JOURNAL OF BACTERIOLOGY, Aug. 1968, p. 374-382
Vol. 96, No. 2

Printed in U.S.A.

Reversal of the Vancomycin Inhibition of Peptidoglycan Synthesis by Cell Walls[1]

RABINDRA K. SINHA[2] AND FRANCIS C. NEUHAUS[3]

Biochemistry Division, Department of Chemistry, Northwestern University, Evanston, Illinois 60201

Received for publication 8 May 1968

Addition of cell walls to the peptidoglycan synthetase-acceptor system containing vancomycin (50 μg/ml) prevented the inhibition by the antibiotic. In addition, the inhibition of incorporation of [^{14}C]muramyl-pentapeptide into peptidoglycan in the presence of vancomycin was reversed by the addition of cell walls to the assay mixture at 60 min. Cell walls previously saturated with vancomycin lost their ability to reverse the inhibition by the antibiotic. The inhibition of peptidoglycan synthesis by ristocetin was partially reversed by the addition of cell walls. The initial stage in peptidoglycan synthesis is catalyzed by phospho-*N*-acetyl(NAc)muramyl-pentapeptide translocase (uridine 5'-phosphate) according to the reaction:

UDP-NAc-muramyl-pentapeptide + acceptor $\rightleftarrows$ acceptor-phospho-NAc-muramyl-pentapeptide + UMP

where acceptor is C_{55}-isoprenoid alcohol phosphate. Vancomycin stimulates the transfer of phospho-NAc-muramyl-pentapeptide to the acceptor, and the addition of cell walls to this assay mixture prevented the stimulation of transfer. In addition to the transfer reaction, the enzyme catalyzes the exchange of [^{3}H]uridine monophosphate (UMP) with UDP-NAc-muramyl-pentapeptide. The exchange reaction is effectively inhibited by vancomycin. For example, 60 μg of vancomycin per ml inhibited the rate of exchange by 50%. Addition of cell walls restored the exchange of UMP with the UMP moiety of UDP-NAc-muramyl-pentapeptide. Thus, cell walls appeared to have a higher affinity for vancomycin than did either the peptidoglycan synthetase-acceptor system or phospho-NAc-muramyl-pentapeptide translocase. These results provide support for the proposal made by Best and Durham that the effective binding of vancomycin to the cell wall could result in the inhibition of transfer of membrane-associated peptidoglycan chains to the growing wall.

Vancomycin inhibits the biosynthesis of bacterial cell walls. The inhibition is accompanied by the accumulation of uridine diphosphate-*N*-acetyl-muramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine (UDP-NAc-muramyl-L-ala-D-isoglu-L-lys-D-ala-D-ala) in *Staphylococcus aureus* (17). This nucleotide and UDP-NAc-glucosamine are precursors of peptidoglycan, the major structural polymer of bacterial cell walls. The site of action of vancomycin has been investigated in several laboratories. Best and Durham (5) proposed that adsorption of vancomycin to acidic groups on the cell wall could inhibit the addition of new cell wall components to the existing peptidoglycan. The inhibition of teichoic acid biosynthesis by vancomycin in *Bacillus subtilis* and *Bacillus licheniformis* has been reported by Burger and Glaser (7). In 1965, Jordan (13) found that the inhibition of cell wall synthesis by vancomycin occurs earlier than that of membrane biosynthesis. It was concluded that the primary effect of vancomycin in intact cells of staphylococci is an inhibition of wall peptidoglycan synthesis and that inhibition of membrane biosynthesis is a secondary effect. Anderson et al. (1-3) established that peptidoglycan synthetase is effectively inhibited by both vancomycin and ristocetin. Moreover, vancomycin also affects phospho-*N*-acetyl-muramyl pentapeptide translocase (23, 24). Chatterjee and Perkins (8)

[1] Presented in part at the 67th Annual Meeting of the American Society for Microbiology, New York, N.Y., 30 April-4 May 1967.

[2] Present address: Bose Institute, Calcutta, India.

[3] Supported by U.S. Public Health Service Research Career Development Program Award 1-K3-AI-6950 from the National Institute of Allergy and Infectious Diseases.

discovered an additional nucleotide in which vancomycin is bound to UDP-NAc-muramyl-pentapeptide when *S. aureus*, *Micrococcus lysodeikticus*, and *Corynebacterium poinsettiae* are grown in the presence of the antibiotic. Recently, these investigators (9) reported that the vancomycin-nucleotide compound causes a greater inhibition of peptidoglycan formation in cell-free systems than a corresponding amount of free vancomycin. It was suggested that the nucleotide-vancomycin adduct may be a necessary intermediate for the function of the antibiotic (9).

Thus, there are a multiplicity of action sites for vancomycin. Inhibition at one or more of these sites could lead to the observed accumulation of UDP-NAc-muramyl-pentapeptide and inhibition of peptidoglycan synthesis. This investigation was undertaken to establish which component, the peptidoglycan synthetase-acceptor system or the cell wall, has the higher affinity for vancomycin. A study of the relative affinities for this antibiotic in in vitro experiments will contribute to our understanding of the primary site of action.

MATERIALS AND METHODS

Materials. D-[^{14}C]alanine was the product of Calbiochem, Los Angeles, Calif. UDP-NAc-glucosamine and Sephadex G-25 were purchased from Sigma Chemical Co., St. Louis, Mo., and Pharmacia Fine Chemicals, Inc., New Market, N.J., respectively. Triton X-100 and Antifoam 66 were the products of Rohm and Haas, Chicago, Ill., and General Electric (Silicone Products Department), Waterford, N.Y., respectively. The plastic beads (styrene-divinyl benzene copolymer, 20 to 50 mesh, 8% cross-linked) were a gift from Dow Chemical Co. *M. lysodeikticus* (ATCC 4698) was purchased from American Type Culture Collection, and *S. aureus* Copenhagen was kindly provided by J. L. Strominger. Vancomycin and ristocetin (Spontin) were given by Eli Lilly & Co., Indianapolis, Ind., and Abbott Laboratories, North Chicago, Ill., respectively. Bacitracin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

UDP-NAc-muramyl-L-ala-D-isoglu-L-lys and UDP-NAc-muramyl-L-ala-D-isoglu-L-lys-D-[^{14}C]ala-D-[^{14}C]-ala were prepared as previously described (16) and by the procedure of Stickgold and Neuhaus (25).

