ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1974, p. 722-728
Vol. 6, No. 6
Printed in U.S.A.
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On the Mechanism of Action of Vancomycin: Inhibition of Peptidoglycan Synthesis in *Gaffkya homari*

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Received for publication 13 August 1974

Vancomycin inhibits the synthesis of peptidoglycan in membrane preparations from *Gaffkya homari* with uridine diphosphate-*N*-acetylmuramyl (UDP-MurNAc)-pentapeptide as substrate, but not with either UDP-MurNAc-tetrapeptide or UDP-MurNAc-tripeptide. These results are correlated with the specificity studies described by Perkins and Nieto for complex formation between the antibiotic and the peptide subunit. It is concluded that the formation of a complex between vancomycin and a postulated cell wall acceptor or between vancomycin and the enzymes involved in peptidoglycan synthesis does not contribute to the inhibitory action of this antibiotic. The mechanism of vancomycin action on peptidoglycan synthesis is clearly different from that of moenomycin and bacitracin. In the presence of these antibiotics, peptidoglycan synthesis is inhibited with both UDP-MurNAc-pentapeptide and -tetrapeptide as substrates. In addition, these results provide additional insight into the mechanism of phospho-MurNAc-pentapeptide translocase. For example, enhancement of the transfer of phospho-MurNAc-peptide from UDP-MurNAc-peptide to undecaprenyl-phosphate at low concentrations of vancomycin is observed with UDP-MurNAc-pentapeptide and not with -tetrapeptide. Complexation of vancomycin with undecaprenyl-diphosphate-MurNAc-pentapeptide, resulting in an ineffective intermediate, would increase the rate of transfer by preventing the reassociation of undecaprenyl-diphosphate-MurNAc-pentapeptide with the enzyme.

Vancomycin is an inhibitor of the biosynthesis of the major structural cell wall polymer, peptidoglycan (10). The mechanism of action of this antibiotic appears to be related to its ability to complex with the D-alanyl-D-alanine moieties that are present in various phases of polymer synthesis (18-20). It has not been established unequivocally in which phase the primary site of action is located. As possible targets of complex formation with vancomycin, the nucleotide-activated precursor, the lipid intermediates, and the postulated cell wall acceptor are considered. Alternatively, inhibition of the peptidoglycan synthetase may be a site of antibiotic action. To define the primary site of action more clearly, the studies presented in this communication were performed.

Membrane fragments of *Gaffkya homari* catalyze a high rate of peptidoglycan synthesis with UDP-MurNAc-pentapeptide and -tetrapeptide (7). These nucleotides differ in their ability to form complexes with vancomycin; only UDP-MurNAc-pentapeptide forms a complex with the antibiotic (16; H. R. Perkins, Biochem. J. **106**:35P). By comparing the effect of vancomycin on peptidoglycan synthesis and on phospho-MurNAc-pentapeptide translocase with these substrates, we can probe some of the possible sites of vancomycin action.

From the data presented, it is concluded that the formation of a complex between the postulated cell wall acceptor and vancomycin or the inhibition of the peptidoglycan synthetase does not contribute to the inhibitory effect of vancomycin. These results also provide further insights into the reaction mechanism of phospho-MurNAc-pentapeptide translocase.

MATERIALS AND METHODS

Materials. Vancomycin and ristocetin (Spontin) were gifts from Eli Lilly and Company and Abbott Laboratories, respectively. Moenomycin (Flavomycin) was given by Farbwerke Hoechst AG, and bacitracin was purchased from Nutritional Biochemicals Corp. The sources of other chemicals and the preparation of substrates and membrane fragments were described previously (6, 7).

