ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1977, p. 396-401

Vol. 11, No. 3
Printed in U.S.A.

Gardimycin, a New Antibiotic Inhibiting Peptidoglycan Synthesis

SERGIO SOMMA,* WILMA MERATI, AND FRANCESCO PARENTI
Research Laboratories, Lepetit S.p.A., Milano, Italy

Received for publication 12 April 1976

Gardimycin, a new antibiotic, at 100 μg/ml, specifically inhibited cell wall synthesis and induced accumulation of uridine 5'-diphosphate-*N*-acetylmuramylpentapeptide in whole cells of *Bacillus subtilis*. The antibiotic was active in a particulate enzyme preparation from *Bacillus stearothermophilus:* 60 μg/ml caused 50%, and 200 μg/ml caused 100%, inhibition of peptidoglycan synthesis. Suppression of peptidoglycan synthesis was accompanied by parallel accumulation of the lipid intermediate. This mechanism of action is discussed in comparison with those of other antibiotics that are known to inhibit bacterial cell wall biosynthesis.

Gardimycin is a new sulfur-containing peptide antibiotic recently isolated from the fermentation broth of *Actinoplanes garbadinensis* sp. nov. (9). It is active against gram-positive bacteria and has very low toxicity. Its chemical characteristics (3) and biological properties (2) have been reported elsewhere. Preliminary experiments indicated that gardimycin interferes with bacterial cell wall biosynthesis. In fact, the drug is inactive on cell wall-lacking bacteria such as *Mycoplasma* and on the L-form of susceptible *Staphylococcus aureus*.

In this paper, a more detailed analysis of its mechanism of action is reported, which confirms the effect of gardimycin on cell wall biosynthesis and indicates that its primary site of action is on the transfer of the disaccharidic pentapeptide unit from the lipid carrier to the acceptor site of the cell wall.

MATERIALS AND METHODS

Bacterial strains. The effects of gardimycin on living cells were studied in *Bacillus subtilis* SB25 (*try*$^-$ *his*$^-$) and PB 556/1 (*thy*$^-$). *Bacillus stearothermophilus* (*calidolactis*) ATCC 10149 was used as the source of particulate enzyme for the studies of in vitro peptidoglycan synthesis.

Bacterial growth. *B. subtilis* was usually grown in flasks at 37°C, with reciprocal shaking, and *B. stearothermophilus* was grown at 55°, in fermenters with forced aeration.

Radiochemicals. [5-^{3}H]uracil, 24.2 Ci/mmol; [2-^{14}C]thymidine, 61 mCi/mmol; [U-^{14}C]phenylalanine, 225 mCi/mmol; L-[U-^{14}C]alanine, 173 mCi/mmol; [1-^{3}H]*N*-acetylglucosamine (GlcNAc), 4 Ci/mmol; and [1-^{14}C]GlcNAc, 58 mCi/mmol; were all obtained from Radiochemical Centre, Amersham, England.

Incorporation of labeled compounds into cell wall and protein fraction. Exponentially growing *B. subtilis* was transferred to Davis minimal medium supplemented with 0.5% glucose, 50 μg of tryptophan per ml, 50 μg of histidine per ml, and the appropriate labeled compound and incubated for 20 min at 37°C. Cells were harvested, washed with buffer [0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4], heated for 5 min, and sonicated for 10 min. Unbroken cells were removed by centrifugation at 6,000 × *g* for 10 min. Total incorporated radioactivity was determined at this stage by precipitating a sample of the sonicated cell suspension with cold 10% (wt/vol) trichloroacetic acid; acid-insoluble material was collected on glass-fiber filters (Whatman GF/C) and washed with ethanol and diethyl ether; dried filters were counted for radioactivity. The radioactivity incorporated in the cell wall or the protein fraction of the cells was determined after fractionation as follows. A sample of sonicated cells was treated with pancreatic ribonuclease and deoxyribonuclease I for 30 min, with trypsin for 2 h, and finally with pepsin at pH 2 for 2 h. Trichloroacetic acid-insoluble material, which consists of cell walls, was collected and the radioactivity was determined.