Enzyme preparations. The preparation of phospho-NAc-muramyl-pentapeptide translocase from *S. aureus* Copenhagen has been previously described (24). The membrane preparation which catalyzes the synthesis of peptidoglycan was isolated from *M. lysodeikticus* (ATCC 4698). The bacteria were grown at 37 C with shaking for 24 hr in medium containing 1% glucose, 0.5% K_2HPO_4, 1% yeast extract, 1% peptone, and 1% of a salt solution containing 4% $MgSO_4 \cdot 7H_2O$, 0.2% $FeSO_4 \cdot 7H_2O$, 0.16% $MnSO_4$, and 0.2% NaCl (4). The yield of bacteria was 7 g (wet weight) per liter. The cells were washed in 0.02 M Tris-chloride (*p*H 7.8) and resuspended to 15% (wet weight) in 0.005 M Tris-chloride (*p*H 7.8) containing 1 M KCl. The cells were disrupted according to the procedure described by Struve et al. (25). The cell walls and unbroken cells were removed at 4,340 × *g* for 30 min, and the particulate enzyme fraction was sedimented by centrifugation at 144,000 × *g* for 30 min. The sediment was suspended in 0.005 M Tris-chloride (*p*H 7.8) containing 1 M KCl, and the centrifugation between 4,340 × *g* for 30 min and 144,000 × *g* for 30 min was repeated four times. The final precipitate was suspended in three times its weight of 0.005 M Tris-chloride (*p*H 7.8) containing 1 M KCl. The enzyme preparation was stored at −196 C.

TABLE 1. *Analysis of cell wall preparation from M. lysodeikticus*[a]

Amino acid (sugar)	This work	Czerkawski et al. (10)	Ghuysen and Salton (11)
Alanine[b]	2.01	1.94	2.98
Glutamic acid	1.00	1.00	1.00
Lysine	1.06	1.02	1.21
Glycine	0.98	1.09	0.91
Muramic acid	0.80	0.76	1.08
Glucosamine	1.00	0.97	0.91

[a] The results are expressed as molar ratios.

[b] A wide variation of alanine-glutamic acid ratios has been reported for *M. lysodeikticus*: Whitney and Grula (27), 2.1; Salton and Pavlik (20), 2.6; Sharon et al. (21), 2.7, in addition to those reported above.

Cell wall isolation. Cell walls from *S. aureus* Copenhagen were prepared according to the method previously described (24). For the preparation of cell walls from *M. lysodeikticus*, the cells were disrupted as described above. Unbroken cells and cell walls were sedimented at 12,000 × *g* for 30 min. The sediment was suspended in 0.005 M Tris-chloride (*p*H 7.8) containing 1 M KCl and centrifuged at 3,000 × *g* for 30 min. The supernatant fraction containing the cell walls was centrifuged at 12,000 × *g* for 10 min. The walls were washed three times with the above buffer containing 1 M KCl. The final precipitate (5.8 g, wet weight) was suspended in 18 ml of 0.005 M Tris-chloride (*p*H 7.8).

The purity of the cell walls was established by analyzing for amino acids, yellow pigmentation, and nucleic acid (18). For amino acid analysis, 0.7 mg (dry weight) of the walls was hydrolyzed in 0.5 ml of 5.7 N HCl under nitrogen for 12 hr at 110 C. The amino acids were determined on the Beckman model 120 amino acid analyzer (Table 1) according to the methods described by Spackman et al. (22). Neither yellow pigmentation nor nucleic acid was detected in the wall preparations.

Assay for phospho-N-acetyl-muramyl-pentapeptide-translocase (UMP). This assay involves the transfer of phospho-*N*-acetyl-muramyl-[^{14}C]pentapeptide to a lipid acceptor according to the following reaction:

UDP-NAc-muramyl-[^{14}C]pentapeptide + acceptor $\rightleftarrows$ acceptor-phospho-NAc-muramyl-[^{14}C]pentapeptide + UMP (1)

The amount of lipid intermediate was measured as a

perchloric acid precipitable fraction as described previously (24). In addition, the translocase has been assayed by the exchange of uridine monophosphate (UMP) with the [^{3}H]UMP moiety of [^{3}H]UDP-NAc-muramyl-pentapeptide according to the procedure described by Heydanek et al. (*in press*).

Assay for peptidoglycan synthesis. This assay measures the incorporation of muramyl-[^{14}C]pentapeptide from UDP-NAc-muramyl-[^{14}C]pentapeptide into peptidoglycan. The assay mixture contained: 0.05 M Tris-chloride (*p*H 7.8), 0.01 M $MnCl_2$, 1.5×10^{-5} M UDP-NAc-muramyl-[^{14}C]pentapeptide (7.5×10^3 counts/min per nanomole), 5×10^{-5} M UDP-NAc-glucosamine, and the membrane preparation (1.1 mg, dry weight) from *M. lysodeikticus* in a total volume of 0.1 ml. The incubation was carried out for 3 hr at 25 C. The reaction was terminated by placing the tube in a boiling-water bath for 2 min, and the suspension was applied to Whatman 3MM paper and developed in a solvent system of isobutyric acid = NH_4OH-water (66:1:33, v/v). The peptidoglycan remained at the origin of the chromatogram. It was excised and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, Ill.). The scintillation fluid was toluene containing 0.3% 2,5-diphenyloxazole. The assay is similar to that described by Anderson et al. (3), with the exception that we have used Mn^{2+} and have omitted adenosine triphosphate (ATP).

