Steric Effects on Penicillin-Sensitive Peptidoglycan Synthesis in a Membrane-Wall System from *Gaffkya homari*†

Carolyn V. Carpenter, Sue Goyer, and Francis C. Neuhaus*

ABSTRACT: Residues 4 and 5 of the pentapeptide moiety, R-Ala[1]-DGlu[2]-Lys[3]-DAla[4]-DAla[5], of peptidoglycan play an important role in the donor phase of cross-linked glycan synthesis. To assess the role of these residues in this phase, a series of UDP-MurNAc-peptides were biosynthesized with residues 4 and 5 replaced singly by either D-α-amino-*n*-butyric acid, D-norvaline, or D-valine. The six nucleotides were compared with UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (reference) in nascent (penicillin-insensitive) peptidoglycan synthesis and in penicillin-sensitive peptidoglycan synthesis. The synthesis of penicillin-sensitive peptidoglycan is catalyzed by membrane-walls isolated from *Gaffkya homari* and would appear to require the concerted action of transglycosylase and transpeptidase. The membrane-wall system shows a high degree of discrimination for the steric substituents, $-CH_3$ and $-CH_2CH_3$, in residue 4. For example, for UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla and -Ala-DGlu-Lys-DAla-DAbu, V_{max}/K_m is 0.19 and 0.95, and V_{max} is 0.03 and 0.52, respectively, of the value for the reference nucleotide. In contrast, for the synthesis of nascent peptidoglycan with these nucleotides V_{max}/K_m is 0.75 and 0.80, and V_{max} is 0.71 and 1.0, respectively, of the value for the reference nucleotide. This trend was also illustrated with the other nucleotides in the time course experiments. These results indicate that the penicillin-sensitive enzyme(s), presumably the transpeptidase, has a higher degree of specificity in the donor phase for D-alanine in residue 4 than for D-alanine in residue 5 in the cross-linking stage of peptidoglycan synthesis.

The biosynthesis of peptidoglycan, the major cell wall structural polymer, requires two nucleotide-activated precursors, UDP-GlcNAc[1] and UDP-MurNAc-pentapeptide. Enzymes intercalated into the membrane matrix utilize these precursors in a cycle of reactions with undecaprenyl phosphate as the carrier (Ghuysen and Shockman, 1973). The cross-linking of the glycan product of this cycle, catalyzed by a penicillin-sensitive transpeptidase, results in the final assembly of the three-dimensional glycan network (Blumberg and Strominger, 1974).

Many of the in vitro membrane systems that have been studied catalyze the synthesis of uncross-linked peptidoglycan, defined as nascent peptidoglycan. In the synthesis of the nascent glycan, the initial enzyme, phospho-MurNAc-pentapeptide translocase, catalyzes the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenyl phosphate (Neuhaus, 1971). The product of this reaction, undecaprenyl-diphosphate-MurNAc-pentapeptide, is the acceptor for GlcNAc derived from UDP-GlcNAc in a reaction catalyzed by GlcNAc transferase (Anderson et al., 1967). In many bacterial strains, incorporation of interpeptide bridge residues and amidation of the α-carboxyl group of the glutamic acid residue completes the synthesis of the lipid disaccharide (Ghuysen and Shockman, 1973). In the final step of nascent peptidoglycan synthesis, transglycosylase catalyzes the $\beta 1 \rightarrow 4$ addition of disaccharide units at the reducing end of the growing glycan chain attached to undecaprenyl diphosphate (Ward and Perkins, 1973). This multienzyme system for the synthesis of nascent peptidoglycan is not affected by the presence of benzylpenicillin (Anderson et al., 1966).

The successful demonstration of cross-linking was first achieved with membrane preparations from *Escherichia coli* (Izaki et al., 1968; Araki et al., 1966; Pollock et al., 1974). Cross-linking of nascent peptidoglycan in this preparation is catalyzed by a penicillin-sensitive transpeptidase. More recently the penicillin-sensitive transpeptidation has been described in membrane fragments from *Bacillus megaterium* (Wickus and Strominger, 1972; Reynolds and Barnett, 1974), *Bacillus stearothermophilus* (Linnett and Strominger, 1974), *Sporosarcinia ureae* (Linnett et al., 1974), and *Streptomyces* spp (Ghuysen et al., 1974).

In 1972 Mirelman and Sharon discovered that membrane-walls isolated from *Staphylococcus aureus* catalyze the synthesis of peptidoglycan that is cross-linked to the preexisting glycan of the wall. This system has also been described in membrane-wall preparations from *Micrococcus luteus* (Mirelman et al., 1972, 1974) and *Bacillus licheniformis* (Ward, 1974). Recent evidence indicates that the newly synthesized material is covalently linked to preexisting peptidoglycan of the wall (Mirelman et al., 1974; Ward and Perkins, 1974). The inhibition of peptidoglycan synthesis in this system by penicillin indicates that transpeptidation plays an important role in the incorporation of glycan into the wall. In the membrane-wall system from *B. licheniformis*, the synthesis of wall peptidoglycan was inhibited 93% in the presence of penicillin. In contrast, the synthesis of wall peptidoglycan with membrane-walls from *M. luteus* was inhibited only 60% in the presence of penicillin. In this system newly synthesized material is incorporated into preexisting cell wall by a penicillin-insensitive process, as well as a penicillin-sensitive process. The model proposed by Mirelman et al. (1972, 1974) suggests that lipid disaccharide units are utilized by the concerted (or

† From the Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60201. *Received January 12, 1976.* This work was supported in part by Grant AI-04615 from the National Institute of Allergy and Infectious Diseases.

