Structures of the Cell Wall Peptidoglycans of *Staphylococcus epidermidis* Texas 26 and *Staphylococcus aureus* Copenhagen. I. Chain Length and Average Sequence of Cross-Bridge Peptides*

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ABSTRACT: In the cell wall peptidoglycans of *Staphylococcus aureus* strain Copenhagen and *Staphylococcus epidermidis* strain Texas 26, all *N*-acetylmuramic acid residues are substituted by peptide subunits, carrying pentapeptides on the ε-amino groups of their lysine residues. These pentapeptides are cross-linked to D-alanine residues in neighboring peptide subunits to the extent of 75 and 66%, respectively, forming oligopeptides of randomly distributed chain length with amino-terminal N^{ϵ}-(pentapeptidyl)-L-lysine at one end and carboxy-terminal L-lysyl-D-alanyl-D-alanine at the other. About 6% of the *S. aureus* cross-bridge pentapeptides are glycyl-seryl-glycyl-glycyl-glycine, the rest being pentaglycine.

In the *S. epidermidis* peptidoglycan, serine is nonrandomly distributed in the cross-bridge pentapeptides, and accounts for about 23, 7, 68, 4, and 3% of their residues, starting from their N termini. Both organisms have a strict alternation of *N*-acetylglucosamine and *N*-acetylmuramic acid in their glycan, and contain a teichoic acid substituted by D-alanyl and *N*-acetylglucosaminyl residues.

Chemical and enzymatic studies (Ghuysen *et al.*, 1966; Muñoz *et al.*, 1966; Jarvis and Strominger, 1967; Tipper *et al.*, 1967a,b) have demonstrated that the cell wall peptidoglycan of *Staphylococcus aureus* strain Copenhagen consists of a glycan (alternating β-1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid) whose muramic acid residues are amide linked to the L-alanine termini of peptide subunits. These peptides consist of the basic tetrapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine, cross-linked by polyglycine bridges between D-alanine and the ε-amino group of lysine in a neighboring peptide unit (Figure 1). Different preparations of these cell walls, after trypsin digestion, also contain 0.03–0.08 mole of serine/mole of glutamic acid but only trace amounts of other amino acids (see below). It has recently been reported that certain coagulase-negative mutants of *S. aureus* strain 8511 contain up to 0.64 mole of serine/mole of glutamic acid in their cell walls while the parent contained only 0.11 mole of serine, and that these mutants had a corresponding deficiency in cell wall glycine content (Korman, 1966). Cell walls of several strains of *Staphylococcus epidermidis* have recently been shown to have total amino acid compositions similar to that of *S. aureus*, but with elevated L-serine contents, the highest reported being that of strain Texas 26 (Browder *et al.*, 1968).

The soluble glycopeptide produced by hydrolysis of cell walls of *S. aureus* strain Copenhagen with the *Chalaropsis* B enzyme (Hash, 1963), and endo-*N*-acetylmuramidase devoid of peptidase activity (Tipper *et al.*, 1964), consists of disaccharides (4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid) from the fragmented glycan linked to the peptide subunits which still retain their polyglycine cross-bridges. This glycopeptide has been fractionated into oligomers of the disaccharide peptide subunit by chromatography on Sephadex G-50 and G-25 columns (Tipper and Strominger, 1968). Except for their D-alanine content, all oligomers had the same amino acid composition as the original cell walls.

About 10% of this glycopeptide consisted of monomer, which, like the nucleotidepentapeptide biosynthetic precursor of the peptidoglycan, had a C-terminal D-alanyl-D-alanine group. This is also true of the C-terminal peptide subunits of all of the oligomers (Tipper and Strominger, 1965, 1968), whereas the other subunits contain only 1 mole of D-alanine since the second is eliminated during the transpeptidation reaction which forms the D-alanyl-glycine cross-links (Tipper and Strominger, 1965; Izaki *et al.*, 1966, 1968). The purpose of this paper is to show that this monomer also carries an N-terminal pentaglycine chain on the ε-amino group of its L-lysine moiety and that the cross-bridges in this organism are therefore probably pentapeptides. Furthermore, the glycopeptide monomers and dimers have also been isolated from cell walls of *S. epidermidis* Texas 26 and have also been shown to carry N-terminal pentapeptidyl-L-lysine chains. These chains contain the L-serine residues of the peptidoglycan, and

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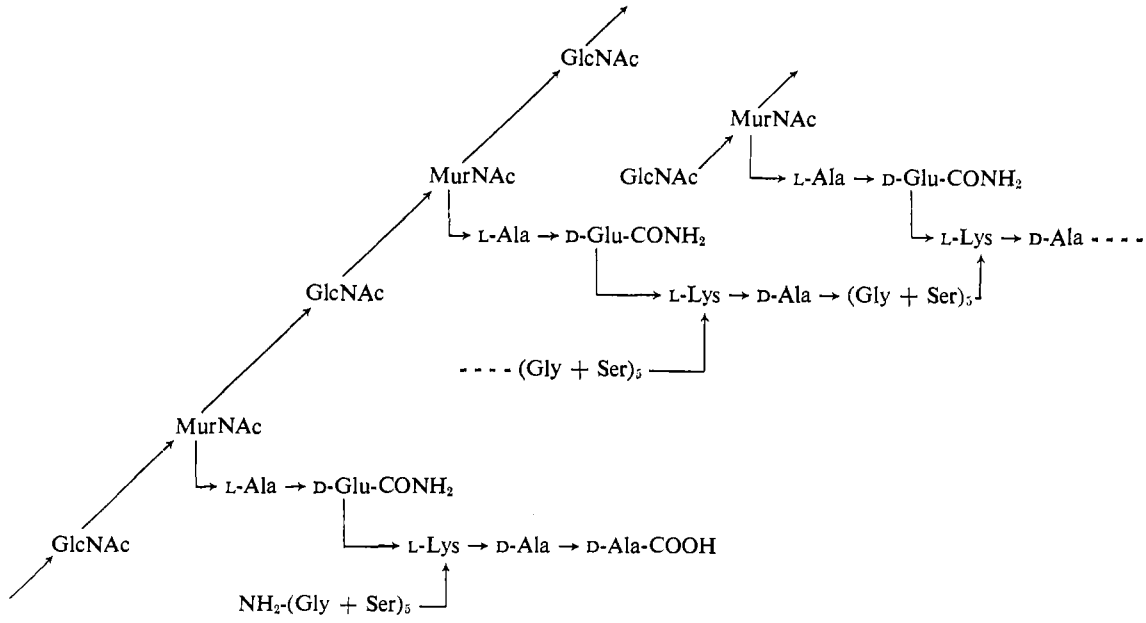

