Biosynthesis of the Peptidoglycan of Bacterial Cell Walls

XII. INHIBITION OF CROSS-LINKING BY PENICILLINS AND CEPHALOSPORINS: STUDIES IN STAPHYLOCOCCUS AUREUS IN VIVO*

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SUMMARY

An uncross-linked monomer (nascent peptidoglycan unit) accumulates in cells of Staphylococcus aureus treated with low concentrations of penicillin G or with ampicillin, methicillin, or cephalothin. The uncross-linked monomer has been isolated, and analyses indicate that it represents a prefabricated subunit of the wall bearing both of the D-alanine residues of its pentapeptide precursor as well as an open pentaglycine chain. Pulse labeling experiments indicate that this uncross-linked unit is a direct precursor of the cross-linked peptidoglycan and that its cross-linking is inhibited by penicillin G. At high concentrations of penicillin G, wall synthesis ceases abruptly and no accumulation of the nascent peptidoglycan units is observed. These data have been obtained in support of the hypothesis that penicillins are substrate analogues of the D-alanyl-D-alanine end of the nascent peptidoglycan units and that they acylate the transpeptidase which catalyzes the cross-linking reaction. The data may also provide an explanation of the paradoxical observation that the killing rate in S. aureus by penicillin G is higher at low concentrations than at high concentrations of the antibiotic. In the presence of low concentrations a weakened wall may be formed, thus rendering the organisms more susceptible to lysis than at high concentrations of penicillin where wall synthesis abruptly ceases.

Investigation of the mechanism by which penicillins kill sensitive organisms started with the discovery of penicillin in 1929, and culminated in 1957 with the theory that penicillin specifically inhibits the biosynthesis of the cell walls of sensitive organisms (2-4). This hypothesis was confirmed in several laboratories by direct isotopic measurements of the effects of several penicillins on cell wall synthesis in both gram-positive and gram-negative organisms (see Reference 5 and other references cited therein). However, although much progress has recently been made in elucidating the complex pathways by which uridine disphosphate acetylmuramyl-pentapeptide and uridine disphosphate D-acetylglucosamine are used for peptidoglycan syntheses by cell-free extracts of penicillin-sensitive, gram-positive organisms (Staphylococcus aureus and Micrococcus lysodeikticus) (see References 6 to 10 and the preceding papers in this series), none of the reactions involved was found to be sensitive to penicillin, even at concentrations several orders of magnitude greater than minimum growth inhibitory concentrations. The product of these biosynthetic reactions in vitro was a linear peptidoglycan in which uncross-linked peptides, retaining both of their COOH-terminal D-alanine residues, were attached to the acetylmuramyl residues of the glycan. In S. aureus the peptide moiety formed in the biosynthetic sequence was a pentapeptide (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) substituted by a pentaglycine chain on the e-amino group of lysine.

However, concurrent studies of cell wall structure had led to the conclusion that the final product of the biosynthetic sequence in vivo was a highly cross-linked material rather than a linear polymer. The solubilized glycopeptide, obtained after treatment of cell walls of S. aureus with an acetylmuramidase, contained only 1.1 D-alanine residues per glutamic acid residue and was a high molecular weight polypeptide substituted by a disaccharide derived from the glycan (11-13). Subsequently, degradation of the peptide with peptidases and chemical synthesis showed that the repeating unit of the polypeptide was L-Ala-D-Glu-L-Lys-D-Ala, i.e. a tetrapeptide lacking one of the D-alanine residues of its pentapeptide precursor, and that in S. aureus the pentaglycine chain was substituted at its carboxyl end on the e-amino group of lysine (13-15). The attachment of the amino end of the pentaglycine chain to the carboxyl group of the terminal D-alanine residue of the tetrapeptide was shown in two ways. In the first place, several bacteriolytic enzymes were found which specifically catalyzed the cleavage of the D-alanylglucosamine linkage in S. aureus or of the linkage of D-alanine to other bridge amino acids in other microorganisms, thus showing directly that the interpeptide bridges were attached to the D-alanine residue of the tetrapeptide (16-18, 14). The other possible point of attachment was the alpha-carboxyl group of D-
glutamic acid, but it was shown that this carboxyl group was present as an amide in the cell walls of S. aureus (19, 20). The γ-carboxyl group of glutamic acid had previously been shown to be attached to the α-amino group of lysine in the main peptide chain (21, 22). Thus, the structural studies made it clear that the terminal β-alanine residue of the pentapeptide present in the biosynthetic precursors of the peptidoglycan must be eliminated in the course of cell wall synthesis and that the point of elimination was also the point of attachment of the interpeptide bridge.

Therefore, it seemed likely that this terminal reaction in cell wall synthesis was a transpeptidation.