Adsorption of vancomycin and ristocetin by cell walls. The adsorption of antibiotics to the cell walls was assayed by a procedure similar to that described by Best and Durham (5). The assay mixture contained 0.05 M Tris-chloride (*p*H 7.8), 0.01 M $MnCl_2$, cell walls, and either 1,000 μg of vancomycin or 500 μg of ristocetin per ml in a total volume of 1 ml. The adsorption was carried out at 25 C for 30 min. The cell walls were removed by centrifugation at $12,000 \times g$ for 5 min. The amount of antibiotic in the supernatant solution was measured spectrophotometrically at 280 mμ.

RESULTS

Synthesis of peptidoglycan. The requirements for the incorporation of radioactivity from UDP-NAc-muramyl-[^{14}C]pentapeptide into peptidoglycan are illustrated in Table 2. The incorporation of NAc-muramyl-pentapeptide was dependent on a divalent cation, UDP-NAc-glucosamine, and membrane fragments. Neither ATP nor glycine or the combination enhanced the formation of product with the enzyme preparation from *M. lysodeikticus*. The addition of lysozyme to the incubation markedly inhibited the incorporation of radioactivity from UDP-NAc-muramyl-pentapeptide into peptidoglycan. These results essentially confirmed those reported by Anderson et al. (3). Since Mn^{2+} was a more effective activator than Mg^{2+} at low concentrations (Fig. 1), 0.01 M Mn^{2+} was used in the routine assay for peptidoglycan synthesis. The kinetics of incorporation into peptidoglycan were compared with the formation of the lipid intermediate (Fig. 2). The structure of the lipid intermediate has been established as lipid-diphosphate-disaccharide-pentapeptide where the lipid moiety is a C_{55}-isoprenoid alcohol (12). The reactions for the synthesis of peptidoglycan are summarized in the discussion. The addition of UMP to the incubation mixture decreased the synthesis of peptidoglycan by inhibiting the first reaction, phospho-NAc-muramyl-pentapeptide translocase (24).

TABLE 2. *Requirements for the synthesis of peptidoglycan*[a]

Additions	Incorporation of [^{14}C] muramyl-pentapeptide
	pmoles/3 hr
Complete	219
− Mn^{2+}	8
− UDP-NAc-glucosamine	3
Boiled enzyme	2
+ ATP (2×10^{-4} M)	175
+ Glycine (3×10^{-4} M)	225
+ Glycine (3×10^{-4} M) + ATP (2×10^{-4} M)	169
+ Lysozyme (1×10^{-4} M)	12
+ UMP (5×10^{-5} M)	10

[a] The complete assay mixture contained: 0.05 M Tris-chloride (*p*H 7.8), 0.01 M $MnCl_2$, 5×10^{-5} M UDP-NAc-glucosamine, 1.5×10^{-5} M UDP-NAc-muramyl-L-ala-D-isoglu-L-lys-D-[^{14}C]ala-D-[^{14}C]ala (7.5×10^3 counts/min per nanomole), and 1.1 mg of enzyme preparation in a total volume of 0.01 ml. The mixture was incubated for 3 hr at 25 C. The reaction was terminated by placing the tubes in a boiling-water bath.

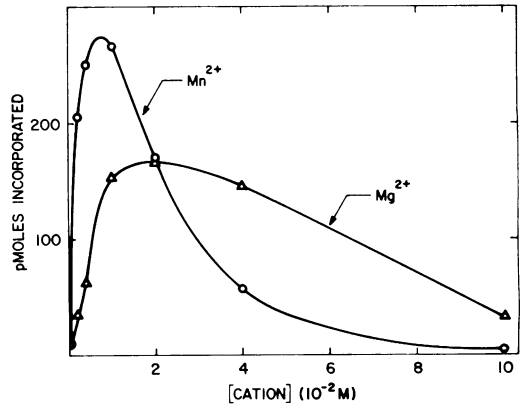

FIG. 1. *Effect of Mg^{2+} and Mn^{2+} on the incorporation of muramyl-[^{14}C]pentapeptide into peptidoglycan. The standard assay was used with 1.4 mg of membranes.*

Effect of antibiotics on peptidoglycan synthesis. The inhibition of peptidoglycan synthesis in membrane preparations from *S. aureus* Copenhagen by vancomycin, ristocetin, and bacitracin

was observed by Anderson et al. (1–3) and confirmed by Struve et al. (23, 24). The effect of vancomycin, ristocetin, and bacitracin on the synthesis of peptidoglycan with our enzyme preparation from *M. lysodeikticus* is shown in Fig. 3. The amount of antibiotic required for 50% inhibition of the incorporation is as follows: ristocetin, 7.5 μg/ml; vancomycin, 20 μg/ml; bacitracin, 11 μg/ml. At a concentration of 50 μg/ml, ristocetin, vancomycin, and bacitracin inhibited 96%, 85%, and 74%, respectively. In similar incubations, Anderson et al. (3) observed that the amount of antibiotic required for 50% inhibition was 15, 10, and 30 μg/ml for ristocetin, vancomycin, and bacitracin, respectively.

Reversal of the inhibition by vancomycin and ristocetin. The effect of walls on the inhibition of incorporation of NAc-muramyl-[^{14}C]pentapeptide into peptidoglycan by vancomycin, ristocetin, and bacitracin is shown in Fig. 4. Addition of cell walls (0.7 mg) to the assay mixture containing vancomycin prevented most of the inhibition by the antibiotic. In contrast, addition of walls (0.7 mg) to an incubation containing bacitracin and ristocetin did not reduce the inhibition to the same extent.

The inhibition of incorporation of NAc-muramyl-[^{14}C]pentapeptide into peptidoglycan by vancomycin can be reversed by the addition of cell walls. Vancomycin at a concentration of 50 μg/ml inhibited the rate of incorporation by 85% (Fig. 3). When cell walls were added after 1 hr to an assay mixture containing vancomycin, the rate of incorporation was restored in proportion to the amount of cell walls that were added (Fig. 5A). For example, in the presence of 0.70 mg of cell walls, the velocity in the system containing vancomycin is 80% of that observed in the absence of vancomycin. When 0.07 and 0.35 mg of cell walls were added at 60 min, the velocities were 16 and 56% of that observed in the absence of vancomycin. Cell walls previously saturated with vancomycin lost their ability to reverse the inhibition by the antibiotic.

