, with the results shown in Table V. In summary, as indicated in Table V, peptide N1 was seryl-glycyl-seryl-glycine and peptide N2 was glycyl-glycyl-seryl-glycine.

The total corrected recovery of glycine and serine was 673 μmoles, which, together with that in the basic peptides (Table I), gives a total recovery of 963 μmoles, 4.69 moles/mole of glutamic acid (205 μmoles, Table I). The recoveries of neutral tri- and tetrapeptides (40.8 and 130.6 μmoles, respectively) were similar to the re-

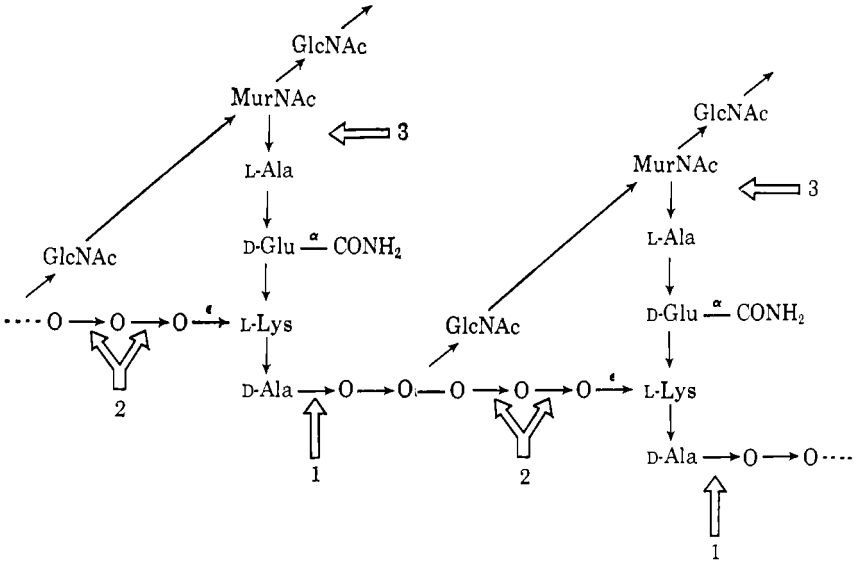

FIGURE 8: Sites of hydrolysis of the peptidoglycans of *S. aureus* and *S. epidermidis* by the *Myxobacter* AL-1 enzyme. The bridge pentapeptides are represented by the circles. The primary site of hydrolysis is at the N terminus of the bridges (1), but secondary hydrolysis within the bridges (2) follows rapidly. Amidase action (3) is very much slower and is inhibited by prior action of an *N*-acetylmuramidase. GlcNAc = *N*-acetylglucosamine; MurNAc = *N*-acetylmuramic acid.

coveries of peptides B2 and B3 (49 and 115 μmoles, respectively), as expected.

Characterization of the Glycan from AL-1 Enzyme Digestion of S. epidermidis Cell Walls. Muramic acid (56 μmoles) in the AL-1 enzyme digest of *S. epidermidis* cell walls was fractionated with the teichoic acid on ECTEOLA-chromatography, while another 30.5 μmoles of muramic acid was not retarded by the ECTEOLA column (Figure 3). However, about 100 μmoles was eluted from the ECTEOLA column with LiCl before the teichoic acid (Figure 3). These fractions were pooled, concentrated, and fractionated by chromatography on Sephadex G-50 with the results shown in Figure 7. Each of the pooled fractions contained approximately equimolar glucosamine and muramic acid, and relatively little peptide (Table VI). Analyses of chain lengths (Table VI) indicated a similar range to that found in *S. aureus* (Tipper *et al.*, 1967) but with a greater preponderance of both long- and short-chain length material. The average chain length calculated from the data in this table is 13 hexosamines, while for fractions 1–4 only, the average chain length is 20 hexosamines, close to the figure of 25 hexosamines found for a similar fraction of the *S. aureus* glycan (Tipper *et al.*, 1967). The total recovery of muramic acid, 189 μmoles, was close to the total recovered glutamic acid (205 μmoles).

A sample (0.2 μmole muramic acid) of glycan fraction II was hydrolyzed in 90 μl of 0.02 M KPO_4 (pH 7.5) with lysotaphin (10 μg). Reducing power release was complete in 40 min, and after reduction with $NaBH_4$, 17% of the glucosamine and 98% of the muramic acid remained, indicating 83% hydrolysis to *N*-acetylmuramyl-*N*-acetylglucosamine disaccharides. The product had the same mobility as 4-*O*-β-*N*-acetylmuramyl-*N*-acetylglucosamine on paper chromatography (*cf.* Table II) and on paper electrophoresis at pH 3.9 (*cf.* Tipper and Berman, 1969). It had the characteristically low reducing power and 30-min Morgan–Elson color of the 1,4-linked disaccharide (Tipper and Strominger, 1966).