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Peptidoglycan synthesis assay. Peptidoglycan synthesis was assayed by determining the incorporation of MurNAc-peptide from UDP-MurNAc-[^{14}C]peptide. The amount of peptidoglycan was determined from the radioactivity that was immobile in the isobutyric acid solvent system (isobutyric acid-concentrated ammonium hydroxide-water, 66:2:33). For the incorporation of MurNAc-[^{14}C]peptides, the reaction mixture contained: 8.3×10^{-4} M UDP-GlcNAc; 0.05 M $MgCl_2$; 8.3×10^{-3} M NH_4Cl; 0.21 M KCl; 6.7×10^{-3} M adenosine 5'-triphosphate; 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0; membrane fragments (94 μg of protein); 250 μg of penicillin per ml; and the indicated concentration of labeled UDP-MurNAc-peptide. After incubation for the indicated time at 25 C, samples were added to equivalent volumes of the isobutyric acid solvent to terminate synthesis. After mixing, a sample was applied to Whatman 3MM and chromatographed in the above solvent system. The origins containing peptidoglycan were assayed for radioactivity. Controls and requirements for peptidoglycan synthesis in this system were described previously (7).

Transfer assay for phospho-MurNAc-pentapeptide translocase. The transfer of phospho-MurNAc-[^{14}C]peptide from UDP-MurNAc-[^{14}C]peptide to undecaprenyl-phosphate was assayed by determining the amount of acid-precipitable radioactivity. The procedure is similar to that described by Hammes and Neuhaus (6). The reaction mixture contained: 0.21 M KCl; 0.05 M $MgCl_2$; 0.16 U of bacterial alkaline phosphatase; 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0; membrane fragments pretreated with UMP (159 μg of protein) and labeled UDP-MurNAc-peptide, in a total volume of 60 μliters. After incubation for 1 min at 25 C, the reaction was terminated by addition of 0.5 ml of 0.3 M $HClO_4$. The precipitate was isolated on a membrane filter (Millipore Corp.), and the amount of transfer was determined as described previously (6).

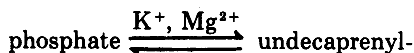
Exchange assay for phospho-MurNAc-pentapeptide translocase. For the determination of the exchange rate, the exchange of [^{3}H]UMP with the unlabeled UMP moiety of UDP-MurNAc-peptide was measured in the following reaction mixture: 0.21 M KCl; 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.8; 0.042 M $MgCl_2$; 3.33×10^{-5} M [^{3}H]UMP (20 counts/min per pmol); membrane fragments (33 μg of protein); and UDP-MurNAc-peptide. The incubation was carried out at 25 C for 10 min. After the reaction was terminated by 2 min of boiling, the amount of exchange was determined by the procedure of Hammes and Neuhaus (6).

Abbreviations. Unless stated otherwise, all abbreviations of amino acid residues denote the L configuration. The omission of the hyphen, i.e., -DAla for -D-Ala-, conforms with the suggestion cited in Biochemistry (5:2485 [1966]). Although not stated, all D-glutamic residues are linked through the γ-carboxyl group to the diamino acid. The abbreviations used are: MurNAc, *N*-acetylmuramyl; GlcNAc, *N*-acetyl-glucosamine; Hsr, homoserine; UDP, uridine diphosphate; UMP, uridine monophosphate.

RESULTS

Effect of vancomycin on phospho-MurNAc-pentapeptide translocase. Phospho-MurNAc-pentapeptide translocase, the initial membrane enzyme in the biosynthesis of peptidoglycan, catalyzes the transfer of either phospho-MurNAc-pentapeptide or phospho-MurNAc-tetrapeptide to undecaprenyl-phosphate according to reaction 1 (6, 7, 14).

UDP-MurNAc-peptide + undecaprenyl-



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

diphosphate-MurNAc-peptide + UMP (1)

At low concentrations of vancomycin, the transfer of phospho-MurNAc-pentapeptide is stimulated and at high concentrations it is inhibited (11, 12, 24, 27). Membrane fragments from *G. homari* also showed the characteristic stimulation and inhibition (Fig. 1A). In contrast, with UDP-MurNAc-tetrapeptide as a substrate, no enhancement of transfer is observed in the presence of vancomycin. At high concentrations, vancomycin inhibits the transfer of phospho-MurNAc-pentapeptide, whereas the transfer of phospho-MurNAc-tetrapeptide is not significantly inhibited. Moenomycin, another inhibitor of peptidoglycan synthesis (8, 11), does not show the characteristic enhancement observed with vancomycin in the presence of UDP-MurNAc-pentapeptide. In the exchange reaction (Fig. 1B), inhibition by vancomycin is observed with UDP-MurNAc-pentapeptide and not with UDP-MurNAc-tetrapeptide.