The inhibition of peptidoglycan synthesis by ristocetin can also be reversed by the addition of cell walls (Fig. 5B). For example, the addition of 0.35 mg and 0.70 mg of cell walls restored the rate of incorporation to 30 and 45% of that observed in the absence of antibiotic. As in the

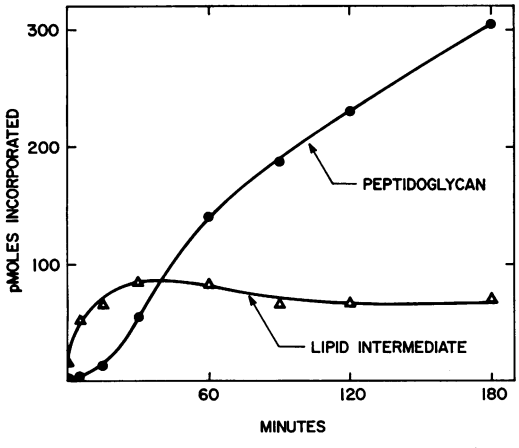

FIG. 2. *Kinetics of incorporation into peptidoglycan and the lipid intermediate. The standard assay was used with 1.1 mg of membranes as described in the text. The lipid intermediate was measured by the method described by Struve et al. (24).*

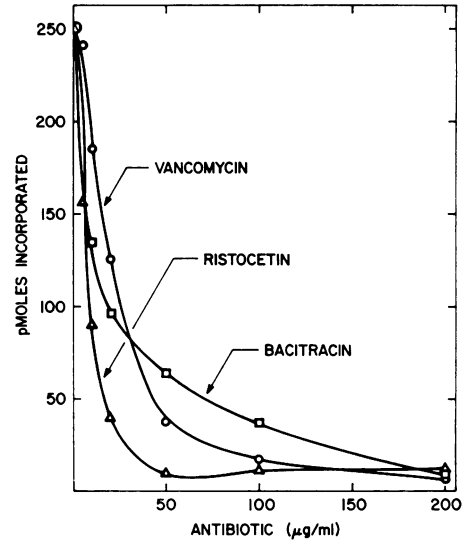

FIG. 3. *Effect of vancomycin, ristocetin, and bacitracin on the incorporation into peptidoglycan. The standard assay was used with 1.1 mg of the enzyme preparation.*

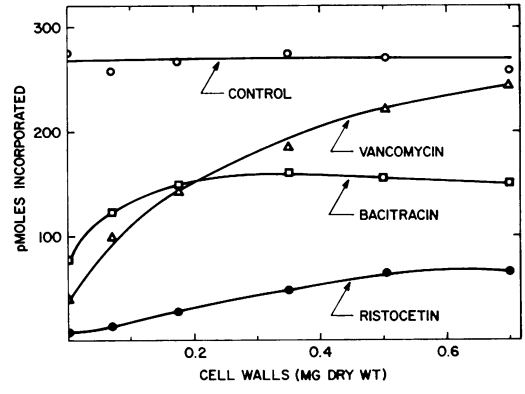

FIG. 4. *Effect of cell-wall concentration on the inhibition by antibiotics. The standard assay was used with 1.1 mg of the enzyme preparation. The following antibiotics were added to the assay mixture: (○) none; (△) vancomycin (50 μg/ml); (●) ristocetin (50 μg/ml); and (□) bacitracin (50 μg/ml).*

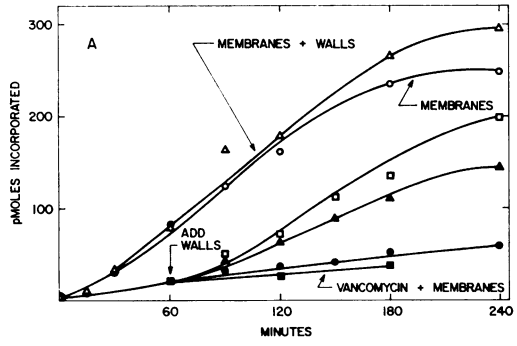

FIG. 5A. *Reversal of the inhibition by vancomycin of peptidoglycan synthesis with cell walls from M. lysodeikticus. The standard assay was used with 1.1 mg of membranes. In the control (△), 0.35 mg of cell walls from M. lysodeikticus was added to the standard assay (○). To the assay mixture containing 50 μg of vancomycin per ml, the following additions of cell walls were made at 60 min: (■) none; (●) 0.07 mg; (▲) 0.35 mg; and (□) 0.7 mg.*

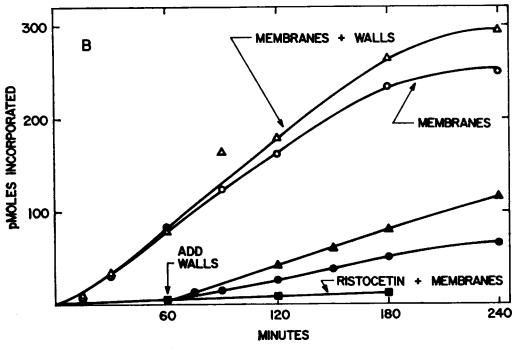

FIG. 5B. *Reversal of the inhibition by ristocetin of peptidoglycan synthesis with cell walls from M. lysodeikticus. The procedure was identical to that described for Figure 5A. To the assay mixture containing 50 μg of ristocetin per ml, the following additions of cell walls were made at 60 min: (■) none; (●) 0.35 mg; (▲) 0.7 mg.*

case of vancomycin, the reversal was dependent on the amount of cell walls added to the assay mixture.