FIGURE 1: Structure of the peptidoglycan of *S. aureus*. Glycan chains of average chain length 12 GlcNAc-MurNAc disaccharides (Tipper *et al.*, 1967a) are interlinked by the polypeptide (average chain length 3.9 peptide units, see below) as shown in the upper part of the figure. The lower part shows an uncross-linked monomer with C-terminal D-alanyl-D-alanine. GlcNAc = *N*-acetylglucocamine; MurNAc = *N*-acetylmuramic acid.

the distribution of L-serine and glycine within these pentapeptides and those of *S. aureus* has been determined by Edman degradation.

Materials and Methods

Organism and Preparation of Cell Walls. A culture of *S. epidermidis* strain Texas 26 was kindly provided by Dr. Henry P. Browder, Mead Johnson Research Center, Evansville, Ind. Cells of this organism in late exponential growth phase were washed once with distilled water at 2° and broken in a Nossal cell disintegrator. Suspensions of cells (10 g wet weight) in water (30 ml) containing glass beads (120 μl, 25 ml) were treated for 3 min at 2–15°. Beads were removed by passage through a coarse glass sinter, and insoluble material was isolated by centrifugation (20 min, 25,000*g*). The pellet was resuspended in water for further breakage. After the fourth disintegration, the pellet from centrifugation was homogeneous and white while the supernatant contained relatively little material absorbing at 280 mμ. A portion (580 mg) of the lyophilized pellet was boiled for 25 min in water (20 ml). These boiled cell walls showed no decrease in turbidity on prolonged incubation at 37° in buffers at pH values from 5 to 9. The same procedure was used for preparation of cell walls of *S. aureus* strain Copenhagen. "Ester-alanine free" cell walls were prepared from boiled cell walls by hydrolysis of samples (200 mg) in 0.1 M sodium pyrophosphate buffer, pH 9.2 (10 ml), for 3 hr at 37° with shaking, followed by three washes with water by centrifugation.

Column Chromatography. Columns of ECTEOLA-cellulose (Bio-Rad Cellex E, 2.3 × 30 cm) were operated at room temperature and were prewashed with 0.5 M LiCl and water before use. Initial elution with water at 0.5 ml/min (250 ml) was followed by a gradient of linearly increasing LiCl concentration from 0 to 0.5 M (800 ml). Columns of Sephadex G-50 and G-25 (fine bead form, Pharmacia Fine Chemicals), 100 × 2.5 cm, connected in series by a polyethylene capillary, were eluted with water at 0.3 ml/min at room temperature. Glycopeptide concentrations were quantitated by reducing power. Since each peptide subunit carries a disaccharide with a molar reducing power 150% of that of an equimolar amount of *N*-acetylglucosamine (see below), reducing power is a measure of the total concentration of disaccharide-peptide subunits, irrespective of the number of subunits in the glycopeptide oligomer.

Edman Degradations. The procedure used was similar to the micromodification of the Konisberg and Hill (1962) procedure previously described (Tipper *et al.*, 1967b). Trifluoroacetic acid was used as the cyclization agent in each cycle. Dried aliquots of glycopeptides (0.5–1.5 μmoles) were dissolved in fresh *N*-ethylmorpholine buffer (100 μl), mixed with phenyl isothiocyanate (10 μl), incubated for 3 hr at 37°, and dried. The residues were carefully extracted twice with benzene (100 μl), dried, dissolved in trifluoroacetic acid (100 μl), sealed, incubated for 1 hr at room temperature, and dried. The residues were redissolved in 0.2 M acetic acid (100 μl) and extracted twice with benzene (100 μl). The aqueous phases were dried and redissolved in 0.2 M acetic acid (100 μl). Aliquots were analyzed for reducing power and for total and N-terminal amino acids. The bulk of the remaining solutions were submitted to the next cycle of degradation. Degradation products were purified by chromatography on columns of Sephadex G-25 (1 × 50 cm) eluted with water at 0.3 ml/min. Products were

TABLE I: Analyses of Cell Walls and Teichoic Acids.[a]

	Lys	Ala	Asp	Gly	Ser	NH_3	GlcN	MurN	PO_4	Glu
S. aureus										
Boiled cell walls	101	302	4	507	8	122	273	85	194	
"Ester-alanine free"	91	220	3	500	6	93	257	97		
TCA–TA	–	35	–	–	–	–	92	2		2
TA–GP	14	58	–	77	–	23	108	14		15
S. epidermidis										
Boiled cell walls	96	358	6	345	94	115	164	81	186	
"Ester-alanine free"	94	238	6	358	100	101	159	94		
TCA–TA	3	51	–	11	4	–	30	1		3
TA–GP	12	72	–	52	14	17	48	14		14