The hypothesis that penicillin must be an inhibitor of the terminal cross-linking reaction in cell wall synthesis was adduced at about the same time in several different laboratories. The rod-shaped cells of Proteus mirabilis are reversibly converted in the presence of penicillin to spherical forms which retain all of the properties of spheres. Martin suggested that the transformation from the rod to the spherical form could be due to loss of rigidity as a consequence of the absence of cross-linking in the spherical form (23), and carried out some experiments with lysozyme lysates of cell walls of the rod and sphere forms which supported this hypothesis (24). Wire and Park (25) carried out double labeling experiments with S. aureus which indicated that the amount of alanine incorporated into the wall in the presence of penicillin was in excess of that incorporated in its absence, and they therefore reasoned that penicillin must be blocking the terminal cross-linking reaction in which β-alanine was eliminated. Tipper and Strominger (19), utilizing techniques which had been developed in the course of structural studies of the cell wall, showed that an uncleaved monomeric unit of the wall, present in small amount in control cultures, increased greatly in the presence of penicillin. This uncleaved unit contained a pentapeptide unit with both β-alanine residues of the precursor and bore an open pentaglycine chain substituted on the ε-amino group of lysine.

Moreover, it was suggested that penicillin was an analogue of the β-alanyl-β-alanine end of the pentapeptide in the uncross-linked precursor of the cell wall (19). Penicillin is a cyclic dipeptide of L-cysteine and D-valine, but molecular models showed a striking resemblance between the antibiotic and the dipeptide, β-alanyl-β-alanine. The CO-N bond in the β-lactam ring of penicillin lies in the same position as the peptide bond involved in the transpeptidation, and it was therefore proposed that penicillin, acting as a substrate analogue of the normal transpeptidation substrate, acylated the transpeptidase and thereby irreversibly inactivated it. This view was considered to be consistent with much earlier studies which indicated that bacterial cells irreversibly bond penicillin, that the binding might be related to the inhibition of growth by penicillin, and that the binding component was a lipoprotein located near the surface of the bacterial cell (26–28).

The present paper is an amplification of preliminary reports (19, 29) of the occurrence of the unlinked unit in the cell wall and of its accumulation induced by penicillin. Furthermore, it is shown that the unlinked unit is a direct precursor of the final cross-linked peptidoglycan of the cell wall.

**Materials and Methods**

Organism—S. aureus, strain Copenhagen, was grown at 37°C in media consisting of 0.5% Bactopeptone (Difco Laboratories, Detroit), 0.5% yeast extract (Difco), 0.2% glucose, and 0.1% K2HPO4. Growth was followed by removal of aliquots (0.2 ml), dilution with water (0.5 ml), and measurement of absorbance at 700 μm in a Zeiss PMQ II spectrophotometer. At maximum growth A260 reached about 1.6. All experiments were performed in the exponential phase of growth at A260 between 0.1 and 0.4. Sterile medium (100 ml) was inoculated from an agar slant of the organism and grown for 16 hours at 37°C. Portions (20 ml) of the stationary culture served as inocula for fresh medium (1000 ml). Cultures were incubated at 37°C in GYrotary incubators (New Brunswick Scientific Company, New Brunswick, New Jersey).

**Enzymes**—Trypsin and pancreatic RNase were purchased from Sigma. The B enzyme from Chalaraopsis, an acetylmuramidase (30, 31), was a gift from Dr. John Hash. *Bacillus cereus* β-lactamase (penicillinase) was a commercial preparation (Neutrogen) from Riker Laboratories, Northridge, California. The L3 enzyme from a flavobacterium species (see below) was a gift from Dr. Kenji Kato.

**Amino Acids and Peptides**—Glycine-14C (uniformly labeled; 106 mCi per mmole) was obtained from Nuclear-Chicago, Des Plaines, Illinois.

**Preparation of Cell Walls and Soluble Glycopeptide**—At the end of incubations, cells were recovered by centrifugation for 5 min at 5,000 × g, washed once in water at 2°C, and finally resuspended in water (30 ml) in the capsule of a Nossal cell disintegrator. They were shaken for a total of 3 min with 25 ml of glass beads (0.2 mm, Fisher Scientific) and separated from the beads by coarse, sintered glass filter. The cell walls were recovered by centrifugation for 20 min at 35,000 × g and washed once with water at 2°C. After resuspension in water (5 ml), the walls were heated in a boiling water bath for 15 min to inactivate any autolytic enzymes. The suspension was cooled and then treated with trypsin (0.5 mg) and RNase (0.5 mg) in 0.1 M K2HPO4, (5.5 ml) for 4 hours at 37°C. After thorough washing with water, the cell walls were resuspended in 0.01 M acetate buffer, pH 4.5 (2 ml), and treated with the *Chalaraopsis* B enzyme (0.08 mg) for 16 hours at 37°C. The entire lysates were applied to columns (0.8 × 15 cm) of Ecteola-cellulose (Cl- form, Cellex-E, Bio-Rad) previously equilibrated with water. The soluble glycopeptides were eluted with water (50 ml), and the glycopeptide-tetrahydroxy acid complexes were subsequently eluted with 0.3 M LiCl (50 ml). The *Chalaraopsis* B enzyme is an endoacetylmuramidase devoid of peptidease activity (30, 31). It catalyzes hydrolysis of all the acetylmuramyl linkages in the cell wall of *S. aureus*. The glycopeptide therefore consists of the intact polypeptide of the peptidoglycan, acylated on its ε-alanine amide termini by the carboxyl group of the disaccharide, 4-O-β-N-acetylmuramyl-N-acetylglucosaminyl (Fig. 1). This glycopeptide was measured in the experiments described below by...
determination of its reducing power. Its reducing equivalent is 1.5 per mole of disaccharide, relative to the reducing power of N-acetylmuramic acid (32). Analyses of many of the glycopeptide fractions described in this paper have confirmed this value for the reducing power (see Table III, below).