Effect of vancomycin and cell walls on phospho-NAc-muramyl-pentapeptide translocase (UMP). In previous studies (24), vancomycin was shown to stimulate the transfer of phospho-NAc-muramyl-[^{14}C]pentapeptide from UDP-NAc-muramyl-[^{14}C]pentapeptide to a lipid acceptor according to the reaction:

UDP-NAc-muramyl-pentapeptide + lipid-phosphate ⇄ lipid-diphosphate-NAc-muramyl-pentapeptide + UMP (2)

The addition of cell walls to the assay mixture containing membrane fragments, UDP-NAc-muramyl-pentapeptide, and vancomycin prevented the stimulation of transfer by vancomycin.

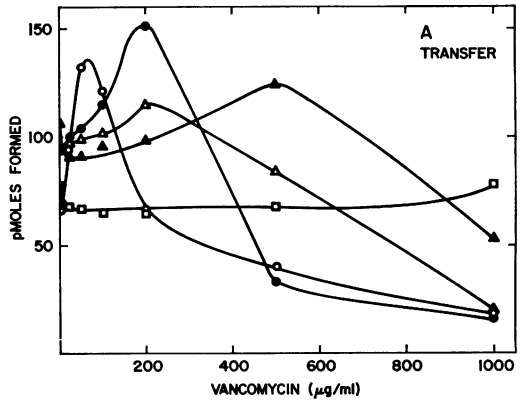

FIG. 6A. *Reversal of the vancomycin effects (transfer assay) on the translocase by cell walls. The reaction mixture for the transfer assay contained 1.5 × 10^{-5} M UDP-NAc-muramyl-[^{14}C]pentapeptide (9.5 × 10^{3} counts/min per nanomole), 0.05 M Tris-chloride (pH 7.8), 0.01 M $MgCl_2$, and 0.55 mg of membrane preparation from S. aureus Copenhagen in a total volume of 0.1 ml. The mixture was incubated for 15 min at 25 C. The following additions of cell walls were made: (○) none, (●) 0.15 mg, (△) 0.30 mg, (▲) 0.60 mg, (□) 1.5 mg.*

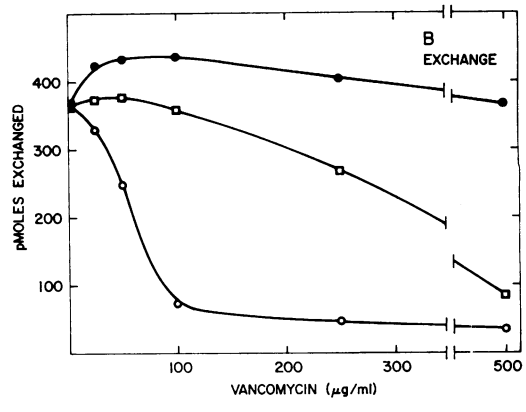

FIG. 6B. *Reversal of the vancomycin effects (exchange assay) on the translocase by cell walls. The reaction mixture for the exchange assay contained 0.05 M Tris-chloride (pH 7.8); 0.01 M $MgCl_2$, 3.2 × 10^{-5} M [^{3}H]UDP-NAc-muramyl-pentapeptide (3.7 × 10^{3} counts/min per nmole), 3.3 × 10^{-4} M UMP, vancomycin, and 0.82 mg of membrane preparation from S. aureus Copenhagen in a total volume of 0.06 ml. The following additions of cell walls were made: (○) none, (□) 0.18 mg, and (●) 0.7 mg. The mixtures were incubated at 25 C for 10 min and the reactions were terminated by placing the tube in a boiling-water bath for 2 min. The amount of [^{3}H]UMP was quantitated by the procedure described by Heydanek et al. (in press).*

The effect of increasing amounts of vancomycin on the translocase is shown in Fig. 6A. With increasing levels of cell walls, the concentration required for maximal stimulation was shifted toward higher concentrations of the antibiotic. At a cell wall concentration of 1.5 mg/0.1 ml, the inhibitory and stimulatory effects of vancomycin were prevented. Vancomycin at low concentrations was observed to enhance the transfer assay (Fig. 6A). In contrast, low levels of the antibiotic inhibited the exchange assay (Fig. 6B). For example, 60 μg of vancomycin per ml inhibited the rate of exchange by 50%. Addition of cell walls restored the exchange of UMP with the UMP moiety of UDP-NAc-muramyl-pentapeptide.

Adsorption of vancomycin and ristocetin to cell walls. Best and Durham (5, 6) described the adsorption of these antibiotics to cell walls isolated from *B. subtilis*. It was concluded that electrostatic interaction between acidic groups on the wall and basic groups on the antibiotic were involved. Mg^{2+} and other cations competed with the antibiotic for the binding sites on the wall (5).

To correlate the reversal of inhibition with the extent of binding, adsorption of ristocetin and vancomycin to walls of *M. lysodeikticus* was measured. Since divalent cations (Mn^{2+}) were present in the assay mixtures for peptidoglycan synthesis, the adsorption of antibiotic was measured in the presence and absence of Mn^{2+} (0.01 M).

With vancomycin (Fig. 7A), 95% of the antibiotic was adsorbed in the absence of Mn^{2+}, whereas in the presence of 0.01 M Mn^{2+}, 82% of the antibiotic was adsorbed from the assay mixture. The concentrations of cell walls required for half-maximal adsorption of 1,000 μg of vancomycin were 0.28 and 0.68 mg/ml in the absence and presence of 0.01 M Mn^{2+}, respectively. With ristocetin (Fig. 7B), 83% of the antibiotic was adsorbed in the absence of Mn^{2+}, whereas in the presence of 0.01 M Mn^{2+}, 62% of the antibiotic was adsorbed from the assay mixture. The concentration of cell walls required for half-maximal adsorption of 500 μg of ristocetin was 0.23 and 2.94 mg/ml in the absence and presence of 0.01 M Mn^{2+}, respectively.