Effect of vancomycin and ristocetin on peptidoglycan synthesis. The synthesis of peptidoglycan is more sensitive to the action of vancomycin and ristocetin than the transfer of phospho-MurNAc-pentapeptide to undecaprenyl-phosphate (1, 2, 12, 24, 26, 27). With membrane preparations from *G. homari*, the synthesis of peptidoglycan is inhibited 50% by vancomycin and ristocetin at 12 and 18 μg/ml, respectively (Fig. 2). In contrast, with UDP-MurNAc-tetrapeptide as a substrate, neither ristocetin nor vancomycin inhibited the synthesis at concentrations up to 1,000 μg/ml. In addition, no effect on the incorporation of MurNAc-tripeptide at high concentrations of vancomycin was detected. These data clearly show that these antibiotics manifest their inhibitory action only when UDP-MurNAc-peptide has a complete pentapeptide subunit.

Effect of moenomycin and bacitracin on the synthesis of peptidoglycan. In contrast to the results with vancomycin and ristocetin, mo-

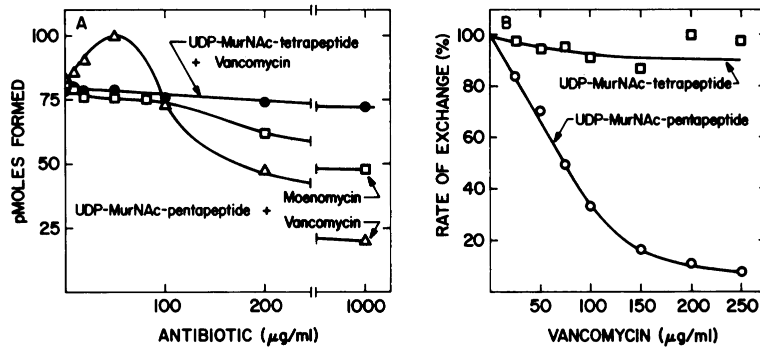


FIG. 1. *Effect of vancomycin and moenomycin on the transfer of phospho-MurNAc-peptide to undecaprenyl-phosphate from UDP-MurNAc-peptide (A) and effect of vancomycin on the exchange reaction (B). The transfer assay was used with 3.7 × 10^{-5} M UDP-MurNAc-pentapeptide (45.9 counts/min per pmol) and 4.0 × 10^{-5} M UDP-MurNAc-tetrapeptide (20.5 counts/min per pmol). The exchange assay was used with 2.7 × 10^{-5} M UDP-MurNAc-peptide.*