Analytical Procedures—Most of the procedures used have been completely described (33). Total amino acids and amino sugars were also determined after hydrolysis for 8 hours in 40 μl of 4 N HCl at 100°. The samples were then diluted directly with 1 ml of buffer and applied to the column of a Beckman-Spinro amino acid analyzer. Lyophilization of hydrolysates was avoided since it causes nonreproducible losses of amino sugars, particularly of muramic acid. Known amounts of amino sugars were hydrolyzed under the standard conditions for the estimation of losses in the procedure used. Bound ammonia was also measured on the amino acid analyzer as the increase in free ammonia after hydrolysis for 3 hours in 4 N HCl at 100°, and by a colorimetric procedure used previously (20). O-Acyl groups were determined by hydroxamate formation as described previously (12).

Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer by one of three procedures. (a) Aliquots (0.1- to 0.5-ml) of aqueous solutions were mixed in counting vials with 10 ml of dioxane containing 100 g of naphthalene, 5 g of 2,5-diphenyloxazole (POO), and 125 mg of 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POP-POP) per liter. (b) Aliquots (0.01- to 0.1-ml) of aqueous solutions were spotted on 1-inch squares of Whatman No. 3MM filter paper, dried, and immersed in 15 ml of toluene containing 4 g of 2,5-diphenyloxazole and 300 μg of 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene per liter. (c) Radioactive zones, detected on thin layer chromatograms by radioautography, were transferred directly to counting vials by a suction device and dispersed in 10 ml of the dioxane solution described above, which in addition contained 50 ml of water per liter.

Gel Filtration—Columns of Sephadex G-50 and G-25, fine grade, bead form (Pharmacia, Uppsala, Sweden), were packed in water and eluted with water at room temperature. The void volume (V₀) was determined from the elution volume of blue dextran (Pharmacia), and the sum of void and interior volumes (V₀ + Vₐ) was determined from the elution volume of NaCl (detected with AgNO₃ solution). Kₐ = (Vₐ - V₀)/V₀, and is a measure of the fraction of the interior volume available to a solute eluted by a volume (Vₐ) of eluent.

RESULTS

Accumulation of Uncross-linked Monomer in Presence of Penicillin—From preliminary experiments it became obvious that accumulation of the uncross-linked monomer could be observed only when growth continued at a normal or near normal rate for some period after the addition of penicillin. This occurred at relatively low penicillin concentrations, under conditions similar to those used previously in which growth did not cease for 1 hour after the addition of penicillin (34; see Fig. 17). At higher penicillin concentrations growth was inhibited more rapidly and little or no accumulation of the uncross-linked monomer was observed.
Cultures of *S. aureus*, strain Copenhagen (500 ml), were prepared and, when turbidity was 20% of maximum, the cultures were harvested by centrifugation in the cold and resuspended in 500 ml of one-tenth strength medium (diluted with water) containing penicillin G at 0.086 μg per ml. An additional flask served as a control. After 5 min, each culture received $^{14}$C-glycine (6.8 μC). Dilute medium was used to minimize dilution of the label while permitting growth under near normal conditions for a short period. The cultures were then quickly warmed to 37°C by swirling in a 60°C water bath and were reincubated at 37°C for 30 min. Growth in all cultures was exponential during this period, with no detectable lag. The rate of growth was about 75% of that in normal, undiluted medium. Previous experiments had shown that growth in the cultures containing penicillin began to deviate from normal at 35 min, and all cultures were therefore harvested at this time.

Cell walls were prepared from each of the cultures. Examination of the products of hydrolysis in 6 N HCl indicated that all of the incorporated radioactivity was $^{14}$C-glycine. The walls were solubilized with the B enzyme from *Chalaraopsis*. The solubilized glycopeptide was then separated from the teichoic acid-glycopeptide complex as described in “Materials and Methods” on a column of Ectocea-cellulose. The solubilized glycopeptides, in 2 ml of water, were applied separately to a column of Sephadex G-25 (95 × 1 cm; $V_0 = 24$ ml; $V_0 + V_1 = 50$ ml). The column was developed with water at a rate of 0.3 ml per min. Fractions of 1.1 ml were collected and analyzed for total reducing power and for radioactivity. The reducing power determination was a measure of all of the material present, including both that synthesized before the addition of penicillin and that synthesized after its addition, while the radioactivity measured only the material synthesized after addition of penicillin. The solubilized glycopeptide was separated by the column into three fractions (Fig. 2), which will be referred to as oligomer ($K_d = 0.0$), dimer ($K_d = 0.2$), and monomer ($K_d = 0.65$) (for analyses, see below). The reducing power measurements indicated that addition of penicillin at low concentrations resulted in a sharp redistribution of the material present, with a marked decrease in the amount of the oligomer fraction and a marked increase in the amount of the monomer fraction (Fig. 2A). Much of the oligomer fraction was undoubtedly material synthesized before the period before addition of penicillin, because the radioactivity measurements indicated an even sharper decrease in the amount of the oligomer fraction at all penicillin concentrations (Fig. 2B). Under the conditions of the experiment the total amount of radioactivity incorporated into all fractions was 50% of the control value at 0.086 μg of penicillin G per ml, and it decreased further at higher penicillin concentrations. Comparison of data obtained at the four penicillin concentrations (Table I) indicated that the greatest accumulation of the monomer was observed at the lowest penicillin concentration used and that as the penicillin concentration increased was the total incorporation was suppressed, with a corresponding decrease in the amount of the monomer.