Discussion

The mode of action of the *Myxobacter* AL-1 peptidase on the cell walls of *S. aureus* (Figure 8; Tipper *et al.*, 1967) has been confirmed by demonstration that the cross-bridges in their peptidoglycan are pentapeptides (Tipper and Berman, 1969), and by isolation, following hydrolysis of these cell walls with the AL-1 enzyme, of tri- and tetraglycine in amounts equal to the amount of N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine, substituted on its lysine ε-amino group by diglycine and glycine, respectively. The isolation in high yield of the same two basic peptides from the AL-1 enzyme digestion of *S. epidermidis* cell walls demonstrates that these walls contain the same peptide sequence. Biosynthetic studies (Petit *et al.*, 1968) have shown that particulate enzyme preparations from cells of both organisms will incorporate the peptide from UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine into peptidoglycan. Isolation of these peptides and neutral di-, tri-, and tetrapeptides of glycine and serine, also demonstrates that the AL-1 enzyme hydrolyses the cell wall peptidoglycan of *S. epidermidis* (whose cross-bridges are also pentapeptides) in exactly the same way as that of *S. aureus*, although there is proportionally less hydrolysis between cross-bridge residues 3 and 4 (Figure 8) in *S. epidermidis*. This is probably due to the prevalence of serine in position 3 of these cross-bridges, since the lack of C-terminal serine in the neutral peptides derived from them indicates that the AL-1 enzyme has relatively low activity against L-seryl-glycine linkages. Analyses of the basic peptides also showed that more than 95% of the fourth

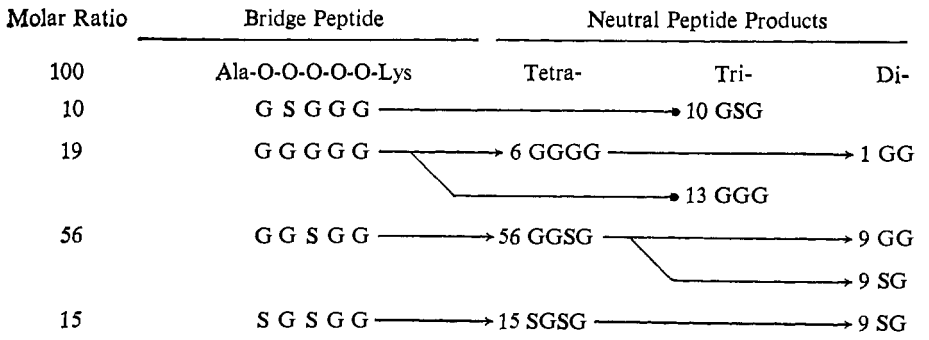

FIGURE 9: Hypothetical sites and extent of hydrolysis by the AL-1 enzyme of four types of pentapeptide cross-bridges in *S. epidermidis*. The recovered pattern of di-, tri-, and tetrapeptides is reproduced. G = glycyl; S = seryl.

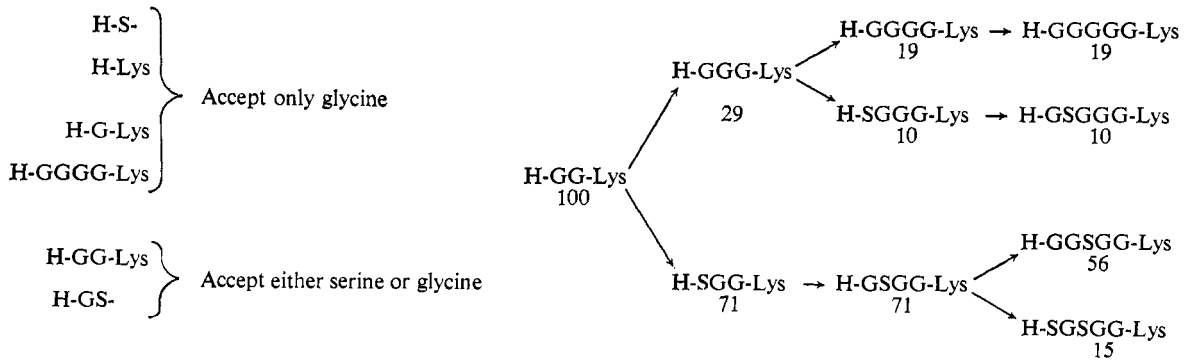

FIGURE 10: Sequential addition biosynthetic scheme. Sequence of acylations of growing N termini of bridge peptides required to give the four types of pentapeptide bridges in the ratios shown in Figure 9. A maximum of 11 enzymes is required, or fewer enzymes with the acceptor specificities outlined on the left.

and fifth cross-bridge residues in *S. epidermidis* are glycine.

It has recently been reported (Kato *et al.*, 1968) that the L_{11} enzyme produced by a *Flavobacterium* hydrolyzes cell walls of *S. aureus* in a similar fashion to the AL-1 enzyme, producing basic peptides and di-, tri-, and tetraglycine. However only partial hydrolysis of D-alanylglycine and *N*-acetylmuramyl-L-alanine linkages occurred. It has also been found that the staphylolytic enzyme produced by a *Pseudomonas* species (Zyskind *et al.*, 1965) hydrolyzes these walls in a very similar fashion, producing di-, tri-, and tetraglycine, but that it has almost no amidase action (M. Lache, W. R. Hearn, J. W. Zyskind, D. J. Tipper, and J. L. Strominger, submitted for publication).