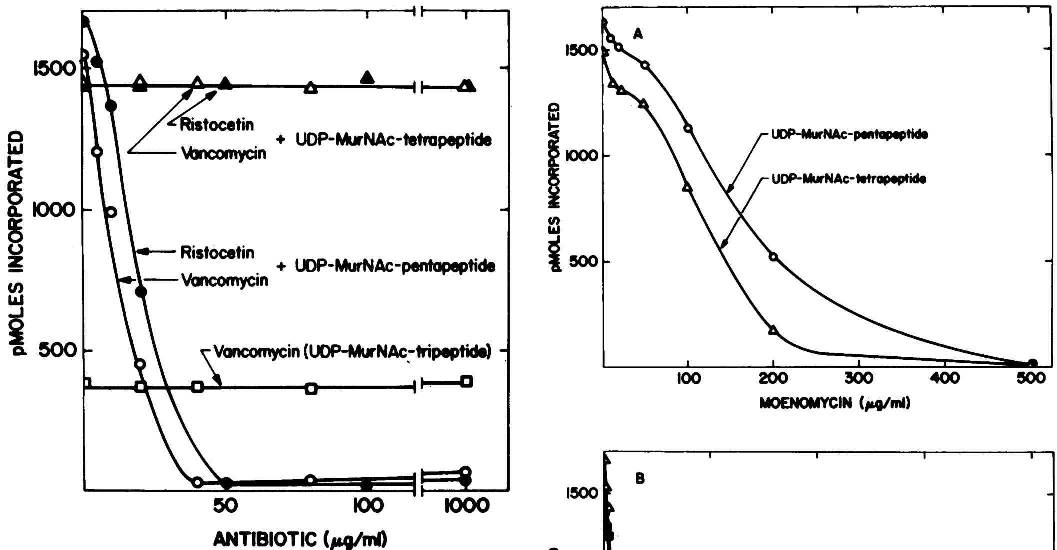


FIG. 2. *Effect of vancomycin and ristocetin on the incorporation of MurNAc-pentapeptide, MurNAc-tetrapeptide, and MurNAc-tripeptide from their UDP-activated precursors into peptidoglycan. The peptidoglycan assay (94 µg of membrane protein, 60-min incubation) was used with either 2.9 × 10^{-5} M UDP-MurNAc-pentapeptide (32.9 counts/min per pmol), 3.0 × 10^{-5} M UDP-MurNAc-tetrapeptide (15.3 counts/min per pmol), or 5.5 × 10^{-5} M UDP-MurNAc-tripeptide (27.7 counts/min per pmol).*

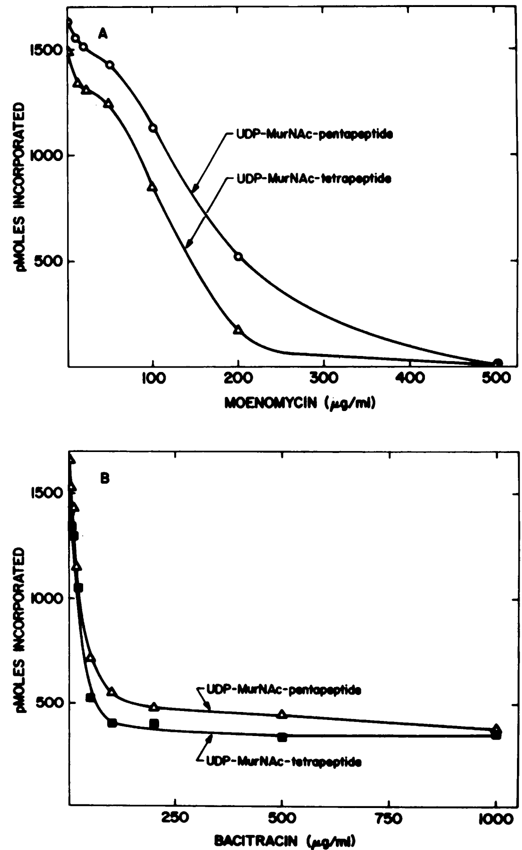


FIG. 3. *Effect of moenomycin (A) and bacitracin (B) on the incorporation of MurNAc-pentapeptide and -tetrapeptide into peptidoglycan. The peptidoglycan assay (94 µg of membrane protein, 60-min incubation) was used with 2.9 × 10^{-5} M UDP-MurNAc-pentapeptide (32.9 counts/min per pmol) and 3.0 × 10^{-5} M UDP-MurNAc-tetrapeptide (15.2 counts/min per pmol).*

enomycin and bacitracin are equally effective as inhibitors with both UDP-MurNAc-pentapeptide and -tetrapeptide (Fig. 3A and B). With moenomycin, 50% inhibition was observed at 105 and 120 µg/ml for UDP-MurNAc-tetrapeptide and -pentapeptide, respectively. Peptidoglycan synthesis is inhibited 50% by bacitracin at 25 µg/ml with both substrates. Thus, the

inhibitory action of these antibiotics is different from that of vancomycin and ristocetin.