![Fig. 2. Filtration on a column of Sephadex G-25 of the soluble glycopeptides obtained from a control culture and from a culture treated with 0.086 μg of penicillin G per ml by lysis of cell walls with acetylmuramidase. See text for details. Measurements of reducing power (A) and of radioactivity incorporated from $^{14}$C-glycine (B) are shown.](http://www.jbc.org/)

### Table I

<table>
<thead>
<tr>
<th>Antibiotic added</th>
<th>Reducing power</th>
<th>Radioactivity incorporated</th>
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<tbody>
<tr>
<td></td>
<td>% total control</td>
<td>% oligomer</td>
</tr>
<tr>
<td>Penicillin G</td>
<td></td>
<td></td>
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<tr>
<td>0.086</td>
<td>72</td>
<td>46</td>
</tr>
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<td>0.15</td>
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<td>0.3</td>
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<td>45</td>
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<td>1.0</td>
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<tr>
<td>Vancomycin, 7.8</td>
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<tr>
<td>Bacitracin, 78</td>
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<td>77</td>
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</table>

* These values are probably artificially high. See the high base line in Fig. 3.
fraction. However, the proportion of the total radioactivity incorporated into monomer was 50 to 60% in all penicillin-containing cultures, compared to only 10% in the control incubation. On the other hand, 75% of the material synthesized in the control incubation was oligomer, while only 20 to 30% was oligomer in the penicillin-treated cultures.

Effects of Bacitracin and Vancomycin—Similar experiments were carried out with the cells exposed to concentrations of vancomycin (7.8 μg per ml) or bacitracin (78 μg per ml) near the minimum growth-inhibitory concentrations for these antibiotics. Vancomycin induced a nearly complete cessation of incorporation of isotope, while a less complete diminution was induced by bacitracin (7.8 μg per ml) or near the oligomer in the penicillin-treated cultures. Bacitracin (Fig. 3 and Table I). These data are compatible with the effects observed during studies of the inhibition of the polymerization reaction in vitro. Examination of the total material present (by reducing power measurements) indicated that both in the presence of vancomycin and in the presence of bacitracin the relative amount of the monomer had decreased, rather than increased as in the presence of penicillin, and that there was no decrease in the amount of oligomer (Fig. 3 and Table I). Thus, although bacitracin and vancomycin inhibited the incorporation of isotope into the monomer fraction as well as into the other fractions, the previously existing monomer fraction was utilized to form a cross-linked peptidoglycan.

These isotopic data are compatible with enzymatic studies, which have indicated that vancomycin inhibits the transfer of disaccharide-peptide units from the lipid intermediate to a cell wall acceptor, while bacitracin inhibits the dephosphorylation of lipid pyrophosphate, the next step in the same reaction cycle (34-36).

A sample of soluble glycopeptide was also treated with the L11 enzyme prior to filtration on Sephadex G-25 in order to obtain materials for reference. This enzyme catalyzes the hydrolysis of glycyglycine and D-alanyl-glycine linkages in the interpeptide bridges of the soluble glycopeptide (16, 17). The reaction was stopped when 65% of the bridges had been hydrolyzed so that all fractions would remain in the lysate. Filtration on Sephadex G-25 revealed the expected distribution of fragments, with a marked increase in the amount of the monomer fraction and a marked decrease in the amount of the polymer fraction, and provided a standard for the position of the monomer on the Sephadex column (Fig. 3).

Pulse Labeling Experiments: Evidence that Uncross-linked Monomer is Precursor of Cross-linked Peptidoglycan—Further experiments of the type described above indicated that a better fractionation of the glycopeptide oligomers could be obtained if the filtrations were carried out on a column of Sephadex G-50 operated in series with a column of Sephadex G-25. The pulse labeling experiments described here were carried out with this technique. Early log phase cells (21) were labeled with 14C-glycine for 30 sec. The suspension was rapidly cooled. The cells were recovered by centrifugation and washed. One sample was saved for analysis, a second was incubated for 3 min at 37°, a third was incubated for 20 min, and a fourth was incubated for 20 min in the presence of 120 μg of penicillin G per ml. After this treatment the cell walls were prepared and solubilized with the B enzyme from Chalaropsis. The soluble glycopeptide was separated from the teichoic acid-glycopeptide complex and then filtered on the G-50 and G-25 columns (Fig. 4). Under the conditions used the total amount of cell wall did not increase greatly and the distribution of monomer, dimer, trimer, tetramer, and higher oligomers (which were separated by the Sephadex columns) did not change greatly (measured by reducing power, Fig. 4).