DISCUSSION

The elucidation of the enzymatic reactions (Fig. 8) involved in the synthesis of peptidoglycan has provided additional loci for testing the action of vancomycin and ristocetin. Strominger and co-workers (1–3) concluded that the last step in the cycle, transfer of the modified disaccharide-hexapeptide dimer from the lipid carrier to the membrane acceptor, is the component most sensitive to ristocetin and vancomycin in *M. lysodeikticus*. The nature of the terminal acceptor associated with the membrane has not been established. Jordan and Reynolds (14) suggested that the in vitro experiments of Strominger and co-workers will not distinguish between the inhibition of peptidoglycan synthetase itself and the blockage of addition sites on the acceptor associated with the membrane.

The effective binding of vancomycin and ristocetin to the wall as described by Best and Durham (5, 6) could result in the inhibition of transfer of membrane-associated peptidoglycan chains to the growing wall. Since vancomycin is readily adsorbed to the wall, Best and Durham (5) suggested that the inhibition by vancomycin of the membrane-associated reactions may be of secondary importance. This proposal is based primarily on the fact that vancomycin reaches

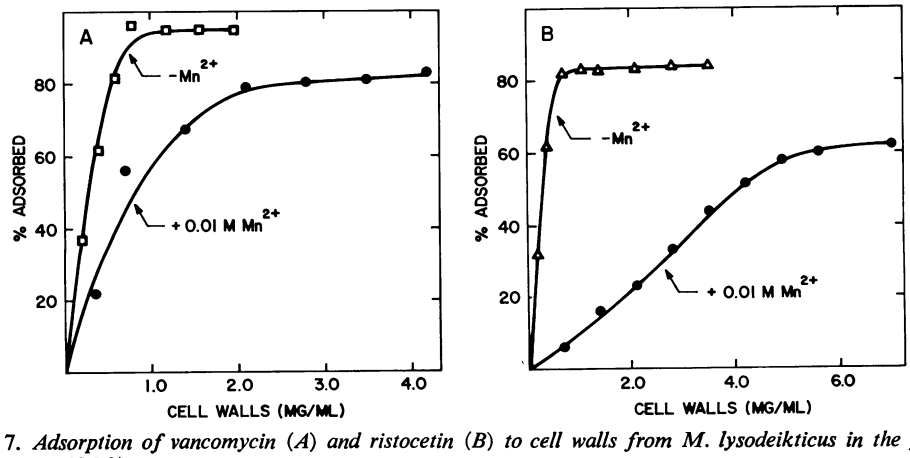

FIG. 7. *Adsorption of vancomycin (A) and ristocetin (B) to cell walls from M. lysodeikticus in the presence and absence of* Mn^{2+}.

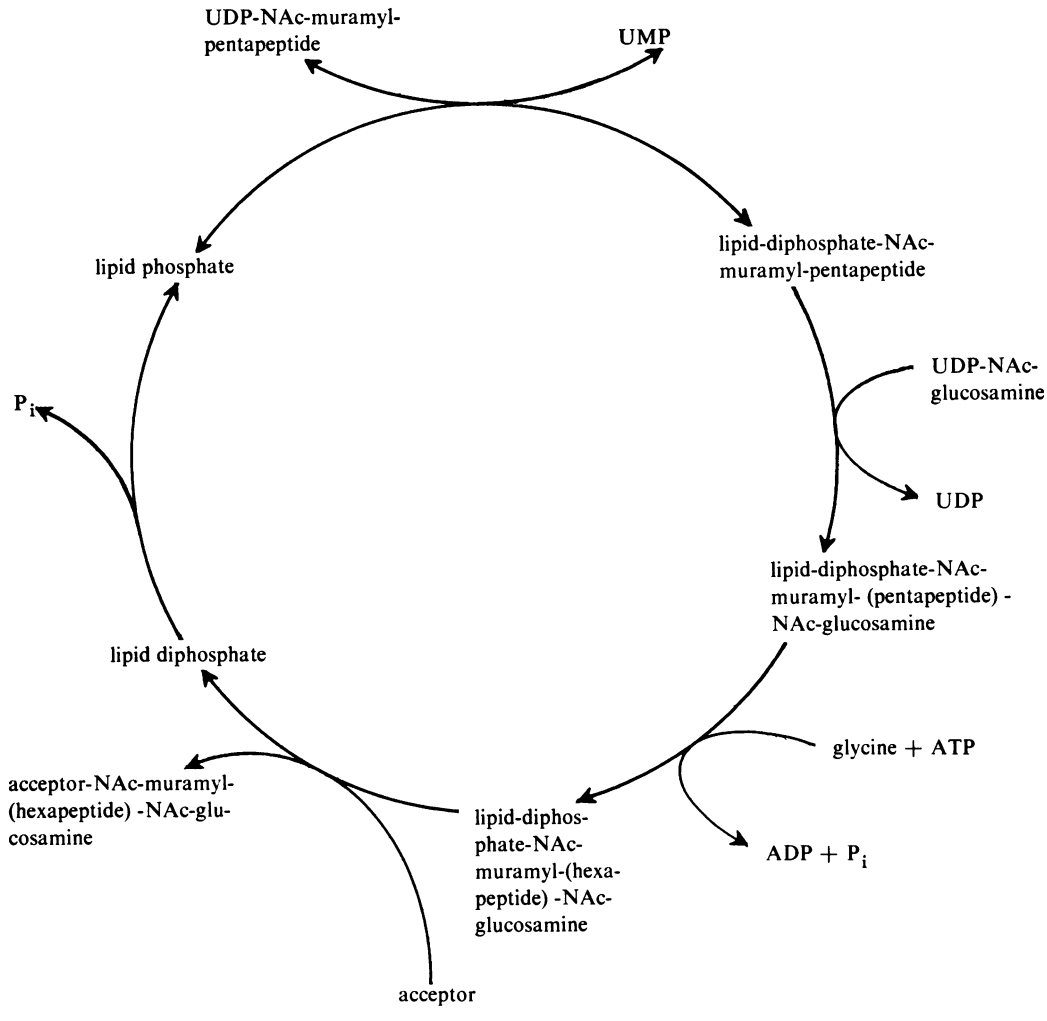

FIG. 8. *Lipid cycle in the biosynthesis of peptidoglycan (15)*

the membrane after the available sites on the cell wall have been saturated.