In addition, the action of moenomycin appears also to differ from that of bacitracin. At high concentrations of antibiotic, peptidoglycan synthesis is inhibited completely with moenomycin. With bacitracin, however, in agreement with its mode of action (23), complete inhibition of in vitro peptidoglycan synthesis was not achieved. Thus, it appears that the mode of action of moenomycin differs from that of vancomycin and ristocetin as well as that of bacitracin.

Specificity of inhibition by vancomycin with analogues of UDP-MurNAc-pentapeptide. Studies on the association of vancomycin with various peptides have indicated features of the peptide that facilitate complex formation (15, 16, 18–20). Since certain analogues of UDP-MurNAc-pentapeptide are utilized for peptidoglycan synthesis (7), it was of interest to compare the sensitivity of the system to vancomycin with different substrates. In Fig. 4, UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla, -MurNAc-Ala-DGlu-Lys-DAla-Gly, -MurNAc-Ala-DGlu-Lys-Gly-DAla, and -MurNAc-Gly-DGlu-Lys-DAla-DAla are compared with increasing concentrations of vancomycin. With three of these nucleotides, the concentration for 50% inhibition is 8 to 9 μg/ml. With UDP-MurNAc-Gly-DGlu-Lys-DAla-DAla, the concentration of vancomycin for 50% inhibition is 4 μg/ml. Thus, the incorporation of MurNAc-pentapeptide with glycine in position 1 is more effectively inhibited by vancomycin than those with L-alanine in this position. Incorporation of MurNAc-peptides with either glycine or D-alanine in positions 4 or 5 are equally sensitive to the action of this antibiotic.

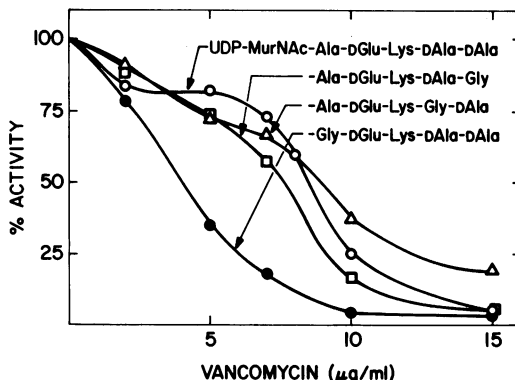


FIG. 4. *Effect of vancomycin on the incorporation of MurNAc-peptides from UDP-MurNAc-peptides into peptidoglycan. The peptidoglycan assay (94 μg of membrane protein) was used with 3.2 × 10^{-5} M UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (32.9 counts/min per pmol), 3.0 × 10^{-5} M UDP-MurNAc-Ala-DGlu-Lys-DAla-Gly (15.4 counts/min per pmol), 3.0 × 10^{-5} M UDP-MurNAc-Ala-DGlu-Lys-Gly-DAla (15.2 counts/min per pmol), 3.1 × 10^{-5} M UDP-MurNAc-Gly-DAla-DAla (19.9 counts/min per pmol).*

DISCUSSION

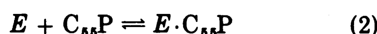
The observation that vancomycin and ristocetin do not inhibit the incorporation of MurNAc-tetrapeptide into peptidoglycan whereas the incorporation of MurNAc-pentapeptide is inhibited provides for a better understanding of the action of these antibiotics on the in vitro synthesis of this polymer. This observation can be correlated with the specificity of vancomycin interaction described by Perkins and Nieto (15, 16, 18–20). Pentapeptide subunits with D-Ala-D-Ala termini bind effectively to vancomycin, whereas peptide subunits that lack the second D-alanine residue do not. Thus, the absence of complex formation with MurNAc-tetrapeptide is consistent with the inability of vancomycin to inhibit incorporation of MurNAc-tetrapeptide from UDP-MurNAc-tetrapeptide into peptidoglycan.