[1] Unless stated, all abbreviations of residues denote the L configuration. The omission of the hyphen, i.e., DAla-DAla, and the abbreviations conform with suggestions cited in *Biochemistry 5,* 2485 (1966). In UDP-MurNAc-pentapeptide the residues are numbered as follows: UDP-MurNAc-Ala[1]-DGlu[2]-Lys[3]-DAla[4]-DAla[5]. Abbreviations used are: MurNAc; *N*-acetylmuramyl; GlcNAc, *N*-acetylglucosamine; UDP, uridine diphosphate; Nva, norvaline; Abu, α-amino-*n*-butyric acid; UMP, uridine monophosphate.

concurrent) action of the transpeptidase and transglycosylase. Thus, nascent peptidoglycan that is synthesized by the membrane preparation need not be a necessary intermediate in the synthesis of cross-linked peptidoglycan in the membrane-wall preparation.

It is the purpose of this paper to define the specificity requirements of the penicillin-sensitive enzyme(s) in the membrane-wall system from *Gaffkya homari* for the DAla-DAla moiety of the peptide subunit of UDP-MurNAc-pentapeptide. Residues 4 and 5 play an important role in the donor phase of cross-linked peptidoglycan synthesis. To assess the role of these residues, a series of UDP-MurNAc-peptides was biosynthesized with residues 4 and 5 replaced singly by either D-α-NH_2-*n*-butyric acid, D-norvaline, or D-valine. The six nucleotides were compared with UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (reference) in nascent peptidoglycan synthesis and in penicillin-sensitive peptidoglycan synthesis. These experiments indicate that the penicillin-sensitive enzyme(s) in the membrane-wall system has a high degree of specificity for D-alanine in residue 4 of the peptide subunit. *G. homari* was chosen for these studies because it has a simple type of peptidoglycan (Nakel et al., 1971) with direct cross-linkages (A 1α type [Schleifer and Kandler, 1972]) between R_4DAla of one peptide subunit and the ϵ-amino group of R_3Lys of a second peptide subunit, and because the specificity profile for nascent peptidoglycan synthesis has been defined with regard to replacement of D-alanine residues by glycine in the peptide subunit (Hammes and Neuhaus, 1974b).

Experimental Procedure

Materials. The DD-dipeptides were synthesized by Dr. H. Plaut, previously associated with the Cyclo Chemical Corp. UDP-[U-^{14}C]GlcNAc (300 mCi/mmol) was purchased from Amersham/Searle Corp. [5-^{3}H]Uridine 5′-monophosphate (17.3 Ci/mmol) was obtained from Schwarz-Mann. Uridine 5′-monophosphate was purchased from Sigma Chemical Co. Sodium dodecyl sulfate was a product of Aldrich Chemical Co. The sources of other chemicals have been previously described (Hammes and Neuhaus, 1974a,b). *G. homari* (ATCC 10400) was maintained in lyophilized samples at −20 °C.

Preparation of Membrane Fragments for Nascent Peptidoglycan Synthesis and Phospho-MurNAc-pentapeptide Translocation. Membrane fragments for the synthesis of nascent peptidoglycan were prepared from *G. homari* according to the procedure described by Hammes and Neuhaus (1974b). When these fragments were used for assaying phospho-MurNAc-pentapeptide translocase, they were preincubated with UMP to generate the maximum amount of undecaprenyl phosphate (Hammes and Neuhaus, 1974a). This procedure lowers the background activity generated by endogenous undecaprenyl-diphosphate-MurNAc-pentapeptide participating in the reverse transfer reaction in the exchange assay.

Preparation of Membrane-Walls for Wall Peptidoglycan Synthesis. G. homari was grown in the medium described by Hammes and Neuhaus (1974b). The cells were grown aerobically (6 l./min) in a New Brunswick I-50 fermentor at 34 °C with a stirring rate of 200 rpm. The cells were harvested in early log-phase growth (three generations, turbidity of 1.0 at 650 nm when read against medium) and washed in 0.02 M Tris-HCl buffer (pH 7.8) containing 1 mM mercaptoethanol. For the disruption of the cells, 40-ml lots of a 15% suspension in this buffer were mixed with 35 g of plastic beads and two drops of antifoam (General Electric, Antifoam 66). The bacteria were disrupted in a Bronwill mechanical cell homogenizer (Braun Model MSK) at 4000 cycles/min for 2 min with cooling by CO_2. Homogenization of the cell suspension for longer than 3 min gave membrane-walls with a marked decrease in the penicillin-sensitive peptidoglycan synthesizing activity. After homogenization, the plastic beads were removed by filtration through a fine-mesh cloth. To remove unbroken cells the suspension was centrifuged at 1100*g* for 10 min. The membrane-walls were sedimented by centrifugation at 7500*g* for 15 min. The pellet was suspended in 0.02 M Tris-HCl buffer (pH 7.8) containing 1 mM mercaptoethanol and 20 mM $MgCl_2$ (TMM buffer), stirred for 30 min at 4 °C, and reisolated by the above procedure. The amount of protein per mg of dry wt is 315 μg. The membrane-wall pellet was resuspended in TMM buffer and stored at −196 °C in small samples. The membrane-walls are used for assaying the synthesis of penicillin-sensitive peptidoglycan that is incorporated into wall material. The method of preparation is similar to that described by Mirelman and Sharon (1972).