After 20 sec of labeling with 14C-glycine, most of the radioactivity in the cell wall was found in the monomer fraction and small amounts were found in each of the higher oligomers (Fig. 4A). After incubation for 3 min the total amount of radioactivity in the cell wall had increased 10-fold by incorporation of 14C-glycine from precursors and endogenous pools which were not removed by the washing procedure, and by this time radioactivity was uniformly distributed among the various oligomers (Fig. 4A). After 20 min of incubation the radioactivity had redistributed itself so that now most of the radioactivity was present in the highest oligomers, although small amounts remained in the monomer (Fig. 4B). The distribution of radioactivity was now similar to the distribution of reducing power, which represents the total previously synthesized glycopeptide. On the other hand, if penicillin G was present from the beginning of the second incubation, the increase of total glycone incorpora-
Incorporation of radioactivity into glycopeptide oligomers after incubation for various times following 30 sec of pulse labeling with $^{14}$C-glycine

These are the quantitative data obtained from the experiment described in the legend to Fig. 4 and in the text. Data are expressed as the percentage in each fraction of the total radioactivity incorporated into glycopeptide at each time. The total amounts of radioactivity incorporated in each experiment, expressed as percentage of the amount incorporated in the 20-min control, were zero time, 10%; 3 min, 66%; and 20 min plus penicillin G, 59%.

<table>
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<th>Time of second incubation</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Trimer</th>
<th>Tetramer</th>
<th>Oligomer</th>
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<tr>
<td>0</td>
<td>40</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>44</td>
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<tr>
<td>3</td>
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<td>11</td>
<td>15</td>
<td>11</td>
<td>8</td>
<td>55</td>
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<tr>
<td>20, + penicillin G</td>
<td>66</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>20</td>
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</table>

Fig. 4. Filtration on tandem columns of Sephadex G-50 and G-25 of the soluble glycopeptides obtained from cells pulse labeled with $^{14}$C-glycine for 30 sec and then incubated for various periods in the presence or absence of penicillin. A culture (21) of S. aureus, strain Copenhagen, at 15% of maximal growth was harvested and resuspended in 100 ml of medium at one-tenth of normal strength at 2°C. The suspension was rapidly warmed to 37°C, $^{14}$C-glycine (14.4 μC) was added, and 30 sec later the culture was rapidly iced and the cells were recovered by centrifugation. After one wash with water at 2°C, the cultures were resuspended in 100 ml of full-strength growth medium. One sample (16 ml) was immediately iced and recovered by centrifugation (zero time control sample). A second sample was rapidly brought to 37°C and the cells were recovered after 3 min of incubation. A third sample of 16 ml was mixed with 160 ml of full-strength growth medium and incubated for 20 min prior to centrifugation, and a fourth sample was similarly treated except that it contained penicillin G, 59%. The extremely small amount of radioactivity which entered the oligomer fractions is particularly evident in quantitative data obtained by pooling and counting the various fractions (Table II). Thus, this pulse labeling experiment showed that radioactivity was initially incorporated as the monomer and that on continued incubation this fraction became incorporated into an oligomer fraction. Moreover, penicillin virtually completely blocked the transformation of monomer into the cross-linked oligomers.

Analyses of Glycopeptide Oligomers—Initially, materials obtained from the experiments shown in Fig. 2 were analyzed. Comparison of the monomer fractions from the penicillin-treated and control cultures (Table III) revealed that both contained 3 alanine residues, 2 of which were $\delta$-alanine residues, 1 of which was COOH-terminal. The presence of the COOH-terminal D-alanine residue was accompanied in each case by the presence of an NH$_2$-terminal glycine residue. By contrast, the polymer fraction had 2 rather than 3 alanine residues, only 1 $\delta$-alanine residue, and contained only trace amounts of any COOH-terminal or NH$_2$-terminal groups. All fractions analyzed contained an amide group. The presence of this amide indicates that the introduction of the amide residue must precede bridge closure and that this reaction is not inhibited by penicillin. Recent studies have indicated that lipid-P-P-disaccharide-pentapeptide is the substrate for the amidation reaction (1).
The analyses in Part A were obtained from the materials in the experiments illustrated in Figs. 2 and 3. The amino acid analyses were carried out by thin layer chromatography, in which the glutamic acid value was more reliable than the lysine. The analyses in Part B were obtained from materials pooled from a number of experiments similar to that shown in Fig. 4. The amino acid and amino sugar analyses were carried out on the amino acid analyzer. Disaccharide was calculated from the reducing power with the use of an N-acetylglucosamine standard, dividing by 1.5 (the reducing equivalent of the disaccharide (32)). NH₂-glycine

Table III

<table>
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<th>Fraction</th>
<th>Disaccharide</th>
<th>Hexosamine</th>
<th>Glucosamine</th>
<th>Muramic acid</th>
<th>Glutamic acid</th>
<th>Lysine</th>
<th>Glycine</th>
<th>Alanine</th>
<th>L-Alanine</th>
<th>n-Alanine</th>
<th>COOH-terminal alanine</th>
<th>NH₂-terminal glycine</th>
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* Number of repeating disaccharide-peptide subunits.

Also contained 72 moles of COOH-terminal glycine per 100 moles of glutamic acid.

Table III shows the analyses of glycopeptide oligomers. The faster moving component in each case was the O-acetylated monomer and the slower moving component was the monomer without an O-acetyl group (see below). Filtration on a Biogel P-2 column similarly revealed that the monomer fractions from the control and the penicillin-treated cultures had nearly identical elution volumes. The molecular weights estimated from the elution volumes were 1100 to 1500. The calculated molecular weight was 1280. The monomer from L₄ digestion (calculated mol wt. 1210) also had the same elution volume on this column.