The unaffected rate of peptidoglycan synthesis in the presence of increasing concentrations of vancomycin with UDP-MurNAc-tetrapeptide cannot be correlated with a specific interaction of vancomycin with one or more of the enzymes in the membrane system. In addition, these observations do not appear to be consistent with a requirement for a postulated cell wall acceptor in the membrane preparation. It has been proposed that this acceptor is incomplete pre-existing peptidoglycan (3, 25) associated with the membrane. Formation of a complex between vancomycin and peptide subunits of this acceptor might be expected to inactivate the system. Recently, Ward and Perkins (29) presented evidence for the extension of peptidoglycan chains that remain attached to the carrier undecaprenyl-phosphate. In their model, the presence of an acceptor would not be required for chain extension. Thus, our results are consistent with the findings of these investigators and those reported by Reynolds (22) for the membrane preparations from *Bacillus megaterium*.

The formation of a complex between the lipid intermediate(s) and vancomycin has been used to interpret the inhibition of peptidoglycan synthesis by this antibiotic (5, 9, 15, 17, 19, 20). It has been proposed that utilization of the lipid intermediate for peptidoglycan synthesis will be inhibited if it is complexed with antibiotic. This

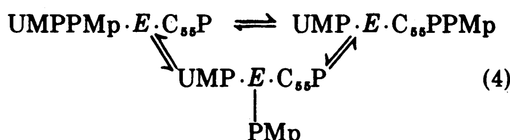
proposal is consistent with an accumulation of lipid intermediate(s) when polymer synthesis is inhibited by vancomycin (12, 22).

The effects of vancomycin on phospho-MurNAc-pentapeptide translocase can be related to the above interpretation. At low concentrations of antibiotic, the transfer of phospho-MurNAc-pentapeptide to undecaprenyl-phosphate is enhanced (9, 26, 27). This enhancement was interpreted as a detergent effect by vancomycin because of certain similarities to the action of surfactants on the enzyme (14). Since no enhancement of the transfer reaction was observed with UDP-MurNAc-tetrapeptide as a substrate, a detergent effect of vancomycin can now be excluded. The enhancement, however, can be correlated with the mechanism proposed by Pless and Neuhaus (21) for the translocase. In the sequential mechanism, both undecaprenyl-phosphate ($C_{55}P$) and UDP-MurNAc-pentapeptide (UMPPMp) bind to the enzyme in the forward direction prior to the release of the first product. It is assumed that either the association of undecaprenyl-phosphate (reaction 2) or the dissociation

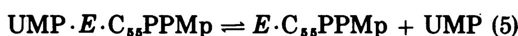


$$E + C_{55}P \rightleftharpoons E \cdot C_{55}P \quad (2)$$

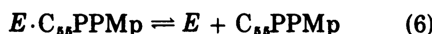
$$E \cdot C_{55}P + UMPPMp \rightleftharpoons UMPPMp \cdot E \cdot C_{55}P \quad (3)$$



$$UMPPMp \cdot E \cdot C_{55}P \rightleftharpoons UMP \cdot E \cdot C_{55}PPMp \quad (4)$$



$$UMP \cdot E \cdot C_{55}PPMp \rightleftharpoons E \cdot C_{55}PPMp + UMP \quad (5)$$



$$E \cdot C_{55}PPMp \rightleftharpoons E + C_{55}PPMp \quad (6)$$

of undecaprenyl-diphosphate-MurNAc-pentapeptide (reaction 6) is rate limiting (21). Thus, complexation of vancomyin with undecaprenyl-diphosphate-MurNAc-pentapeptide resulting in an ineffective intermediate, would increase the rate of transfer by preventing the reassociation of undecaprenyl-diphosphate-MurNAc-pentapeptide with the enzyme. Alternatively, association of vancomycin with the enzyme-bound lipid intermediate may enhance dissociation and, therefore, increase the rate of transfer. The inhibition of transfer at higher concentrations of antibiotic may be due to complex formation between UDP-MurNAc-pentapeptide and vancomycin.