A different sample of sonicated cells was incubated with lysozyme in the presence of 0.05% (wt/vol) sodium dodecyl sulfate for 1 h and then with ribonuclease and deoxyribonuclease for 30 min, and the radioactivity of the trichoroacetic acid-insoluble material, which represents "cellular protein," was determined. All the enzymes were used at a concentration of 1 mg/ml, and the incubation temperature was 37°C.

The specificity of the fractionation procedure was checked as in previous experiments by determining the origin of the radioactivity found in the two fractions obtained from cells grown in the presence of [^{14}C]phenylalanine and [^{3}H]GlcNAc as protein and cell wall precursors, respectively.

Extraction and determination of uridine nucleotide in cells. Cell wall precursors were extracted from exponentially growing *B. subtilis* SB25 incubated in Pennassay (PY) broth (Difco) as described

by Garret (4). *N*-acetylhexosamine was determined by the procedure of Reissig et al. (10).

Identification of the accumulated cell wall precursors. To an exponentially growing culture of *B. subtilis* SB25 in PY medium containing 22% (wt/vol) sucrose, 0.4 μCi of [^{3}H]GlcNAc per ml and gardimycin at 200 μg/ml were added. After a further 40-min incubation, the cells were harvested and washed with 22% sucrose in 0.1 M Tris-hydrochloride, pH 7.9. Then, nucleotides were extracted and separated by descending chromatography on Whatman no. 1 filter paper, with a mixture of 95% ethanol and 1 M ammonium acetate, pH 7 (7.5:3), for 16 h. After drying, the paper strip was cut into 0.5-cm segments, and the radioactivity on each segment was counted.

Preparation of uridine 5'-diphosphate *N*-acetyl[^{14}C]muramylpentapeptide (UDP-MurNAc-pentapeptide). Cells of *B. subtilis* SB 25 were treated as described above, except that 0.1 μCi of [^{14}C]GlcNAc per ml and 100 μg of vancomycin per ml were used. The pooled nucleotides were applied to a Dowex 1 column (Cl^- form), 100 to 200 mesh, and eluted with a linear gradient of NaCl (from 0.05 to 0.3 M) in 0.01 N HCl. A main peak absorbing at 260 nm and containing most of the radioactivity was obtained. The fractions making up this peak were pooled and lyophilized. The product thus obtained was homogeneous by paper chromatography and had a specific activity of 104,000 cpm/μmol.

Preparation of particulate enzyme. Cells of *B. stearothermophilus* were grown at 55°C in a medium containing 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 0.25% (wt/vol) K_2HPO_4, and 0.1% (wt/vol) glucose, harvested in the middle of the exponential phase, washed, suspended in buffer (0.05 M Tris-hydrochloride, pH 7.9, and 0.01 M Mg acetate), and frozen. The frozen-cell paste was ground in a homogenizer with an equal volume of Superbrite (Minnesota Mining & Manufacturing) for three periods of 5 min while being cooled in ice. The Superbrite was removed by filtering on a sintered-glass funnel. The cell suspension was centrifuged twice at 6000 × *g* for 10 min, and the supernatant was centrifuged at 38,000 *g* for 20 min. Then, the pellet was washed twice and resuspended in buffer. The suspension was stored under liquid nitrogen.

In vitro synthesis of peptidoglycan. The reaction mixtures contained 100 mM Tris-hydrochloride, pH 7.9, 25 mM $MgCl_2$, 0.5 mM UDP-GlcNAc, 0.5 mM [^{14}C]UDP-MurNAc-pentapeptide and the particulate enzyme at a concentration of 8 mg of protein per ml (determined with the Folin-Ciocalteau reagent). Final volume was 100 μl. After 40 min of incubation at 30°C, the entire reaction mixture was spread on a 3-cm line at the origin of a strip of Whatman no. 1 chromatographic paper and dried with hot air. Chromatograms were developed in a descending direction with a mixture of isobutyric acid-1 M NH_4OH (5:3). According to Strominger et al. (13), the synthesized peptidoglycan remains at the point of origin, while the substrate and intermediate move down.

Radioactivity measurement. Filter disks and paper segments were counted in a Philips liquid scintillation analyzer with Instagel (Packard) as scintillation fluid; chromatograms were analyzed by a Packard chromatography scanner, and the distribution of radioactivity was determined from the peak areas.