[a] Data for cell walls are expressed as moles per mole of total glutamic acid. These walls had been "heat killed" to destroy autolytic activity, but had not been digested with trypsin. Other amino acids present in trace amounts (less than 3 moles/100 moles of glutamic acid) were arginine, threonine, leucine, isoleucine, and valine. Ester-alanine-free cell walls were prepared by hydrolysis for 3 hr at pH 9.2, 37°, as described in Methods. Data for teichoic acids are expressed as moles per 100 moles of total phosphate. TCA–TA is teichoic acid isolated from 10% trichloroacetic acid extracts of cell walls (see text) and TA–GP is the covalently linked complex of teichoic acid and glycopeptide produced by hydrolysis with the *Chalaropsis* B enzyme and eluted with LiCl from ECTEOLA (see text). The recovery of cell wall phosphate in the TA–GP fractions was over 90% (see text), while the recovery of *S. aureus* and *S. epidermidis* TCA–TA was 69 and 56%, respectively. Data for hexosamines are corrected for hydrolytic losses. – indicates not determined. GlcN, glucosamine; MurN, muramic acid.

quantitated by reducing power, since the ratio of reducing power to peptide remained constant throughout the degradation (see below).

Analytical Procedures. Determinations of total amino acid contents after hydrolysis for 18 hr at 105° in 6 N HCl and of hexosamines after hydrolysis for 16 hr at 100° in 3 N HCl were performed using a Beckman-Spinco amino acid analyzer. D- and L-alanine, reducing power, and N- and C-terminal amino acids were determined as previously described (Ghuysen *et al.*, 1966). The yield of dinitrophenylserine was low (40–60% of theoretical) and somewhat variable. Standards of free serine, L-seryl-glycine, and L-seryl-glycyl-glycine (Cyclo Chemicals Corp.) were used. N-Terminal serine was also quantitated from the yield of formaldehyde on oxidation for 10 min at 25° in excess 0.001 M IO_4^- in 0.01 M NaOAc (pH 4.5) using seryl-glycine as a standard. Formaldehyde was determined as previously described (Ghuysen *et al.*, 1966). Data presented are the averages of several determinations by the two procedures. Morgan–Elson determinations (30 min) were performed as described previously (Tipper, 1968a). Under these conditions, peptide-substituted disaccharide, 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, has a ratio of reducing power to 30-min Morgan–Elson color of 2.8, using *N*-acetylglucosamine as the standard for both procedures. For the tetrasaccharide formed from two β-1,4-linked disaccharide units, the ratio is about 25. Thus, this ratio is a sensitive measure of the extent of *N*-acetylmuramidase action. It is also characteristic of this disaccharide (when peptide substituted), because it cannot form Morgan–Elson chromogen until its glycosidic linkage is hydrolyzed, following an initial β elimination of its D-lactyl-peptide substituent (Tipper, 1968a). Glucose was determined with glucose oxidase using a micromodification of the glucostat procedure (Worthington Biochemical Corp.). Samples of cell walls (0.1 mg) were hydrolyzed for 8 hr in 3 N HCl at 105°, neutralized with NaOH, and centrifuged to remove insoluble material. Determinations were made on aliquots (10 μl) of the supernatants (80 μl). Hydrolysis of glucose in the presence of 0.1 mg of cell walls had no effect on subsequent color production with the glucostat reagents.

Results

Analyses of Cell Walls. The results of analyses of hydrolysates are presented in Table I. Both cell walls contained 0.48 μmole of total glutamic acid/mg and about twice as much total phosphate. Boiling of the walls in water liberated some free alanine, presumably from the

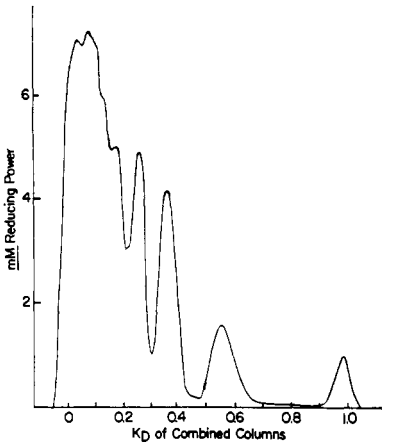

FIGURE 2: Fractionation of *S. aureus* glycopeptide on Sephadex G-50 and G-25 columns (100 × 2.5 cm). Details are given in Methods. $K_D = (V_e - V_0)V_i$, where V_e is the elution volume, V_0 (375 ml) is the elution volume of Blue Dextran (Pharmacia), and $V_0 + V_i$ (850 ml) is the elution volume of NaCl. Aliquots (1 μl) of the fractions (5 ml) were analyzed for reducing power. The small peak of totally included material at $K_D = 1$ is free GlcNAc, presumably released from the reducing end groups of the glycan (which are almost entirely GlcNAc; Tipper, 1969a) by the *Chalaropsis* B enzyme. Fractions pooled were: K_D = 0–0.13 (oligomer), 0.13–0.21 (pentamer and tetramer), 0.22–0.3 (trimer), 0.3–0.45 (dimer), and 0.48–0.65 (monomer).