Thus, the experiments presented in this paper were initiated in an attempt to establish whether the peptidoglycan synthetase-acceptor system or the cell wall has the higher affinity for vancomycin. In addition to peptidoglycan synthetase, the initial enzyme of the cycle, phospho-NAc-muramyl-pentapeptide translocase, has been tested for the reversal of the effects by vancomycin.

The addition of cell walls to an incubation mixture containing vancomycin prevented the inhibition of incorporation of NAc-muramyl-[^{14}C]pentapeptide into peptidoglycan. In the case of ristocetin and bacitracin, the inhibition was partially prevented. Moreover, if cell walls were added at 60 min to an incubation mixture containing vancomycin, the inhibition of incorporation was reversed. The extent of reversal was dependent on the concentration of cell walls added to the incubation. Thus, cell walls appear to have a higher affinity for vancomycin than does the peptidoglycan synthetase-acceptor system associated with the membranes. Although the concentration of walls required for reversal appears high (7 mg/ml), it must be emphasized that a higher concentration of membranes (11 mg/ml) must be incorporated into the incubation mixture to observe the activity of peptidoglycan synthetase. On a percentage of dry weight comparison, the ratio of wall to membrane in *M. lysodeikticus* is approximately one (18, 19). Thus, the concentration of walls that were added to an incubation mixture to observe reversal of the antibiotic effects is not excessive.

Best and Durham (5) observed that Mg^{2+} and other cations compete with vancomycin for binding sites on the cell wall. This observation was confirmed in the present investigation with walls from *M. lysodeikticus*. An attempt was made to correlate the reversal of inhibition of incorporation of NAc-muramyl-[^{14}C]pentapeptide into peptidoglycan by walls with the extent of binding of vancomycin to cell walls. However, it was not possible to perform the adsorption assay for vancomycin in the presence of membranes. Thus, the concentrations of walls required to adsorb a given amount of antibiotic was less in the absence of membranes than in the presence. In the presence of 0.01 M Mn^{2+}, only 82% of the vancomycin was adsorbed from the assay mixture. The maximal reversal of inhibition by vancomycin was 80% (Fig. 5A). These observations are consistent with the experiments described by Best and Durham (5). They observed that only 80% of the vancomycin could be adsorbed from solution. (These results were established in the absence of divalent cations.) Additional cell walls could not adsorb the remaining 20%. On the basis of these results, it was suggested (5) that vancomycin may exist as two components in an 80:20 ratio.

The first reaction in the cycle, phospho-NAc-muramyl-pentapeptide translocase, is also affected by vancomycin (24). The addition of low levels of vancomycin to the assay mixture results in an enhancement of transfer while increasing concentrations result in an inhibition of the transfer reaction. In contrast, if the exchange assay is used, a pronounced inhibition is observed at low concentrations of vancomycin. Heydanek et al. (*in press*) proposed that the transfer of phospho-NAc-muramyl-pentapeptide to the C_{55}-isoprenoid alcohol phosphate precedes via an enzyme-phospho-NAc-muramyl-pentapeptide intermediate according to the following reaction sequence:

$$E + UMPPPMp \rightleftarrows EUMPPPMp \quad (3)$$

$$EUMPPPMp \rightleftarrows EPMp + UMP \quad (4)$$

$$EPMp + Acceptor \rightleftarrows Acceptor\text{-}PMp + E \quad (5)$$

where E is enzyme, UMPPPMp is UDP-NAc-muramyl-pentapeptide, and acceptor is C_{55}-isoprenoid alcohol phosphate. The stimulation-inhibition observed in the transfer assay (reactions 3, 4, and 5) results from a combination of the detergent and inhibitory effects of the antibiotic. Thus, inhibition in the transfer assay is only observed at high concentrations of vancomycin (500 μg/ml). In contrast, the exchange reaction (reactions 3 and 4) is inhibited 50% by 60 μg/ml of antibiotic. For comparison, the peptidoglycan synthetase-acceptor system is inhibited 50% by 20 μg of vancomycin per ml. Since the concentrations of antibiotic required to inhibit these enzymes differ by only threefold, the translocase may still be considered as a potential site of vancomycin action. It is apparent from the results that cell walls prevent the enhancement of transfer and the inhibition of exchange activity.

When *S. aureus* is grown in the presence of either vancomycin (17) or ristocetin (26), the major nucleotide that accumulates is UDP-NAc-muramyl-pentapeptide. In addition, lower amounts of UDP-NAc-muramyl-L-ala were observed to accumulate in both cases. Chatterjee and Perkins (8) observed an additional nucleotide in which vancomycin is bound to UDP-NAc-muramyl-pentapeptide. However, Best, Sinha, and Neuhaus (*unpublished data*) were not able to detect a vancomycin-nucleotide adduct when vancomycin was added to a culture of *S. aureus* Copenhagen. The synthesis of this complex is effected by incubating vancomycin and UDP-NAc-muramyl-pentapeptide (H. R. Perkins, Biochem. J. **106**:35P, 1968). This observation has been confirmed by Best, Sinha, and Neuhaus (*unpublished data*). Although the exact role of the vancomycin-nucleotide complex has not been established, this type of complex formation may provide the key to the mechanism of vancomycin action. The minimal structure that is required for complex formation is D-isoglu-L-lys-D-ala-D-ala (H. R. Perkins, Biochem. J. **106:1** 35P, 1968). This structure corresponds to the noncross-linked terminus where new peptidoglycan is being added. The binding of vancomycin to this sequence in the wall could prevent addition of new peptidoglycan strands according to the proposal of Best and Durham (5).

ACKNOWLEDGMENTS

We thank Rosemary Linzer and Judy Domin for amino acid analyses on a Beckman Spinco amino acid analyzer that was supported by Public Health Service grant HE-11119 from the National Heart Institute.

This investigation was supported by the Public Health Service grant AI-04615 from the National Institute of Allergy and Infectious Diseases and by a Public Health Service training grant GM-626 from the Division of General Medical Science.