Preparation of UDP-MurNAc-pentapeptides. UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla was isolated from *Staphylococcus aureus* Copenhagen as previously described (Hammes and Neuhaus, 1974a). Analogues of UDP-MurNAc-pentapeptide were synthesized enzymatically according to the method of Neuhaus and Struve (1965). The reaction mixture contained 0.015 M Tris-HCl (pH 7.8), 0.05 M $MgCl_2$, 5 mM ATP (neutralized with NaOH), 1 mM UDP-MurNAc-Ala-DGlu-Lys (prepared from *S. aureus* Copenhagen by the procedure of Hammes and Neuhaus, 1974a), 10 mM dipeptide, and 9.2 mg of partially purified UDP-MurNAc-Ala-DGlu-Lys:DAla-DAla ligase (ADP) (55–70% ammonium sulfate fraction; Neuhaus, 1962a) from *Streptococcus faecalis* (ATCC 8043) in a total volume of 2 ml. The mixture was incubated for 60 min at 37 °C. The reaction was terminated by heating the mixture at 100 °C for 2 min. Denatured protein was removed by centrifugation and the pellet was washed once with 1 ml of water. The supernatant fractions were filtered on a column of Sephadex G-25 as described by Stickgold and Neuhaus (1967), and the fractions containing the UDP-MurNAc-peptides were pooled and concentrated. UDP-MurNAc-pentapeptide was separated from unreacted UDP-MurNAc-tripeptide by paper chromatography in solvent A. The nucleotide-pentapeptide band was eluted with 0.01 M NH_4HCO_3, pH 8, after which the preparation was desalted by gel filtration on Sephadex G-25. A portion of the final product was hydrolyzed for determination of the amino acid composition. The molar ratios of the amino acids were consistent with the expected values and agreed with the results of work by Neuhaus and Struve (1965).

Assay for Phospho-MurNAc-pentapeptide Translocase: Exchange Assay. The exchange assay, performed by the method of Hammes and Neuhaus (1974a), measures the exchange of [^{3}H]UMP with the unlabeled UMP moiety of UDP-MurNAc-pentapeptide (eq 1).

$$[^3\text{H}]\text{UMP} + \text{UDP-MurNAc-pentapeptide} \overset{K^+,\,Mg^{2+}}{\rightleftharpoons} [^3\text{H}]\text{UDP-MurNAc-pentapeptide} + \text{UMP} \quad (1)$$

Exchange activity was calculated according to the first-order rate equation reported by Struve et al. (1966). The rate of exchange, R, is presented as moles exchanged per liter per min.

Assay for Nascent Peptidoglycan Synthesis. The synthesis of nascent peptidoglycan was determined as previously described (Hammes and Neuhaus, 1974b). Membrane fragments catalyze the penicillin-insensitive formation of uncross-linked peptidoglycan. This material is defined as nascent

peptidoglycan. Formation of product is measured by the incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc into a product chromatographically immobile in solvent A. The labeled product is sensitive to lysozyme, and the incorporation of [^{14}C]GlcNAc is dependent on the presence of UDP-MurNAc-pentapeptide.

Assay for Penicillin-Sensitive Peptidoglycan Synthesis. Penicillin-sensitive peptidoglycan synthesis was assayed after the procedure of Ward (1974). Membrane-walls catalyze the synthesis of penicillin-sensitive peptidoglycan incorporated into walls (Mirelman et al., 1972, 1974; Ward, 1974; Ward and Perkins, 1974). The amount of peptidoglycan synthesized is measured by the incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc into walls isolated from the reaction mixture by repeated washing with 2% sodium dodecyl sulfate, 1 M NaCl, and water. The reaction mixture contained the following: membrane-walls (160 μg of protein), 50 mM Tris-HCl buffer (pH 7.8), 50 mM magnesium acetate, 10 mM NH_4Cl, 4 mM ATP (neutralized with NaOH), 4×10^{-4} M UDP-[^{14}C]-GlcNAc (5.5 cpm/pmol), and UDP-MurNAc-pentapeptide in a total volume of 135 μl. After incubation at 25 °C for the indicated times, the reaction was terminated by heating the mixture for 2 min at 100 °C. To the mixture 1 ml of water was added, and the mixture was centrifuged for 3 min in a Beckman Microfuge B. The supernatant fraction was discarded and the wall fraction was isolated from the membrane-walls by washing the pellet successively with 2% sodium dodecyl sulfate (3×1 ml), 1 M NaCl (2×1 ml), and water (3×1 ml). The wall pellet was suspended in 1.5 ml of water and assayed for radioactivity.

The total incorporation of [^{14}C]GlcNAc into walls includes both the penicillin-sensitive and the penicillin-insensitive incorporation. In order to measure the penicillin-sensitive component, the total incorporation was corrected for the penicillin-insensitive component. All determinations of v_0 for the Lineweaver-Burk plots (Figure 3) reflect the penicillin-sensitive incorporation of [^{14}C]GlcNAc into the wall. Since the incorporation is characterized by a lag period, v_0 is calculated from the amount of penicillin-sensitive incorporation observed between 15 and 30 min.

Analytical Procedures. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Descending paper chromatography was performed on Whatman No. 3 MM paper in isobutyric acid–concentrated NH_4OH–H_2O (66:2:33 v/v), solvent A. Radioactivity from the chromatograms was counted in toluene containing 0.3% 2,5-diphenyloxazole and 0.025% 1,4-bis[2-(5-phenyloxazolyl)]benzene. Radioactivity in aqueous samples was measured in the scintillation fluid described by Patterson and Greene (1965). Amino acids were analyzed on a Durrum amino acid analyzer Model D-500 after hydrolysis of the peptides in 6 N HCl for 12 h at 100 °C. For the hydrolysis of UDP-MurNAc-peptides containing valine in R_4 and R_5, the hydrolysis procedure was for 18 h at 110 °C.