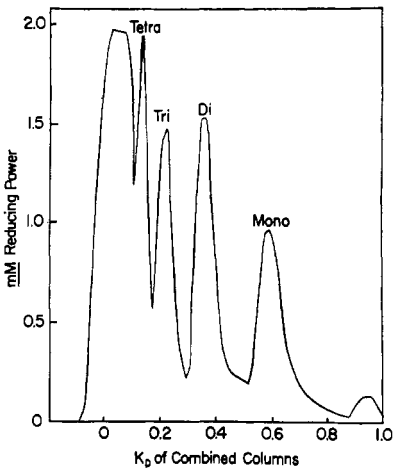

FIGURE 3: Fractionation of *S. epidermidis* glycopeptide on Sephadex G-50 and G-25 columns. Details as in Figure 2. Fractions pooled were: K_D = 0–0.13, oligomer; 0.13–0.18, tetramer; 0.18–0.29, trimer; 0.29–0.42, dimer; and 0.51–0.68, monomer.

ester-linked residues of the teichoic acids. The rest of this alanine (respectively, 80 and 120 moles per 100 moles of glutamic acid in *S. aureus* and *S. epidermidis* cell walls) was liberated on hydrolysis at pH 9.2 at 37° (*cf.* Tipper *et al.*, 1967a), together with small amounts of glycine, aspartic acid, and lysine (about 8, 3, and 3 moles per 100 moles of glutamic acid, respectively, in both preparations). The alanine released by alkaline hydrolysis had the D configuration in both cases. The residual total alanine contents are consistent with the presence of 25% D-alanyl-D-alanine end groups in *S. aureus* and 35% in *S. epidermidis*, as indicated by the degree of peptide cross-linking (see below). Aspartic acid was the only "trace" amino acid other than serine consistently present in significant amounts in all preparations of both *S. aureus* and *S. epidermidis* cell walls (*cf.* Table I). It was not present, however, in the purified glycopeptides produced from either organism by digestion with the *Chalaropsis* B enzyme (see below), and so is not a minor component of their peptidoglycans.

Analyses of the peptidoglycan of *S. aureus* (Table I and below) show its average subunit to contain (approximately), isoglutamine, 1.0; lysine, 1.0; alanine, 2.25; glycine, 4.9; serine, 0.1; *N*-acetylglucosamine, 1.0; and *N*-acetylmuramic acid, 1.0 residues. It also contains 0.6 residue of *O*-acetyl (Tipper *et al.*, 1965) giving a molecular weight for the subunit of 1208. There are 2 moles of organic (teichoic acid) phosphate/mole of glutamic acid in both cell walls (Table I), and the *S. aureus* teichoic acid contains 1 mole of *N*-acetylglucosamine and 0.4 mole of alanine per phosphate (Table I). A teichoic acid subunit containing 2 moles of the sodium salt of *N*-acetylglucosaminylribitol phosphate and 0.8 mole of alanine has a molecular weight of 935. The sum of peptidoglycan plus teichoic acid is 2143, so that 1 mg of cell wall with this composition would contain 0.47 μmole of each subunit, as found, indicating the absence of large amounts of other polymers. The cell wall should thus contain 3 moles of glucosamine/mole of glutamic acid. This discrepancy is unexplained.

The *S. epidermidis* peptidoglycan subunit (Table I and below) is similar in composition, containing more alanine (2.35 residues) and serine (1.0 residue) and less glycine (4.0 residues) giving a molecular weight of 1242. Its teichoic acid, containing (per phosphate) about 0.6 mole of alanine but only 0.35 mole of *N*-acetylglucosamine (Table I and below), has a subunit molecular weight (based on 2 moles of the sodium salt of glycerophosphate/subunit, see below) of 615. The sum of peptidoglycan plus teichoic acid is 1857, so that 1 mg of cell wall should contain 0.54 μmole of each subunit, somewhat higher than that found (0.48). In these walls, the expected ratio of glucosamine to glutamic acid is 1.7, as found. Neither cell wall preparation contained significant quantities of glucose (less than 0.1 mole/mole of glutamic acid) even though *S. epidermidis* strains usually contain a glucosylglycerol-type teichoic acid (Davison and Baddiley, 1963).

The serine content of the *S. epidermidis* cell walls is somewhat lower than that previously reported for this strain (H. P. Browder, private communication). Browder, however, found that cell wall serine contents varied with growth conditions. Variation in the serine content of different preparations of *S. aureus* strain Copenhagen cell walls from 2 to 8 moles per 100 moles of total glutamic acid has been found in this laboratory.

Hydrolysis of Cell Walls with the Chalaropsis B Enzyme. A. *S. aureus* CELL WALLS. Boiled *S. aureus* strain Copenhagen cell walls (1.5 g) were incubated with *Chalaropsis* B enzyme (a gift from Dr. J. Hash, Vanderbilt University School of Medicine, Nashville, Tenn., 5 mg) in 0.01 M NaOAc, pH 4.5 (30 ml). Aliquots were analyzed for release of reducing power, which was maximal at 8 hr (1.1 mmoles). After 48 hr, insoluble mate-