A second set of analyses was carried out on pooled oligomers recovered from several experiments as shown in Fig. 4. These analyses (Table III) confirmed the supposition that the various fractions were monomer, dimer, trimer, tetramer, and higher oligomers. Particularly noteworthy are the values for COOH-terminal alanine and NH₂-terminal glycine, which were nearly equivalent and close to the theoretical values of 1.0, 0.5, 0.33, 0.25, and less than 0.20 for these various fractions. All fractions had, per mole of glutamic acid, 1 mole each of disaccharide, L-alanine, amide ammonia, and lysine, and 5 moles of glycine. Each oligomer of chain length n monomer units also contained 2 + 1/n moles of total alanine and 1 + 1/n moles of total n-alanine per mole of glutamic acid.

Separation of Glycopeptide Oligomers by Thin Layer Chromatography—Aliquots of the glycopeptide oligomers (up to 1 μmole) containing about 5000 cpm of ¹⁴C-glycine were spotted as 1-cm zones on plates of Silica Gel G (thickness, 0.25 mm). The plates were developed for 4 to 8 hours in isobutyric acid-1 N NH₄OH (3:5) at room temperature. After drying for 2 hours in a stream of air at room temperature development was repeated.

After drying again at 40° for 1 hour, radioautograms were prepared with Kodak Royal Blue x-ray film by exposure for 48 hours (Fig. 5). Two components were seen in the monomer fraction, corresponding to the O-acetylated and nonacetylated monomers (mobility of the O-acetylated monomer, 1.16 relative to the nonacetylated monomer). Three fractions were present in the dimer, presumably corresponding to dimers containing 0, 1, or 2 O-acetyl groups (mobilities, 0.98, 0.78, and 0.57, relative to the nonacetylated monomer). The trimer gave rise to four zones (mobilities, 0.80, 0.64, 0.47, and 0.37) and the tetramer to several poorly resolved zones, the slowest of which was presumably the nonacetylated tetramer (mobility, 0.21).

An effort was made to de-O-acetylate these compounds by treatment with 5 M ammonia at 37°. However, all fractions were decomposed by treatment with ammonia, both monomer fractions giving rise to zone with a relative mobility of 1.25, the dimer to a compound with mobility 1.06, trimer to one with mobility 1.0, and tetramer to one with mobility 0.9 (Fig. 5). Subsequent study (37) showed that the materials formed in the present study correspond to lactyl peptide monomer, dilactyl peptide dimer, trilactyl peptide trimer, and tetralactyl peptide tetramer, respectively. The β elimination reaction has also been observed in two other laboratories (38, 39).

In another experiment, cell walls were prepared and incubated in 10 ml of 0.01 M sodium methoxide at 60° for 2 hours in order to remove all acetyl groups. After this treatment the walls were recovered by centrifugation, washed in dilute acetic acid and water, and finally solubilized with the B enzyme from Chalio-
Untreated Alkali-treated

FIG. 5. Separation of glycopeptide oligomers by thin layer chromatography. See the text for details. Left, untreated oligomers; right, oligomers treated with 5 M ammonia at 37°C. A radioautogram of the thin layer plate is shown. It is evident that some byproducts are also formed in the alkali treatment. See Fig. 8 for separation of glycopeptide oligomers obtained after cell walls had been treated with sodium methoxide at 60°C to remove O-acetyl groups prior to lysis with the acetylmuramidase.

Effect of Penicillin Concentration on Incorporation of 14C-Glycine into Glycopeptide Oligomers and Irreparable Nature of Lesion in Cell Wall Synthesis Induced by Penicillin—With the technique of examining the various oligomers by thin layer chromatography, it was possible to carry out a number of experiments relatively easily which would have been exceedingly difficult to carry out with the Sephadex columns. In order to examine the effect of different concentrations of penicillins on accumulation of the monomer, cells were incubated with concentrations of penicillin G between 0.05 and 20 μg per ml during 20 min with 14C-glycine present in the medium. At the end of this time cell walls were prepared, de-O-acetylated, and solubilized with the B enzyme from Chalaraopsis. After separation from teichoic acid-glycopeptide complex, the glycopeptide fragments were then separated by thin layer chromatography. After radioautography, areas corresponding to the various oligomers were scraped into vials for counting in the liquid scintillation spectrometer. This experiment (Fig. 6d) confirmed the earlier indications that accumulation of the monomer occurred only at low penicillin concentrations. At concentrations above 0.8 μg per ml accumulation decreased, and at 20 μg per ml there was an actual decrease in the amount of monomer formed rather than an increase.1

Some of the decrease in incorporation into monomer at high penicillin concentrations could be due to the formation of soluble peptidoglycan products containing a large proportion of monomer (compare Reference 40), which might not be attached to the cell wall and hence would be lost during the preparation of the walls. In recent experiments, the amount of such soluble products expressed as percentage of the total 14C-glycine incorporated into control cell walls was 7% in control cells and 12 to 13% in cells labeled in the presence of 0.1, 1.0, or 10 μg of penicillin G per ml. This soluble material obtained from penicillin-treated cells was almost completely degraded to monomer by treatment with the Chalaraopsis B enzyme. Conversion to monomer of the material obtained from control cells was much less complete. Thus, although formation of soluble peptidoglycan was increased by penicillin, the amount formed was not large and was not increased by increasing penicillin concentrations. It cannot, therefore, provide an explanation of the much larger decrease in formation of total glycopeptide or of the decreasing increment of monomer observed at increasing penicillin G concentrations (Table I and also Fig. 6).
oligomers formed was decreased at all penicillin concentrations used, and at concentrations above 0.8 µg per ml the formation of oligomers was practically eliminated.