Results

Synthesis of Penicillin-Sensitive Peptidoglycan. The requirements for the synthesis of penicillin-sensitive peptidoglycan by membrane-walls from *G. homari* are summarized in Table I. The incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc requires UDP-MurNAc-pentapeptide and ATP, and is greatly enhanced by the addition of NH_4^+ and Mg^{2+}. As illustrated in Figure 1, at least 95% of the incorporation is sensitive to the action of benzylpenicillin. The amount of antibiotic required to inhibit the incorporation by 50% is 0.3 μg/ml. The product is rendered 99% soluble by the action of lysozyme. On the basis of the penicillin and lysozyme sensitivities and on the comparison to the systems described by Mirelman et al. (1972, 1974) and Ward (1974), it was assumed that the newly synthesized peptidoglycan is covalently cross-linked to the preexisting peptidoglycan of the wall. This system would appear to require the concerted action of transglycosylase and transpeptidase. Our assay measures the incorporation of [^{14}C]GlcNAc into sodium dodecyl sulfate resistant material (wall). Thus, the assay does not measure the synthesis of nascent peptidoglycan associated with the membrane fragments of the membrane-wall system.

TABLE I: Requirements for the Incorporation of [^{14}C]GlcNAc into Walls of *G. homari*.[a]

Additions	Incorporation (pmol/15 min)
Complete	664
−UDP-MurNAc-pentapeptide	15
−NH_4^+	85
−Mg^{2+}	80
−ATP	17
+Benzylpenicillin	49
Complete, lysozyme	31
Boiled membrane-wall fragments	2.4

[a] The reaction mixture contained: 0.05 M Tris-HCl, pH 7.8; 0.05 M magnesium acetate; 0.01 M NH_4Cl; 4 mM ATP (neutralized with NaOH); 4×10^{-4} M UDP-[^{14}C]GlcNAc (5.5 cpm/pmol); 1×10^{-4} M UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla; benzylpenicillin where indicated at a concentration of 20 μg/ml; and 160 μg of protein (membrane-walls) in a total volume of 135 μl. After incubation for 15 min at 25 °C, the reaction was terminated by heating the mixture for 2 min at 100 °C. One milliliter of water was added to each tube, and the samples were treated with sodium dodecyl sulfate as described in the assay for penicillin-sensitive peptidoglycan synthesis (Experimental Procedure). To test for lysozyme sensitivity, 200 μg of lysozyme was added to the inactivated reaction mixture and this mixture was incubated for 60 min at 37 °C. The amount of [^{14}C]GlcNAc incorporated was determined as described for the complete system.

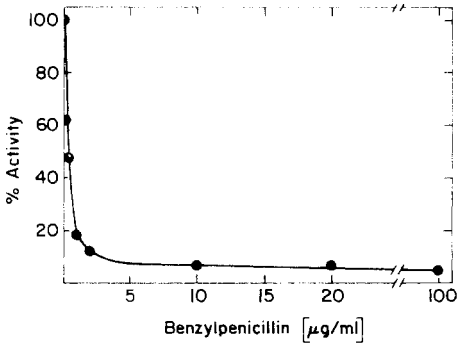

FIGURE 1: Effect of benzylpenicillin on the incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc into walls of *G. homari*. The assay for penicillin-sensitive peptidoglycan synthesis was used with increasing concentrations of benzylpenicillin.

[^{14}C]GlcNAc Incorporation into Nascent and Wall Peptidoglycan Using Analogues of UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (R_4, R_5). The time courses of [^{14}C]GlcNAc incorporation into nascent peptidoglycan with each of the six analogues and the reference nucleotide are illustrated in Figure 2A. With the reference nucleotide, 1780 pmol of [^{14}C]GlcNAc was incorporated in 30 min into the lysozyme-sensitive product

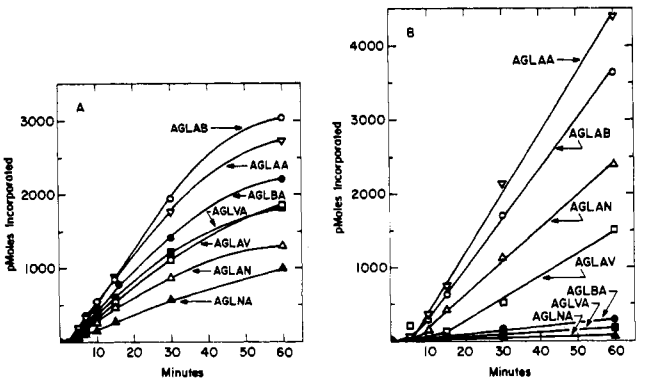

FIGURE 2: Time courses of incorporation of [^{14}C]GlcNAc with analogues of UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (R_4, R_5) into nascent peptidoglycan (A) and total incorporation into wall peptidoglycan (B). In A the assay for nascent peptidoglycan was used with 99 μg of membrane protein and 1×10^{-4} M of the indicated UDP-MurNAc-peptide. In B the reaction mixture described in Table I was used with 160 μg of protein (membrane-wall) and 1×10^{-4} M of the indicated UDP-MurNAc-peptide. At the indicated time, the reaction was terminated and the amount of incorporation was determined as described in Table I. The abbreviations are: AGLAA, UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla; AGLAB, UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu; AGLBA, UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla; AGLVA, UDP-MurNAc-Ala-DGlu-Lys-DVal-DAla; AGLAV, UDP-MurNAc-Ala-DGlu-Lys-DAla-DVal; AGLAN, UDP-MurNAc-Ala-DGlu-Lys-DAla-DNva; AGLNA, UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla.