rial (21 mg, dirt and protein) was removed by centrifugation (20 min at 25,000*g*) and the clear supernatant was fractionated on ECTEOLA-cellulose). Water eluted a peak of reducing power (0.8 mmole, glycopeptide), free of phosphate, and 0.1–0.4 M LiCl eluted a peak of reducing power (0.3 mmole) associated with phosphate (1.8 mmoles, teichoic acid–glycopeptide complex). A portion (0.67 mmole) of the glycopeptide was fractionated on columns of Sephadex G-50 and G-25 with the results shown in Figure 2. The peaks were pooled as indicated and their analyses and reducing power contents are given in Table II. Their N-terminal glycine, D-alanine, and total and C-terminal alanine contents are consistent with the chain lengths indicated in Table II, and also with their relative peak positions (Figure 2). The recovery of reducing power was virtually complete. When the unfractionated glycopeptide (0.1 μmole of reducing power) was reduced overnight in 0.05 M $NaBH_4$ (50 μl), hydrolysates of the product retained about 1 mole of glucosamine/mole of glutamic acid. However, they contained no muramic acid, showing that the *N*-acetylmuramidase action of the *Chalaropsis* B enzyme had been complete. This was confirmed by the ratios of reducing power to 30 min. Morgan–Elson color for the glycopeptide fractions, all of which fell in the range 2.5–3.0, characteristic of 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid-peptide (see Methods). The absence of any peptide-free oligosaccharides or of tetrasaccharide-substituted peptides demonstrates that peptide substitution of the *N*-acetylmuramyl moieties in this peptidoglycan was complete. Thus the average chain length of the peptide (3.9 subunits) can be calculated from the relative amounts of reducing power in the oligomers, using the chain lengths given in Table II.

B. *S. epidermidis* CELL WALLS. Boiled *S. epidermidis* cell walls (400 mg) were hydrolyzed with *Chalaropsis* B enzyme (1.5 mg) in 0.05 M NaOAc (12 ml, pH, 4.5) at 37°. Aliquots (0.1 μl) were analyzed for reducing power liberated, which reach a maximum of 260 μmoles at 10 hr. After 40 hr, the products were centrifuged as above, giving a pellet (26 mg after lyophilization) again consisting of dirt and protein and devoid of hexosamines. The supernatant was fractionated on a column of ECTEOLA-cellulose as described above. Water eluted a peak of reducing power (200 μmoles) and 0.3 M LiCl eluted a peak of reducing power (60 μmoles) associated with phosphate (320 μmoles) (teichoic acid–glycopeptide complex). The whole of the water eluate (glycopeptide) was fractionated on Sephadex G-50 and G-25 as described above, as shown in Figure 3. The fractions were pooled as indicated, and the amounts in each pooled fraction are given in Table II. Their analyses are given in Table III, and chain lengths deduced from these analyses and the relative peak positions are given in Table II. The recovery of total reducing power (212 μmoles) was complete. No peptide-free oligosaccharides were produced, and the reducing power and 30-min Morgan–Elson determinations are consistent with the presence of 1 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide/mole of glutamic acid in each oligomer. Thus all muramic acid residues are linked to peptide, as in the *S. aureus* peptidoglycan.

TABLE II: Yields of Oligomers from G-50–G-25 Chromatography of *S. aureus* and *S. epidermidis* Glycopeptides and Analyses of *S. aureus* Oligomers.[a]

	S. aureus						*S. epidermidis*		
	μmoles of Reducing Power	% of Total	Chain Length	Total Alanine	Total D-Alanine	N-Terminal Glycine	C-Terminal Alanine	μmoles of Reducing Power	% of Total	Chain Length
Monomer	45	6.8	1	290	190	110	90	32	15.1	1
Dimer	99	14.8	2	251	160	40	40	33	15.6	2
Trimer	80	12.0	3	236	140	30	30	32	15.1	3
"Tetramer"	202	30.2	5	226	110	25	20	30	14.2	4.5
Oligomer	242	36.2	10	220	100	15	10	85	40.0	9

[a] Fractions were pooled as indicated in Figures 2 and 3. The "tetramer" fraction in the *S. aureus* glycopeptide also contains material of higher molecular weight. Analyses of the *S. aureus* glycopeptide oligomers are given as moles per 100 moles of total glutamic acid. Each contained 1 mole of disaccharide (by both reducing power and 30-min Morgan–Elson procedures), lysine, ammonia, and L-alanine, about 5 moles of glycine and 0.05 mole of serine per mole of glutamic acid. No other amino acids were detectable (*cf.* Tipper and Strominger, 1968, Table III). Chain lengths are deduced from the analyses as follows: an oligomer of chain length *n* disaccharide-peptide subunits, with C-terminal D-alanyl-D-alanine, should contain $100/n$ moles of C-terminal alanine, $100/n$ moles of N-terminal glycine (plus serine), $100(2 + 1/n)$ moles of total alanine, and $100(1 + 1/n)$ moles of D-alanine per 100 moles of glutamic acid.

TABLE III: Analyses of *S. epidermidis* Glycopeptide Oligomers.[a]

	Alanine						N Terminal					
	Lys	Total	D	L	Gly	Ser	NH_3	GlcN	MurN	Gly	Ser	Disaccharide
Monomer	98	298	182	130	340	98	115	80	82	78	8	102
Dimer	95	257	152	105	352	105	112	89	102	48	7	85
Trimer	88	245	142	85	322	95	103	79	100	32	5	90
Tetramer	87	242	124	100	339	99	135	72	103	28	3	93
Oligomer	90	226	114	105	339	98	135	72	95	9	3	97
Unfractionated	90	238			330	95	121	74	72			96

[a] Data are expressed as moles per 100 moles of total glutamic acid. The ratio of reducing power to 30 min Morgan-Elson color was characteristic of GlcNAc–MurNAc disaccharide in all fractions (see Methods). No other amino acids were detected in any fraction. Chain lengths were deduced as for the *S. aureus* oligomers (Table II). MurN = muramic acid; GlcN = glucosamine.