A duplicate set of cells was washed free of penicillin and ^14C-

Fig. 6. Incorporation of ^14C-glycine into glycopeptide oligomers in the presence of various concentrations of penicillin G, and the effect of subsequent incubation in the absence of penicillin G. A culture (2000 ml) of S. aureus, strain Copenhagen, was harvested in exponential growth phase at 29°C of maximum growth, resuspended in medium diluted to one-tenth strength (1050 ml) at 29°C, and quickly warmed to 37°C. Aliquots (170 ml) were transferred to 250-ml flasks containing penicillin G at various concentrations. One flask without antibiotic served as control. After 5 min of shaking, ^14C-glycine (1.5 µCi) was added to each flask; cells were harvested 20 min later. The soluble glycopeptides were prepared from the de-O-acetylated cell walls as described in the text. Glycopeptides in water (10 ml) were analyzed for radioactivity and reducing power. The specific radioactivities of the soluble glycopeptides relative to the control were 95, 80, and 49% at 1.4, 2.4, and 32 µg of penicillin G per ml, respectively. Aliquots (50 ml) of the solutions (1 ml) were subjected to thin layer chromatography. Data are expressed as the amount of radioactivity in each fraction relative to the amount in the equivalent control fraction.

glycine after the 20-min incubation and then incubated for 70 min in normal medium. The cell wall oligomers were then prepared and fractionated as described above. During the 70-min incubation the cells began to grow after a short lag, and turbidity doubled during this second incubation. Nevertheless, there was little change in the distribution of the ^14C-oligomers synthesized during the earlier, 20-min incubation in the presence of penicillin (Fig. 6B). In particular, the accumulated ^14C-monomer remained uncleaved during the second incubation period. Thus, it seemed clear that monomer introduced in large amount into the cell wall in the presence of penicillin was irreversibly uncleaved and subsequent incubation of the cells in the absence of penicillin under conditions in which growth occurred did not result in cross-linking of the previously introduced monomer units.

Effects of Other Penicillins on Formation of Cell Wall Oligomers—Similar experiments were carried out with methicillin, ampicillin, and cephalothin. With these substances accumulation of the monomer was also readily shown, together with a decrease in the formation of higher oligomers at all concentrations tested (Figs. 7 and 8). Total glycopeptide synthesis was reduced to a minimum of 40% of control levels (see legend to Fig. 7), and the decrease in accumulation of the monomer occurred at the highest concentrations of antibiotics examined in these experiments.

Failure of Phenoxyethyl Dipeptides to Inhibit Growth of S. aureus—Since penicillin G is presumed to be acting as an analogue of acyl-d-alanyl-d-alanine in inhibiting the cross-linking reaction, it
seemed possible that phenacetyl dipeptides might also inhibit growth to some extent. Phenacetyl-β-alanyl-α-alanine, and phenacetyl-α-alanyl-β-alanine were prepared with the use of phenacetyl chloride to acylate the corresponding dipeptides. The growth of S. aureus, strain Copenhagen, was measured in flasks which contained 2 mM concentrations of each of the peptide derivatives, in a flask containing 0.4 μg of penicillin G per ml (i.e. about 0.001 mM), and in a flask containing both penicillin G and phenacetyl-α-alanyl-β-alanine. No deviation of growth from the normal rate was observed in the flask containing the phenacetyl dipeptides, and the presence of phenacetyl-β-alanyl-β-alanine together with penicillin G did not reverse the inhibition of growth induced by this antibiotic. Similar results have recently been reported for studies in which various phenacetylglycyl-amino acid derivatives structurally related to penicillin G were used (41).

Failure of Penicillinase to Catalyze Hydrolysis of Phenacetyl Dipeptides—It has been suggested (19) that penicillinase may be the transpeptidase released from its site of membrane attachment and differing from transpeptidase in that the penicilloyl residue to the amino end of the pentaglycine bridge is formed can further react with water so that a hydrolysis of penicillin is the end result. It was therefore of interest to examine the possibility that penicillinase might also catalyze the hydrolysis of peptides related to the natural cell wall substrate. However, no hydrolysis of phenacetylglucylglycine, phenacetyl-β-malanyl-β-alanine, or phenacetyl-α-malanyl-β-alanine was observed during a 3-hour incubation with an amount of penicillinase which would catalyze the hydrolysis of 8.4 μmoles of penicillin G in less than 1 min. Similarly, penicillinase did not catalyze the hydrolysis of the terminal β-alanine residue of UDP-acetylglucosyl-pentapeptide labeled with 14C-α-alanyl-β-alanine. No inhibition of the penicillinase-catalyzed hydrolysis of penicillin G by the phenacetyl dipeptides was observed.