Since the cross-bridges of *S. epidermis* are pentapeptides, the neutral tetrapeptides must all have been derived from residues 1 to 4 of these pentapeptides, and must therefore have been derived from three different kinds of bridges; pentaglycine, Gly-Gly-Ser-Gly-Gly, and Ser-Gly-Ser-Gly-Gly. Since the AL-1 enzyme hydrolyzes tetraglycine almost exclusively to diglycine and very little free glycine was produced in the hydrolysis, the neutral tripeptides must have been derived from residues 1 to 3 of cross-bridge pentapeptides of two types, pentaglycine and Gly-Ser-Gly-Gly-Gly. This is confirmed by the similar recoveries of total neutral tripeptides and of basic peptides containing N-terminal diglycine. Since the AL-1 enzyme does not hydrolyze triglycine and little basic peptide with 3 moles of glycine plus serine was produced, most of the neutral dipeptides must have been derived from hydrolysis of tetrapeptides. Based on these assumptions, the hydrolysis of the cross-bridges in the peptidoglycan of *S. epidermidis* can be envisaged as in Figure 9. The ratios of recovered neutral peptides are reproduced by assuming just four different cross-bridge sequences in the proportions shown. The average per cent of serine at each position would then be 15, 10, 71, 0, and 0, close to the figures of 23, 7, 68, 4, and 3 derived from Edman degradation of the glycopeptide monomers (Tipper and Berman, 1969). The total serine and glycine per glutamic acid are 0.96 and 4.04, respectively, close to the over-all amino acid composition of the cell walls (0.97 and 3.47, respectively). The failure to isolate Gly-Ser-Gly-Gly is unexplained.

The incorporation of glycine into the *S. aureus* peptidoglycan has been studied by Matsuhashi *et al.* (1967), and studies of the incorporation of glycine and serine into the *S. epidermidis* peptidoglycan were made at the same time as the structural studies reported here, and have recently been described (Petit *et al.*, 1968). Although the crude membrane particle preparations that contain these activities contain ribosomes, and the aminoacyl-tRNAs are obligatory intermediates in synthesis of the cross-bridges, this process is totally resistant to all the antibiotic inhibitors of ribosome function, and all attempts to identify peptidyl-tRNA during peptido-

glycan synthesis have failed. It is therefore possible that these pentapeptides are synthesized by sequential acylation of the N terminus, starting at the ε-amino group of lysine. Since the studies presented in this and the preceding paper show that the products are of constant length and have a few preferred sequences (the presence of a few per cent of other lengths and sequences cannot be ruled out), it remains to be shown how these lengths and sequences are determined. A sequential addition scheme with eleven specific enzymes (or fewer less specific enzymes) is shown in Figure 10. The proportions of the various products should vary with the availability of aminoacyl-tRNA precursors, and this has been demonstrated *in vitro* by Petit *et al.* (1968). The variability of the serine content of the cell walls of this organism has been demonstrated *in vivo* by H. P. Browder (personal communication). Petit *et al.* (1968) also showed that while glycine incorporation was independent of the presence of seryl-tRNA, serine incorporation was to a large extent dependent upon the simultaneous presence of glycyl-tRNA, in accord with the sequential scheme shown in Figure 10.

Except for the replacement of some glycine by L-serine, the cell wall peptides of *S. aureus* and *S. epidermidis* appear to be identical. Hydrolysis of both cell walls with the AL-1 enzyme produced an undegraded glycan stripped of 90% of its peptide. These had similar size distributions, and parts of both were linked to teichoic acid. Hydrolysis of both cell walls by the *Chalaropsis* B enzyme resulted in complete hydrolysis of *N*-acetylmuramyl linkages and production of 4-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid disaccharides (Tipper and Berman, 1969). Hydrolysis of the isolated glycan with the endo-*N*-acetylglucosaminidase of lysostaphin (Browder *et al.*, 1965) resulted in the production of 4-*O*-(*N*-acetylmuramyl)-*N*-acetylglucosamine disaccharides, as in the case of *S. aureus* glycan (Tipper *et al.*, 1967). Assuming that these two glycanases are specific for the β linkages found in *S. aureus* glycan (Tipper and Strominger, 1966), then the two glycans have identical structures.

The teichoic acids of both organisms are of the *N*-acetylglucosaminyl-polyol phosphate type, and apart from the presence of ribitol in the *S. aureus* teichoic acid and glycerol in the *S. epidermidis* teichoic acid, the only gross difference in total cell wall structures of these organisms detected in these present studies is in the glycine to serine ratio of the cross-bridge peptides. The genetic complements of these organisms concerned with cell wall synthesis must be very similar, and are possibly related by a few mutations, as in the *S. aureus* mutants isolated by Korman (1967), which by cell wall structure could be *S. epidermidis* strains. Such mutations might alter the intracellular proportions of seryl-tRNA and glycyl-tRNA or affect one of the glycine-adding enzymes involved in the scheme that is shown in Figure 10, where addition of L-serine is a possible alternative.

Acknowledgments

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