TABLE IV: Sephadex G-25 Purification of Edman Degradation Products: Recoveries of Reducing Power.[a]

Starting Material	Cycles of Degradation	μmoles of Reducing Power: Total Eluted from G-25	μmoles of Reducing Power: Total in Pooled Fractions
S. aureus monomer	2	90	63
	4	60	39
S. epidermidis monomer	2	106	90
	4	80	46
S. epidermidis dimer	2	92	72
	4	24	19

[a] Data are given as per cent of the reducing power of the starting material and are corrected for the fraction of the products of each degradation cycle removed for analyses.

After reduction of the unfractionated glycopeptide, no muramic acid was released on hydrolysis. The *Chalaropsis* B enzyme thus hydrolyzes all of the *N*-acetylmuramyl linkages of *S. epidermidis* cell walls, and at about the same rate as it hydrolyzes the β-1,4-linkages of *S. aureus* cell walls. A portion of the unfractionated glycopeptides from *S. aureus* and *S. epidermidis* (0.15 μmole of reducing power each) was hydrolyzed separately for 6 hr at 37° in 0.02 M NaOAc, pH 5.2 (100 μl), containing 50 μg of *N*-acetylmuramyl-L-alanine amidase from *Streptomyces albus* G (Ghuysen *et al.*, 1962). After electrophoresis of the products at pH 3.9, a single fluorescent spot was visible on heating with NaOH (Sharon, 1964). It had mobility identical with 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, but distinct from that of 4-*O*-β-*N*-acetylmuramyl-*N*-acetylglucosamine (*cf.* Tipper and Strominger, 1966). After paper chromatography in butanol–acetic acid–water (3:1:1, v/v), NaOH again gave a single spot with the same mobility as the former disaccharide. No spot corresponding to its tetrasaccharide dimer was visible. Thus the *S. epidermidis* glycan contains alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, probably linked β-1,4. From the reducing power and assumed chain lengths of the *S. epidermidis* glycopeptide fractions (Table II), an average chain length of 2.8 disaccharide-peptide units is derived.

Teichoic Acid Compositions. The teichoic acid in cell walls of *S. aureus* strain H (Baddiley *et al.*, 1962) and Copenhagen (Sanderson *et al.*, 1962) is a phosphodiester-linked polymer of 4-*O*-(*N*-acetylglucosaminyl)-D-ribitol 1 (5)-phosphate. Teichoic acid was prepared from 100 mg of walls of strain Copenhagen by hydrolysis with 10% trichloroacetic acid (4 ml) for 2 hr at 60° followed by centrifugation and precipitation of the supernatant with ethanol (12 ml). Analyses of this product, of

TABLE V: Analyses of Products of Edman Degradation of *S. aureus* Glycopeptide Monomer.[a]

Cycles of Degradation	0	1	2	3	4	5
Recovery of total glutamic acid	(100)	92	102	98	92	100
Total alanine	290	310	310	300	290	320
Total glycine	500	405	315	220	145	65
Δ[b]glycine		100	90	95	75	80
Total N terminal	110	110	120	130	120	110

[a] Data are expressed as moles per 100 moles of glutamic acid. *S. aureus* monomer (1.6 μmoles) was subjected to five cycles of Edman degradation as described in Methods. The second and fourth cycle products were purified by chromatography on Sephadex G-25. Successively larger aliquots (20, 25, 32, and 40 μl) of the solutions (100 μl) of the products were removed for analyses at each stage. Data for the individual N-terminal amino acids are presented in Figure 4. The recovery of total glutamic acid, expressed as per cent of the amount in the undegraded monomer, is corrected at each stage for aliquots removed for analyses, and at stages 2 and 4 for the ratio of pooled to total eluted reducing power during purification on Sephadex G-25 (see Table IV). [b] Δ signifies "decrease in."

TABLE VI: Analyses of Products of Edman Degradation of *S. epidermidis* Glycopeptide Monomer.[a]

Cycles of Degradation	0	1	2	3	4	5
Recovery of total glutamic acid	(100)	102	96	90	112	102
Total alanine	290	280	280	300	300	
Total glycine	350	295	195	170	95	40
Total serine	100	85	70	15	10	10
Total serine + glycine	450	380	265	185	105	50
Δ[b]total (serine + glycine)		70	115	80	80	55
Δ[b]serine/Δ(serine + glycine) × 100		21	13	70	6	0
Total N terminal	90	130	80	110	100	130

[a] Data are expressed as moles per 100 moles of total glutamic acid. *S. epidermidis* monomer (1.6 μmoles) was subjected to five cycles of Edman degradation as described in Methods. The product of each cycle was analyzed as described in Table V. Data for individual N-terminal amino acids are plotted in Figure 5. [b] Δ signifies "decrease in."

the original cell walls and of the teichoic acid–glycopeptide complex isolated from them after hydrolysis with the *Chalaropsis* B enzyme (Table I) show that each had an excess of glucosamine over muramic acid roughly equal to their phosphate content. All three also contained an excess, per mole of phosphate, of about 0.3 mole of D-alanine in excess of their glutamic acid content. This excess D-alanine is released from the cell walls by mild alkali hydrolysis, and so is the ester-linked D-alanine of the teichoic acid (Baddiley *et al.*, 1962).

S. epidermidis cell walls also contained about 2 moles of phosphate/mole of peptidoglycan subunit (Table I). Teichoic acid was prepared from these walls with trichloroacetic acid as described above. This material, the original cell walls, and the teichoic acid–glycopeptide complex derived from them, all contained, per mole of phosphate, about 0.3 mole of glucosamine and 0.5 mole of alanine in excess of the amounts contained in their peptidoglycan components (Table I). Thus in contrast to the almost fully glycosylated *S. aureus* teichoic acid, only about 30% of the polyol phosphate residues in this teichoic acid carry *N*-acetylglucosaminyl residues. The extent of esterification by D-alanine is, however, somewhat higher.