**Failure of 14C-Penicillin G to be Incorporated into Cell Wall Peptidoglycan**—Another possibility which needed to be excluded was that the penicilloyl transpeptidase might transfer the penicilloyl residue to the amino end of the pentaglycine bridge and thereby prevent cross-linking. The isolation of the uncross-linked monomer containing glycine chains with free amino ends indicated that no considerable penicilloylation could have occurred, and the following experiment established that no penicilloylation could be detected even when 14C-penicillin G was used. The culture (1000 ml) was incubated with 14C-penicillin G (103 μg, 0.28 μmole; specific activity, 15.7 μCi per μmole). The penicillin G was added during exponential growth, and incubation was continued for 4 hours. The cell walls prepared from this culture contained only 1100 cpm (i.e. about 70 μmoles of 14C-penicillin G). The cell walls were solubilized with the B enzyme from Chalaropsis and fractionated on a column of Sephadex G-25 as described in the legend to Fig. 2. The uncross-linked monomer (10 μmoles) represented 32% of the total glycopeptide (31 μmoles) but contained not more than 2% of the radioactivity which had been present in the cell wall (i.e. less than 1 μmole). The remainder of the radioactivity was associated with the high molecular weight material obtained in the fractionation. The extremely small amount of 14C-penicillin G present may be ascribed to some unspecific reaction, and the absence of radioactivity in the monomer fraction excludes the possibility that a significant transfer of penicilloyl residues to peptidoglycan occurred in this organism.

**DISCUSSION**

Thus, it is clear that in the presence of low concentrations of penicillin G an uncross-linked monomer, which may be referred to as a nascent peptidoglycan unit, accumulates. Pulse labeling experiments have clearly shown that this uncross-linked unit is a precursor of the final cross-linked product and that penicillin blocks its subsequent cross-linking. The accumulation of the uncross-linked material is also induced by ampicillin, cephalothin, and methicillin. At higher concentrations of penicillin G cell wall synthesis is brought to a more abrupt halt and no accumulation of the uncross-linked monomer is observed. It has also been established that the uncross-linked unit, once it is present in the wall to a large extent, cannot subsequently be cross-linked when the penicillin is removed from the culture and growth is allowed to proceed again. We suggest the following interpretation of these findings. Normally, uncross-linked units are introduced into a growing peptidoglycan at sites on the exterior of the membrane, and may be immediately cross-linked (presumably to a peptide on a second newly introduced peptidoglycan unit at a neighboring site for glycan polymerization) before a second unit is introduced. At low concentrations of
penicillin it is possible that cell wall synthesis “stutters,” that is, cross-linking is partially inhibited and cell wall synthesis continues for a time with the introduction of a certain number of cross-linked as well as uncross-linked units. However, in the process of this abnormal type of cell wall synthesis, the uncross-linked units become spatially removed from the membrane-bound enzymes which catalyze cross-linking and therefore, on re-establishment of normal growth and cell wall synthesis by removal of penicillin, these units can no longer become cross-linked. At high concentrations of penicillin G, when cross-linking is totally inhibited, cell wall synthesis is brought to an abrupt stop for reasons which are not clear, no stuttering occurs, and hence little or no accumulation of the uncross-linked units can be observed.

This interpretation may provide an explanation of the zone phenomenon originally described by Eagle (42) and subsequently studied by other investigators (e.g. Reference 43). Eagle observed in S. aureus that the killing rate paradoxically was higher at a low penicillin concentration (1 pg per ml) than at a high penicillin concentration (100 pg per ml). Lederberg and St. Clair (43) observed the same phenomenon with Escherichia coli. In plates containing sucrose agar and a gradient of penicillin, a zone was observed at a relatively low penicillin concentration in which virtually no organism survived either as cells or as protoplasts. At higher penicillin concentrations, however, the colonies of E. coli survived as protoplast colonies. In view of the data presented here these observations could be explained as follows. At low concentrations of penicillin, cell wall synthesis stutters and a weakened wall is synthesized. Under these conditions the organisms are fragile and are more readily killed by various environmental influences. At high penicillin concentrations, however, cell wall synthesis ceases abruptly. The protoplast remains protected by its original wall, which has been only slightly damaged by a small amount of synthesis after the addition of penicillin. It is therefore better able to survive than the organism in which a weakened wall has been propagated.

Many facets of the physiology of wall growth remain unexplained. One obvious one is the fact that cells of P. mirabilis, although converted to spheres by the presence of penicillin, continue to grow and multiply as spheres indefinitely. These cells contain all the normal constituents of the cell wall. However, the units are not cross-linked to each other, thus accounting for the transformation of the rigid rod-shaped wall to a less rigid sphere (23, 24). On the other hand, the spherical organism, S. aureus, ceases to grow abruptly in the presence of high concentrations of penicillin when cross-linking is inhibited, and at low concentrations of penicillin it can continue to grow only for a relatively brief period, during which it synthesizes a partially cross-linked wall. The reason for this striking difference in the growth physiology of these organisms is not apparent.

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Biosynthesis of the Peptidoglycan of Bacterial Cell Walls: XII. INHIBITION OF CROSS-LINKING BY PENICILLINS AND CEPHALOSPORINS: STUDIES IN STAPHYLOCOCCUS AUREUS IN VIVO

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