RESULTS

Effect on growth and viability. The minimal inhibitory concentration of gardimycin against *B. subtilis* SB25 is 100 μg/ml. Addition of the antibiotic at this concentration to a growing culture causes an immediate decrease in absorbance, indicating cell lysis (Fig. 1). The effect of gardimycin on cell viability is shown in Fig. 2. When the antibiotic is added at 200 μg/ml to a growing culture, an irreversible loss of viability takes place. At a lower concentration (50 μg/ml), however, there is a resumption of growth after 1 h of incubation. A similar effect of subinhibitory concentrations has already been reported for other antibiotics that interfere with cell wall biosynthesis (5). Addition of gardimycin (200 μg/ml) to resting cells deprived of a carbon source, however, does not affect cell viability.

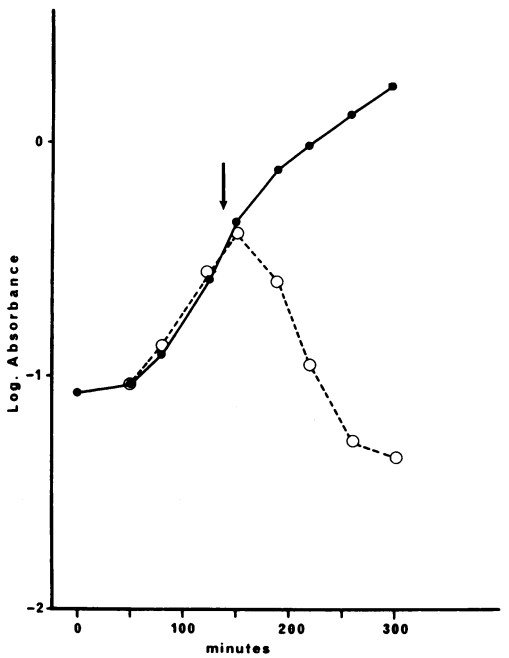

FIG. 1. *Effect of gardimycin on growth. Two cultures of B. subtilis were incubated in PY medium, and the growth was determined by absorbance at 595 nm. At the time indicated by the arrow, gardimycin was added to one of the cultures to a final concentration of 100 μg/ml. Symbols: (●) Control, (○) gardimycin.*

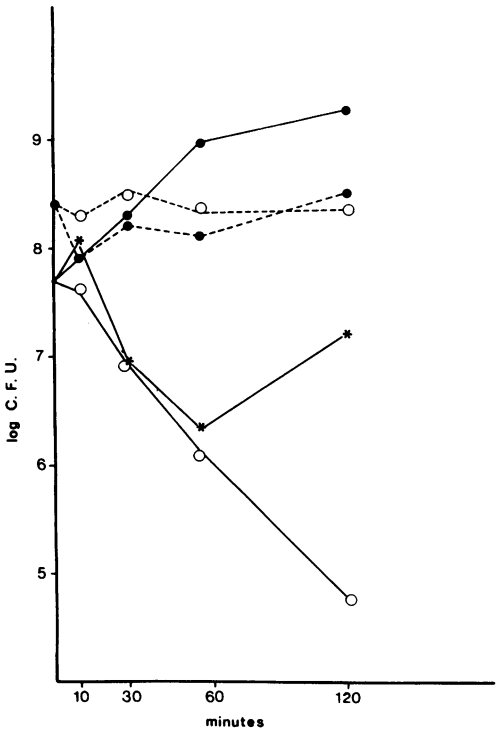

FIG. 2. *Effect of gardimycin on cell viability. The solid line indicates the number of colonies grown on an agar plate after suitable dilution of a culture of B. subtilis growing in PY medium. The dashed line indicates the number of colonies formed after cell washing and incubation in buffer containing 0.15 M NaCl, 0.1 M Tris-hydrochloride (pH 7.9), and 0.01 M $MgCl_2$. Symbols: (●) Control; (*) gardimycin, 50 μg/ml; (○) gardimycin, 200 μg/ml.*

Effect on macromolecular synthesis in vivo. To identify the cellular macromolecular synthesis primarily affected by gardimycin, the rate of incorporation of radioactive precursors was measured in the presence or absence of the antibiotic.