When the reference nucleotide was replaced by either UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu or -MurNAc-Ala-DGlu-Lys-DAbu-DAla, 1880 and 1410 pmol of [^{14}C]GlcNAc were incorporated into peptidoglycan, respectively. Although replacement of D-alanine in either residue 4 or 5 by D-norvaline or D-valine results in a reduction of activity, the system has a low degree of selectivity between a substituent in either residue 4 or 5.

In Figure 2B the time courses for [^{14}C]GlcNAc incorporation into walls catalyzed by the membrane-wall system with UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla and six analogues are presented. With the reference substrate, 2130 pmol of [^{14}C]GlcNAc was incorporated into sodium dodecyl sulfate resistant material (walls) in 30 min. When the reference substrate was replaced by UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu, -MurNAc-Ala-DGlu-Lys-DAla-DNva, or -MurNAc-Ala-DGlu-Lys-DAla-DVal, 1710, 1130, and 513 pmol of [^{14}C]GlcNAc were incorporated, respectively. In contrast, when D-alanine was replaced in residue 4, a large decrease in activity was observed. For example, replacement by D-α-NH_2-n-butyric, D-norvaline, or D-valine reduced the amount of incorporation to 153, 52, and 111 pmol, respectively. Thus, replacements of D-alanine in residue 4 have a large inhibitory effect on the total incorporation into wall peptidoglycan.

Penicillin Sensitivity of Analogue Incorporation. Inhibition of peptidoglycan synthesis in the membrane-wall system by penicillin varied with the analogue of UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla tested. As illustrated in Table II, incorporation of [^{14}C]GlcNAc into wall peptidoglycan was inhibited 94% by penicillin in the presence of the reference nucleotide (see also Figure 1). Similar sensitivities to this antibiotic were observed with UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu, -MurNAc-Ala-DGlu-Lys-DAla-DNva, and -MurNAc-Ala-DGlu-Lys-DAla-DVal. Replacement of D-alanine in residue 4 by either D-α-amino-n-butyric acid, D-norvaline, or D-valine reduced the percentage inhibition to 63, 22, and 52%, respectively. Thus, replacements in residue 4 yield a higher fraction of penicillin-insensitive incorporation. Whereas 8% of the incorporation with UDP-MurNAc-Ala-DGlu-Lys-DAla-DNva is penicillin-insensitive, 78% of the incorporation with -MurNAc-Ala-DGlu-Lys-DNva-DAla is penicillin-insensitive. Correction of the total [^{14}C]GlcNAc incorporation for the penicillin-insensitive component gives the amount of penicillin-sensitive incorporation. The corrected results reveal that UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla gives 1% of the activity observed with -MurNAc-Ala-DGlu-Lys-DAla-DNva, and that -MurNAc-Ala-DGlu-Lys-DAbu-DAla gives 6% of the activity observed with -MurNAc-Ala-DGlu-Lys-DAla-DAbu.

Comparison of V_{max} and V_{max}/K_m for Nascent Peptidoglycan Synthesis and for Penicillin-Sensitive Peptidoglycan Synthesis. In the synthesis of penicillin-insensitive (nascent) peptidoglycan, addition of steric substituents to either residue 4 or 5 decreases the value for V_{max}/K_m (Table III). For example, V_{max}/K_m for UDP-MurNAc-pentapeptide (R_4-DAbu) and UDP-MurNAc-pentapeptide (R_5-DAbu) is 0.75 and 0.80, respectively, of that for the reference substrate. V_{max}/K_m for UDP-MurNAc-pentapeptide (R_4-DNva) and UDP-MurNAc-pentapeptide (R_5-DNva) are 0.24 and 0.33 of that for the reference substrate. These results, together with those obtained with UDP-MurNAc-pentapeptide (R_4-DVal) (0.37) and R_5-DVal (0.70), indicate that the system has only limited ability to discriminate between steric substituents in residues 4 and 5.

In Figure 3B, the Lineweaver-Burk plots for the reference nucleotide, UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu and

TABLE II: Penicillin Sensitivity of [^{14}C]GlcNAc Incorporation into Walls in the Presence of Analogues of UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla.

Substrate	Incorporation			Inhibition by Penicillin (%)
	Total[a] (pmol)	Penicillin Insensitive[b] (pmol)	Penicillin Sensitive (pmol)	
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla	1630	98	1530	94
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu	1250	89	1160	93
UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla	115	42	73	63
UDP-MurNAc-Ala-DGlu-Lys-DAla-DNva	1010	80	930	92
UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla	46	36	10	22
UDP-MurNAc-Ala-DGlu-Lys-DAla-DVal	257	49	208	81
UDP-MurNAc-Ala-DGlu-Lys-DVal-DAla	101	48	53	52

[a] The reaction mixture described in Table I was incubated for 30 min, and the amount of [^{14}C]GlcNAc incorporation was measured. [b] For penicillin-insensitive incorporation, 10 μg/ml of benzylpenicillin was added to the reaction mixture.