Samples (0.1 mg) of the two acid-extracted teichoic acids were hydrolyzed for 3 hr at 100° in 2 N HCl and dried. Paper chromatography in butanol–ethanol–water–concentrated NH_3 (40:10:49:1, v/v, upper phase) and detection with $AgNO_3$ or periodate–Schiffs reagent (Baddiley *et al.*, 1956) showed anhydroribitol in the *S. aureus* hydrolysate, but only glycerol in the *S. epidermidis* hydrolysate. This was confirmed by alkaline hydrolysis followed by hydrolysis with *Escherichia coli* alkaline phosphatase, which again liberated glycerol but not ribitol or *N*-acetylglucosaminylribitol from the *S. epidermidis* teichoic acid. This teichoic acid thus resembles that isolated from a strain of *S. albus* which consisted of polyglycerolphosphate, about one-third of whose residues carried *N*-acetylgalactosamine residues, the rest carrying D-alanyl residues (Ellwood *et al.*, 1963).

Edman Degradation of Glycopeptide Oligomers. In a preliminary experiment, *S. epidermidis* monomer (0.4 μmole) was subjected to four cycles of Edman degradation with no attempt at purification between cycles. The recovery of components (other than glycine) and of total reducing power was 97 and 96%, after the first and second cycles, respectively. However, recovery fell off rapidly in subsequent cycles. It was therefore necessary in subsequent experiments to repurify the products of degradation after every other cycle. Glycopeptide monomers from *S. epidermidis* and *S. aureus* and glycopeptide dimer from *S. epidermidis* were subjected to five cycles of degradation as described in Methods. The products in each case were purified after the second and fourth cycles of degradation by chromatography on columns of Sephadex G-25 (1 × 30 cm) eluted with water. Glycopeptide could be quantitated by reducing power, which was unaffected by the degradation procedure. On such a column, glycopeptide monomer has

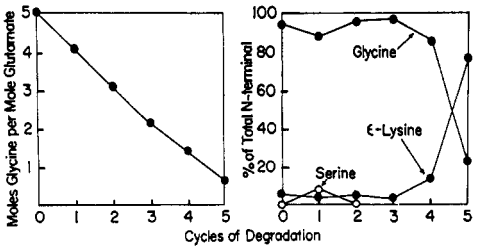

FIGURE 4: Edman degradation of *S. aureus* glycopeptide monomer. Data (from Table V) on the total glycine contents of the products of each successive cycle are plotted on the left, and data on the per cent of the total N terminal (*cf.* Table V) in individual amino acids is plotted on the right (G, glycine; S, serine). Significant amounts of N-terminal serine were found only in the product of the first cycle of degradation. After dinitrophenylation, hydrolysis, and ether extraction of other DNP derivatives, ε-DNP-lysine was determined by thin-layer chromatography of the remaining solution. Cochromatography with unidentified minor DNP derivatives probably accounts for much of the color attributed to ε-N-terminal lysine in columns 0, 1, 2, and 3.

a K_D of about 0.45 and dimer has a K_D of 0.15. Accumulated pigmented by-products were eluted later. Only fractions in the appropriate regions were pooled, with the result that while recovery of reducing power from the monomers, as determined by total eluted reducing power (Table IV) was good, only part of this material was used for subsequent degradation cycles (Table IV). After each cycle of degradation, the products were analyzed for total and N-terminal amino acids with the results shown in Tables V–VI and VII and Figures 4–5 and 6. The recovery of total glutamic acid at each stage (Tables V, VI, and VII) was excellent except during the third cycle of degradation of the dimer, when unexplained losses occurred, probably during lyophilization.

In each case, there was a loss of approximately 1 mole of glycine and serine at each cycle, with an abrupt appearance of ε-aminolysine (0.75–0.9 mole/mole of glutamic acid) after the fifth cycle, indicating actual chain lengths of five glycine plus serine residues in these N-terminal peptides. The residual glycine in the monomers was low (0.6 and 0.5 mole per mole of glutamic acid) and reflects the accumulation of products of incomplete degradation throughout the five degradation cycles. The initial and residual glycine plus serine in the dimer approximated the theoretical figures of 10 and 5 moles per 2 moles of glutamic acid, respectively. In all three glycopeptides, the ratios of lysine and alanine to glutamic acid remained constant throughout the degradations, except for a slight decrease (10%) in lysine at the fifth cycle, in accord with the N-terminal data.

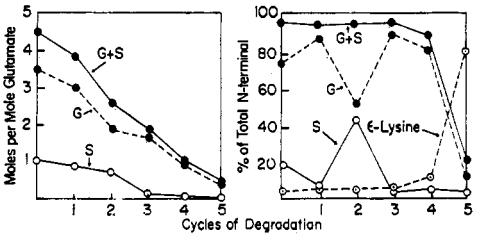

FIGURE 5: Edman degradation of *S. epidermidis* monomer. Data (from Table VI) on the total glycine (G) and serine (S) contents of the products of successive cycles are plotted on the left. Data on the per cent of the total N terminal (*cf.* Table VI) in individual amino acids is plotted on the right.