We thank Gary K. Best, William G. Struve, and Menard G. Heydanek for discussions and methods.

LITERATURE CITED

1. Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger. 1965. Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide: presumed membrane transport intermediates in the biosyn-

thesis of bacterial cell walls. Proc. Natl. Acad. Sci. U.S. *53*:881–889.
2. Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger. 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. II. Phospholipid carriers' in the reaction sequence. J. Biol. Chem. **242**:3180–3190.
3. Anderson, J. S., P. M. Meadow, M. A. Haskin, and J. L. Strominger. 1966. Biosynthesis of the peptidoglycan of bacterial cell walls. Arch. Biochem. Biophys. **116**:487–515.
4. Beers, R. F., Jr. 1955. Submerged culture of *Micrococcus lysodeikticus* for large-scale production of cells. Science **122**:1016.
5. Best, G. K., and N. N. Durham. 1965. Vancomycin adsorption to *Bacillus subtilis* cell walls. Arch. Biochem. Biophys. **111**:685–691.
6. Best, G. K., and N. N. Durham. 1966. Adsorption of the ristocetins to *Bacillus subtilis* cell walls. Antimicrobial Agents and Chemotherapy—1965, p. 334–338.
7. Burger, M. M., and L. Glaser. 1964. The synthesis of teichoic acids. I. Polyglycerophosphate. J. Biol. Chem. **239**:3168–3177.
8. Chatterjee, A. N., and H. R. Perkins. 1966. Compounds formed between nucleotides related to the biosynthesis of bacterial cell wall and vancomycin. Biochem. Biophys. Res. Commun. **24**:489–494.
9. Chatterjee, A. N., J. B. Ward, and H. R. Perkins. 1967. Synthesis of mucopeptide by L-form membranes. Nature **214**:1311–1314.
10. Czerkawski, J. W., H. R. Perkins, and H. J. Rogers. 1963. A study of the composition and structure of the cell wall mucopeptide of *Micrococcus lysodeikticus*. Biochem. J. **86**:468–474.
11. Ghuysen, J. M., and M. R. J. Salton. 1960. Acetylhexosamine compounds enzymically released from *Micrococcus lysodeikticus* cell walls. I. Isolation and composition of acetylhexosamine and acetylhexosamine-pentide complexes. Biochim. Biophys. Acta **40**:462–472.
12. Higashi, Y., J. L. Strominger, and C. C. Sweeley. 1967. Structure of a lipid intermediate in cell wall peptidoglycan synthesis: A derivative of a C_{55} isoprenoid alcohol. Proc. Natl. Acad. Sci. U.S. **57**:1878–1884.
13. Jordan, D. C. 1961. Effect of vancomycin on the synthesis of the cell wall and cytoplasmic membrane of *Staphylococcus aureus*. Can. J. Microbiol. **11**:390–393.
14. Jordan, D. C., and P. E. Reynolds. 1967. Vancomycin, p. 102–116. *In* D. Gottlieb, and P. L. Shaw (ed.), Antibiotics: mechanism of action, vol. 1, Springer-Verlag, Heidelberg.
15. Katz, W., M. Matsuhashi, C. P. Dietrich, and J. L. Strominger. 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. IV. Incorporation of glycine in *Micrococcus lysodeikticus*. J. Biol. Chem. **242**:3207–3217.
16. Neuhaus, F. C., and W. G. Struve. 1965. Enzymatic synthesis of analogs of the cell wall precursor. I. Kinetics and specificity of uridine diphospho - N - acetylmuramyl - L - alanyl - D-glutamyl-L-lysine:D-alanyl-D-alanine ligase (adenosine diphosphate) from *Streptococcus faecalis* R. Biochemistry **4**:120–131.
17. Reynolds, P. E. 1961. Studies on the mode of action of vancomycin. Biochim. Biophys. Acta **52**:403–405.
18. Salton, M. R. J. 1964. The bacterial cell wall. Elsevier Publishing Co., Amsterdam.
19. Salton, M. R. J. 1967. Bacterial membranes, p. 77. *In* B. D. Davis, and L. Warren (ed.), The specificity of cell surfaces. Prentice-Hall, Inc., Englewood Cliffs, N.J.
20. Salton, M. R. J., and J. G. Pavlik. 1960. Studies of the bacterial cell wall. VI. Wall composition and sensitivity to lysozyme. Biochim. Biophys. Acta **39**:398–407.
21. Sharon, N., T. Osawa, H. M. Flowers, and R. W. Jeanloz. 1966. Isolation and study of the chemical structure of a disaccharide from *Micrococcus lysodeikticus* cell walls. J. Biol. Chem. **241**:223–230.
22. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30**:1190–1206.
23. Struve, W. G., and F. C. Neuhaus. 1965. Evidence for an initial acceptor of UDP-NAc-muramyl-pentapeptide in the synthesis of bacterial mucopeptide. Biochem. Biophys. Res. Commun. **18**:6–12.
24. Struve, W. G., R. K. Sinha, and F. C. Neuhaus. 1966. On the initial stage in peptidoglycan synthesis. Phospho-N-acetyl-muramyl-pentapeptide translocase (uridine monophosphate). Biochemistry **5**:82–93.
25. Stickgold, R. A., and F. C. Neuhaus. 1967. On the initial stage in peptidoglycan snythesis. Effect of 5-fluorouracil substitution on phospho-N-acetyl-muramyl-pentapeptide translocase (uridine 5'-phosphate). J. Biol. Chem. **242**: 1331–1337.
26. Wallas, C. H., and J. L. Strominger. 1963. Ristocetins, inhibitors of cell wall synthesis in *Staphylococcus aureus*. J. Biol. Chem. **238**:2264–2266.
27. Whitney, J. G. and E. A. Grula. 1964. Incorporation of D-serine into the cell wall mucopeptide of *Micrococcus lysodeikticus*. Biochem. Biophys. Res. Commun. **14**:375–381.