TABLE III: Comparison of Nascent Peptidoglycan Synthesis and Penicillin-Sensitive Peptidoglycan Synthesis.[a]

Substrate	Nascent Peptidoglycan Synthesis			Penicillin-Sensitive Peptidoglycan Synthesis		
	V_{max}[b]	K_m (μM)	(V_{max}/K_m) (l. min^{-1} × 10^6)	V_{max}[c]	K_m (μM)	(V_{max}/K_m) (l. min^{-1} × 10^6)
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla	133	65	2.0	147	69	2.1
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu	139	87	1.6	77	38	2.0
UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla	95	65	1.5	4.4	11	0.40
UDP-MurNAc-Ala-DGlu-Lys-DAla-DNva	95	143	0.66			
UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla	48	100	0.48			
UDP-MurNAc-Ala-DGlu-Lys-DAla-DVal	91	65	1.4			
UDP-MurNAc-Ala-DGlu-Lys-DVal-DAla	52	71	0.73			

[a] V_{max} and V_{max}/K_m are used as parameters to compare the effectiveness of an analogue with that of the reference substrate. The ratio V_{max}/K_m, the reciprocal slope of the Lineweaver-Burk plot, represents the effectiveness of a substrate at low substrate concentration. V_{max} reflects the effectiveness of a substrate at high substrate concentration (Hammes and Neuhaus, 1974b). The data for UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla, UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu, and UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla are from Figure 3A and B. [b] V_{max} = pmol min^{-1} per 99 μg of protein. [c] V_{max} = pmol min^{-1} per 160 μg of protein.

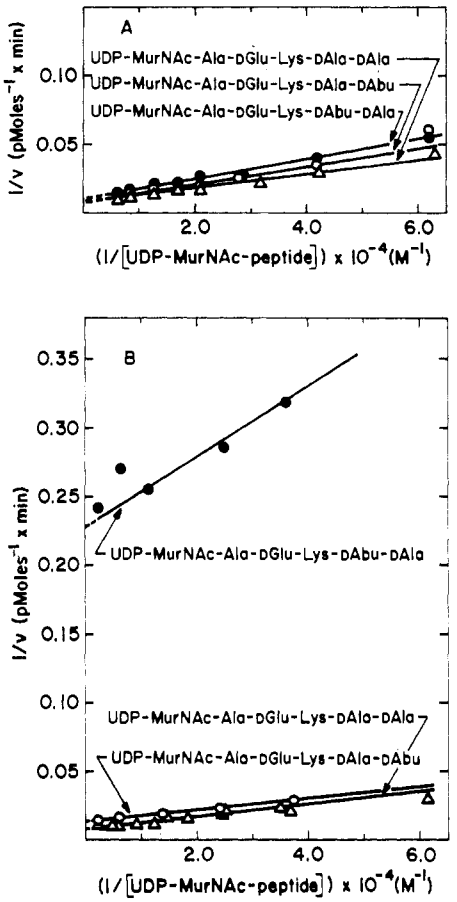

FIGURE 3: Lineweaver-Burk plots for the incorporation of [^{14}C]GlcNAc into nascent peptidoglycan (A) and penicillin-sensitive peptidoglycan (B). In A the assay for nascent peptidoglycan synthesis was used with 99 μg of membrane protein, and in B the assay for penicillin-sensitive peptidoglycan synthesis was used with 160 μg of protein (membrane-wall). In A and B increasing concentrations of UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (△), UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu (○), and UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla (●) were compared.

-MurNAc-Ala-DGlu-Lys-DAbu-DAla are presented for penicillin-sensitive wall peptidoglycan synthesis. Each determination of velocity was corrected for the penicillin-insensitive component of wall peptidoglycan synthesis. V_{max}/K_m and V_{max} for UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla are 0.19 and 0.03 of that observed for the reference nucleotide, whereas V_{max}/K_m and V_{max} for UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu are 0.95 and 0.52 of that observed for the reference nucleotide. The comparison of the Lineweaver-Burk plots in Figure 3A for nascent peptidoglycan synthesis with those in Figure 3B clearly illustrates the high degree of discrimination for the replacement of D-alanine by D-α-NH_2-n-butyric acid in position 4 in the membrane-wall system.

Specificity Profile of Phospho-MurNAc-pentapeptide Translocase with Analogues of UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (R_4, R_5). The initial membrane reaction in the biosynthesis of peptidoglycan is catalyzed by phospho-MurNAc-pentapeptide translocase according to the reaction:

UDP-MurNAc-pentapeptide

$$+ \text{undecaprenyl phosphate} \overset{Mg^{2+},\,K^{+}}{\rightleftharpoons}$$

undecaprenyl diphosphate-MurNAc-pentapeptide + UMP (2)

A comparison of the specificity profile of this enzyme with those of the two peptidoglycan synthesizing systems would allow an evaluation of the contribution of this enzyme to the specificity determinants of the glycan synthesizing systems. In Table IV the values of R_{max}, K_m, and R_{max}/K_m are summarized for each of the analogues and the reference nucleotide. In every case R_{max}/K_m for the analogue of UDP-MurNAc-pentapeptide is larger than that for the reference substrate. For example, R_{max}/K_m for UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla and UDP-MurNAc-Ala-DGlu-Lys-DAla-DNva is 3.3 and 2.3-fold higher, respectively, than that observed for UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla. At high substrate concentrations, R_{max} varies from 0.44 (UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla) to 1.22 (UDP-MurNAc-Ala-DGlu-Lys-DAla-DVal) of that for the reference substrate. From these results it would appear that translocase does not make a significant contribution in discriminating against the analogues examined in this paper.

Discussion

Six analogues of UDP-MurNAc pentapeptide with residues 4 and 5 replaced singly by either D-α-amino-n-butyric acid, D-norvaline, or D-valine were compared with the reference substrate, UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla, in the synthesis of peptidoglycan. The synthesis of this polymer was

TABLE IV: Specificity Profile of Phospho-MurNAc-pentapeptide Translocase.