TABLE VII: Analyses of Products of Edman Degradation of *S. epidermidis* Glycopeptide Dimer.[a]

Cycles of Degradation	0	1	2	3	4	5
Recovery of total glutamic acid	(100)	89	95	30	28	26
Alanine	480	502	496	504	490	485
Glycine	728	658	536	516	418	364
Serine	196	162	174	96	108	92
Serine + glycine	924	820	710	612	520	456
Δ[b](serine + glycine)		104	110	98	86	70
Δ[b]serine/ Δ(serine + glycine) $\times$ 100		33	0	67	0	6
Total N terminal	110	100	80	90	80	80

[a] Data are expressed as moles per *200* moles of total glutamic acid. *S. epidermidis* dimer (1.9 μmoles) was subjected to five cycles of Edman degradation as described in Methods. The products of each cycle were analyzed as described in Table V and the data for individual N-terminal amino acids are plotted in Figure 6. [b] Δ signifies "decrease in."

Discussion

Analysis of the products of hydrolysis by the *Chalaropsis* B enzyme of the *S. aureus* and *S. epidermidis* cell walls demonstrates that, in both, all muramic acid residues are substituted by peptide and all *N*-acetylmuramyl linkages are susceptible to the enzyme, giving rise in each case to disaccharides, with identical properties. The glycans in these peptidoglycans therefore probably have identical structures. The teichoic acids in these two organisms are also similar in being polymers of polyol, phosphate, glucosamine, and D-alanine.

During biosynthesis of cell wall peptidoglycan, complete disaccharide-peptide subunits are built up on nucleotide and lipid carriers from which they are transferred to the growing glycan chains, presumably to their nonreducing *N*-acetylglucosamine end groups at sites on the exterior surface of the membrane (*cf.* Strominger *et al.*, 1967). In both *S. aureus* and *S. epidermidis*, cross-linking of these nascent subunits by transpeptidation (*cf.* Tipper and Strominger, 1968) probably occurs at the same time. The results of fractionation of the *Chalaropsis* B enzyme digests show that this process was somewhat less efficient (66%) in the particular culture of *S. epidermidis* employed in these studies, than in the culture of *S. aureus* (75% efficient). Analyses of the fractionated products from both organisms (Tables II and III) indicate that in each glycopeptide oligomer, the

TABLE VIII: Distribution of Serine in the N-Terminal Pentapeptides of *S. epidermidis* Glycopeptide Monomers and Dimers.[a]

Residues from the N Terminus		0	1	2	3	4
S. epidermidis Monomer	(A)	21	13	70	6	2
	(N)	20	6	(43)	5	6
S. epidermidis Dimer	(A)	33	0	67	0	4
	(N)	20	8	(45)	4	6
Average		23	7	68	4	4

[a] Data are expressed as % of the total glycine and serine at each position. N: = data from N-terminal determinations (Figures 5 and 6), A: = data from total amino acid determinations (Table VI and VII). The N-terminal serine data are less reliable, and are almost certainly underestimates at higher values, as indicated by the low yields of total N terminals in the second cycle products (Tables VI and VII).

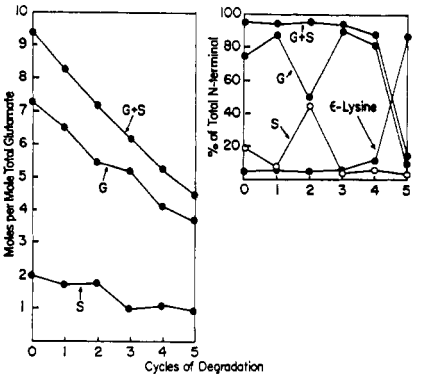

FIGURE 6: Edman degradation of *S. epidermidis* dimer. Data (from Table VII) on the total glycine (G) and serine (S) contents of the products are plotted on the left, and data on the per cent of the total N terminals (*cf.* Table VII) in the individual amino acids is plotted on the right.

C-terminal subunit carries an intact D-alanyl-D-alanine residue. Both organisms thus lack the D-alanine carboxypeptidases found in several other organisms (Izaki and Strominger, 1968). Apart from their D-alanine content, all oligomers have N-terminal glycine (or serine) and the same relative contents of amino acids as the original cell walls. They therefore carry glycine-serine peptides of average chain length 5 on the ε-amino groups of their N-terminal subunits. Assuming that these N-terminal peptides on the glycopeptide monomer and dimer fractions are representative, the data from their Edman degradation indicate that probably all the peptide cross-bridges in both organisms are pentapeptides, a fact not established by any previously published studies.

The N-terminal data indicate that serine occurs only in the second residue (from the N-terminal end) of the *S. aureus* strain Copenhagen pentapeptide cross-bridges, and that this organism therefore has two types of bridges, pentaglycine (92–96%) and glycyl-seryl-glycyl-glycyl-glycine (4–8%). One cycle of Edman degradation of the *S. aureus* glycopeptide dimer also liberated a small amount of N-terminal serine, absent in the original dimer and also absent in its second cycle product, thus confirming this conclusion.

The N-terminal and total amino acid data both indicate that much of the serine in *S. epidermidis* peptidoglycan occurs in the third residue of its pentapeptide cross-bridges, and most of the rest in the first residue, with a smaller amount in the second, and relatively little in the fourth and fifth residues (Table VIII). The sum of these amounts gives a total serine content of 105 moles/100 moles of glutamic acid, in good agreement with the amino acid analysis of these glycopeptide oligomers (Table III) and the whole cell walls (Table I). This nonrandom distribution of serine within these pentapeptides indicates that, as in *S. aureus*, relatively few different cross-bridge sequences may occur in this organism, a conclusion verified by analyses of partial hydrolysis products presented in the second paper in this series (Tipper, 1969b) in which the relevance of these findings to the possible mechanisms of biosynthesis of these cross-bridges is discussed.

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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*

Donald J. Tipper†

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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† A preliminary account of these findings has been published (Tipper, 1968b).