The results with the exchange assay are also consistent with this interpretation. The exchange of UMP with the UMP moiety of UDP-MurNAc-pentapeptide is inhibited 50% by 75 μg of vancomycin per ml and, in contrast to the transfer assay, no stimulation is observed. As in the case of the transfer assay, no effect by vancomycin is observed with UDP-MurNAc-tetrapeptide. The exchange reaction, which requires catalytic amounts of either undecaprenyl-phosphate or undecaprenyl-diphosphate-MurNAc-pentapeptide, is inhibited when it is depleted of these compounds.

The effect of alanine replacement by glycine in positions 1, 4, and 5 of UDP-MurNAc-pentapeptide on the sensitivity of the peptidoglycan-synthesizing system to vancomycin was examined. These results can be compared with the binding profile described by Perkins and Nieto (16, 18). For example, the association constant for diacetyl-Lys-DAla-Gly and diacetyl-Lys-Gly-DAla is significantly smaller than that observed for diacetyl-Lys-DAla-DAla. Our results with the peptidoglycan-synthesizing system, however, do not indicate a significant difference in the degree of inhibition with UDP-MurNAc-pentapeptide with or without replacement of D-alanine by glycine in either position 4 or 5. On the other hand, the incorporation of MurNAc-Gly-DGlu-Lys-DAla-DAla was twofold more sensitive to the inhibitory action of vancomycin when compared with UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla. Thus, the amino acid residue in position 1, as well as the D-Ala-D-Ala terminus, of the peptide subunit would appear to play a role in the interaction of antibiotic and the peptide subunit. This observation can be correlated with the fact that complex formation between vancomycin and UDP-MurNAc-pentapeptide was found to occur more easily from certain corynebacteria than from staphylococci (17). The peptide subunits of the nucleotides from these corynebacteria have the sequence Gly-DGlu-Hsr-DAla-DAla.

For comparison, two additional antibiotics, moenomycin and bacitracin, were tested with UDP-MurNAc-pentapeptide and UDP-MurNAc-tetrapeptide in the peptidoglycan-synthesizing system. With both antibiotics no difference was detected between the incorporation of MurNAc-pentapeptide and MurNAc-tetrapeptide. These results distinguish the action of these antibiotics from that of vancomycin and ristocetin. Bacitracin is a specific inhibitor of the enzymatic dephosphorylation of undecaprenyl-pyrophosphate (23). Thus, peptidoglycan synthesis in the presence of this antibiotic proceeds to an extent equivalent to the concentration of undecaprenyl-phosphate (22, 23). The data in this paper are consistent with this

proposal, and it is calculated that approximately three cycles in which the lipid carrier has participated were accomplished in the absence of bacitracin. With membrane preparations from *Micrococcus lysodeikticus*, the number of cycles was approximately four to six (23). The number of cycles for membrane preparations from *B. megaterium* was three (22). High concentrations of moenomycin, on the other hand, resulted in complete inhibition of peptidoglycan synthesis. Thus, the action of moenomycin is clearly different from that of bacitracin as well as vancomycin and ristocetin.

An additional class of high-affinity binding sites for vancomycin exists on the cell wall (4). Adsorption to these sites is believed to interfere with the addition of new wall components (nascent peptidoglycan) to the pre-existing peptidoglycan. In fact, binding studies revealed that most of the bound vancomycin is adsorbed to the cell wall. With *M. lysodeikticus*, Perkins and Nieto (17) estimated that 10% of the iodovancomycin was in the protoplast-membrane fraction. Thus, only a fraction of the antibiotic penetrates to the membrane to exert the effects found with the in vitro peptidoglycan-synthesizing system. Since our in vitro studies do not include the incorporation of nascent peptidoglycan into pre-existing peptidoglycan of the cell wall (13, 28), we can not exclude that wall sites may play a role in the in vivo action of this antibiotic.

ACKNOWLEDGMENTS

We thank Sue Goyer for excellent technical assistance, John C. Swenson for helpful discussions, and Harold R. Perkins for comments on the manuscript.

This investigation was supported in part by Public Health Service grant AI-04615 from the National Institute of Allergy and Infectious Diseases. W.P.H. was a visiting scholar and recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

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