Substrate	R_{max}[a] (M min^{-1} × 10^6)	K_m (μM)	(R_{max}/K_m) (min^{-1} × 10^2)
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla	0.95	62	1.5
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu	1.14	50	2.3
UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla	0.65	15	4.3
UDP-MurNAc-Ala-DGlu-Lys-DAla-DNva	0.79	23	3.4
UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla	0.44	9	4.9
UDP-MurNAc-Ala-DGlu-Lys-DAla-DVal	1.22	68	1.8
UDP-MurNAc-Ala-DGlu-Lys-DVal-DAla	1.17	53	2.2

[a] R_{max} = mol exchanged $l.^{-1}$ min^{-1} per 15 μg of protein.

examined in two membrane preparations, membranes and membrane-walls, in an attempt to define features that influence the synthesis of nascent peptidoglycan and cross-linked peptidoglycan. These analogues were also examined in the exchange reaction catalyzed by phospho-MurNAc-pentapeptide translocase in order to define its specificity determinants more clearly for comparison with those in peptidoglycan synthesis.

The membrane-wall system shows a high degree of discrimination for the steric substituents, $-CH_3$ and $-CH_2CH_3$, in residue 4. For example, V_{max}/K_m for UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla is 0.19 of that observed for the reference substrate, whereas V_{max}/K_m for UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu is 0.95. At high concentrations of UDP-MurNAc-peptide, V_{max} for UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAla is 0.06 of that observed for UDP-MurNAc-Ala-DGlu-Lys-DAla-DAbu. In contrast, for the synthesis of nascent peptidoglycan the ratio of V_{max} for these nucleotides is 0.68. This trend was also illustrated in the time course experiments for the other analogues. For example, UDP-MurNAc-Ala-DGlu-Lys-DNva-DAla is 1% as active as UDP-MurNAc-Ala-DGlu-Lys-DAla-DNva. In the synthesis of nascent peptidoglycan, however, D-norvaline in position 4 gave 73% of the activity observed for this residue in position 5. It would appear that the specificity profile of the membrane-wall system reflects the specificity determinants exerted by the penicillin-sensitive enzyme(s), presumably the transpeptidase. Thus, on the basis of these results, the transpeptidase has a higher degree of specificity in the donor phase for D-alanine in residue 4 and a lower degree of specificity for D-alanine in residue 5.

Specificity studies on the penicillin-sensitive acyl-DAla-DAla carboxypeptidase, uncoupled transpeptidase from *Streptomyces* spp, show similar specificity determinants against residues 4 and 5 of the donor substrate, diacetyl-Lys^3-$DAla^4$-$DAla^5$ (reference peptide)[2] (Leyh-Bouille et al., 1972). For example, when glycine or D-leucine replaces D-alanine in residue 4, V_{max}/K_m for carboxypeptidase R39 is zero when compared with the reference peptide. When glycine or D-leucine replaces D-alanine in position 5, V_{max}/K_m is 9.8% and 78%, respectively, of that observed for the reference peptide. It was concluded that the carboxypeptidase exhibited considerable specificity for a C-terminal LR^3-$DAla^4$-D-amino $acid^5$ sequence (Ghuysen et al., 1974; Blumberg and Strominger, 1974). In the proposed enzyme intermediate for carboxypeptidase (transpeptidase) it is residue 4 that is covalently linked to the enzyme nucleophile. Thus, there is a correlation between the donor profile of this enzyme and that established for the synthesis of penicillin-sensitive peptidoglycan incorporation into walls.

These results together with other results from this laboratory (Neuhaus, 1962a,b; Neuhaus and Struve, 1965; Hammes and Neuhaus, 1974a,b) have defined some of the specificity determinants in the biosynthesis of peptidoglycan that ensure the successful assembly of this polymer. For example, D-alanine:D-alanine ligase (ADP), the enzyme responsible for the synthesis of the terminal dipeptide in UDP-MurNAc-pentapeptide, has a high specificity for D-amino acids in the N-terminal site and a low specificity for D-amino acids in the C-terminal site (Neuhaus, 1962a,b). In contrast to D-alanine:D-alanine ligase (ADP), UDP-MurNAc-Ala-DGlu-Lys:DAla-DAla ligase (ADP) has a low specificity for amino acids in the N-terminal residue and a high specificity for amino acids in the C-terminal residue (Neuhaus and Struve, 1965). Thus, the two enzymes cooperate to ensure that DAla-DAla is the major dipeptide that is added to the nucleotide precursor, UDP-MurNAc-Ala-DGlu-Lys. There are certain growth conditions in which an analogue can replace the D-alanine residues. For example, growth of *S. aureus* Copenhagen in the presence of high concentrations of glycine results in the partial replacement of both stereoisomers of alanine by glycine (Hammes et al., 1973). Specificity studies on phospho-MurNAc-pentapeptide translocase indicate that this enzyme has a key role in selecting analogues of UDP-MurNAc-pentapeptide with alanine residues replaced by glycine (Hammes and Neuhaus, 1974a). It will be of interest to know what effect replacement of alanine by glycine has in residues 4 and 5 of the pentapeptide on the transpeptidase-dependent peptidoglycan synthesizing system. In a second example with either *S. faecalis* R or *Lactobacillus delbrueckii* grown on a pyridoxal deficient medium, Snell et al. (1955) observed that D-α-amino-*n*-butyric acid was the only amino acid that could satisfy the requirement for D-alanine. D-α-Amino-*n*-butyric acid is about 3.5% as effective as D-alanine in promoting growth of *L. delbrueckii*. In enzyme specificity studies with D-alanine:D-alanine ligase (ADP), it was shown that DAbu-DAbu was synthesized at 0.26 of the rate observed for DAla-DAla (Neuhaus, 1962a,b). DAbu-DAbu is a poor substrate in the in vitro synthesis of UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAbu catalyzed by UDP-MurNAc-Ala-DGlu-Lys:DAla-DAla ligase (ADP). From the specificity profile in this paper, it is presumed that UDP-MurNAc-Ala-DGlu-Lys-DAbu-DAbu can be utilized poorly as a substrate for the synthesis of cross-linked peptidoglycan. These results correlate with the poor growth promoting activity observed with D-α-amino-*n*-butyric acid in vitamin B_6 deficient *L. delbrueckii*.

The specificity profile described in this paper for the mem-

[2] Ghuysen et al. (1974) number this sequence as L-R^3-$DAla^2$-D-amino $acid^1$.

brane-wall system would appear to reflect that determined by the transpeptidase. This enzyme, therefore, plays an important role in discriminating analogues for incorporation into peptidoglycan. These results and those presented by Ghuysen and co-workers, as well as the specificity profiles of the other enzymes in this biosynthetic pathway define a rational basis for designing potential analogues of D-alanine that inhibit the assembly of cross-linked, functional peptidoglycan.

Acknowledgment

We thank Dr. Walter Hammes for suggestions regarding the preparation of the membrane-wall system from *G. homari*.

References

Anderson, J. S., Matsuhashi, M., Haskin, M. A., and Strominger, J. L. (1967), *J. Biol. Chem. 242,* 3180.

Anderson, J. S., Meadow, P. M., Haskin, M. A., and Strominger, J. L. (1966), *Arch. Biochem. Biophys. 116,* 487.

Araki, Y., Shimada, A., and Ito, E. (1966), *Biochem. Biophys. Res. Commun. 23,* 518.

Blumberg, P. M., and Strominger, J. L. (1974), *Bacteriol. Rev. 38,* 291.

Ghuysen, J.-M., Leyh-Bouille, M., Frère, J.-M., Dusart, J., Marquet, A., Perkins, H. R., and Nieto, M. (1974), *Ann. N.Y. Acad. Sci. 235,* 236.

Ghuysen, J.-M., and Shockman, G. D. (1973), in Bacterial Membranes and Walls, Leive, L., Ed., New York, N.Y., Marcel Dekker, p 37.

Hammes, W., Schleifer, K. H., and Kandler, O. (1973), *J. Bacteriol. 116,* 1029.

Hammes, W. P., and Neuhaus, F. C. (1974a), *J. Biol. Chem. 249,* 3140.

Hammes, W. P., and Neuhaus, F. C. (1974b), *J. Bacteriol. 120,* 210.

Izaki, K., Matsuhashi, M., and Strominger, J. L. (1968), *J. Biol. Chem. 243,* 3180.

Leyh-Bouille, M., Nakel, M., Frère, J.-M., Johnson, K., Ghuysen, J.-M., Nieto, M., and Perkins, H. R. (1972), *Biochemistry 11,* 1290.

Linnett, P. E., and Strominger, J. L. (1974), *J. Biol. Chem. 249,* 2489.

Linnett, P. E., Roberts, R. J., and Strominger, J. L. (1974), *J. Biol. Chem. 249,* 2497.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mirelman, D., Bracha, R., and Sharon, N. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3355.

Mirelman, D., Bracha, R., and Sharon, N. (1974), *Ann. N.Y. Acad. Sci. 235,* 326.

Mirelman, D., and Sharon, N. (1972), *Biochem. Biophys. Res. Commun. 46,* 1909.

Nakel, M., Ghuysen, J.-M., and Kandler, O. (1971), *Biochemistry 10,* 2170.

Neuhaus, F. C. (1962a), *J. Biol. Chem. 237,* 778.

Neuhaus, F. C. (1962b), *J. Biol. Chem. 237,* 3128.

Neuhaus, F. C. (1971), *Acc. Chem. Res. 4,* 297.

Neuhaus, F. C., and Struve, W. G. (1965), *Biochemistry 4,* 120.

Patterson, M. S., and Greene, R. C. (1965), *Anal. Chem. 37,* 854.

Pollock, J. J., Nguyen-Distèche, M., Ghuysen, J.-M., Linder, R., and Salton, M. R. J. (1974), *Ann. N.Y. Acad. Sci. 235,* 225.

Reynolds, P. E., and Barnett, H. J. (1974), *Ann. N.Y. Acad. Sci. 235,* 269.

Schleifer, K. H., and Kandler, O. (1972), *Bacteriol. Rev. 36,* 407.

Snell, E. E., Radin, N. S., and Ikawa, M. (1955), *J. Biol. Chem. 217,* 803.

Stickgold, R. A., and Neuhaus, F. C. (1967), *J. Biol. Chem. 242,* 1331.

Struve, W. G., Sinha, R. K., and Neuhaus, F. C. (1966), *Biochemistry 5,* 82.

Ward, J. B. (1974), *Biochem. J. 141,* 227.

Ward, J. B., and Perkins, H. R. (1973), *Biochem. J. 135,* 721.

Ward, J. B., and Perkins, H. R. (1974), *Biochem. J. 139,* 781.

Wickus, G. G., and Strominger, J. L. (1972), *J. Biol. Chem. 247,* 5297.