As shown in Fig. 3, the addition of gardimycin (200 μg/ml) to a growing culture immediately suppressed the incorporation of GlcNAc, a specific precursor of peptidoglycan. The syntheses of deoxyribonucleic acid, ribonucleic acid, and protein, measured as incorporation of thymine, uracil, and phenylalanine, respectively, were only slightly affected for the first 20 min, thus suggesting that cell wall synthesis is the primary target of the drug. To ascertain that the target of gardimycin is peptidoglycan synthesis and not merely GlcNAc uptake or utilization, the distribution of labeled alanine, a component of both peptidoglycan and protein, between the cell wall and the protein fractions of bacteria grown in the presence or absence of gardimycin was determined. As a control, the same distribution was also determined in bacteria grown in the presence of the protein synthesis inhibitor, tetracycline. The results of such an experiment are shown in Fig. 4.

Gardimycin did not reduce the amount of alanine incorporated into the protein fraction as compared with the control culture, whereas it strongly decreased its incorporation into the cell wall. As expected, tetracycline inhibited alanine incorporation into the protein fraction, but not into the cell wall fraction.

Accumulation of cell wall precursors. Most antibiotics interfering with peptidoglycan synthesis cause an endocellular accumulation of UDP nucleotides linked to *N*-acetylaminohexoses.

As shown in Fig. 5, such accumulation also occurs in a growing bacterial culture treated with 50 μg of gardimycin per ml. Figure 6 shows the relationship between the antibiotic concentration and the amount of *N*-acetylaminohexose accumulated over a 30-min period of incubation. To identify the accumulated precursors, the nucleotides extracted from cells grown in the presence of [^{3}H]GlcNAc plus 200 μg of gardimycin per ml were co-chromatographed with UDP-NAc[^{14}C]Mur-pentapeptide, obtained from cells treated with vancomycin. Only one main peak of tritium radioactivity was found, with an R_f identical to that of the ^{14}C radioactivity peak, thus indicating that UDP-NAcMur-pentapeptide was the main cell wall precursor accumulated in the presence of gardimycin. The target of the antibiotic is, therefore, the peptidoglycan polymerization process.

Effect on in vitro synthesis of peptidoglycan. The effect of gardimycin on peptidoglycan biosynthesis was studied with a particulate enzyme preparation from *B. stearothermophilus*, capable of synthesizing cross-linked peptidoglycan (11). Gardimycin inhibits the incorporation of labeled MurNAc-pentapeptide into cross-linked peptidoglycan, while causing an accumulation of a phospholipid intermediate (Fig. 7). The same results were obtained when labeled UDP-GlcNAc was used, thus suggesting that the accumulated phospholipid intermediate contains the disaccharide unit GlcNAc-MurNAc-pentapeptide. The effect of gardimycin was compared, in the same experiment, with that of the known peptidoglycan synthesis inhibitors vancomycin and bacitracin. All three antibiotics inhibited precursor utilization. Although vancomycin caused a reduction of pepti-

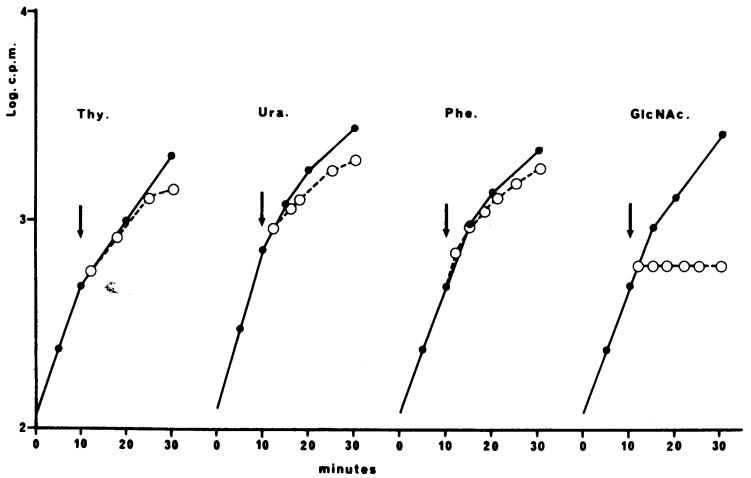

FIG. 3. *Effect of gardimycin on the incorporation of macromolecule precursors into whole cells of B. subtilis 556/1. Cells were grown in Davis minimal medium supplemented with 0.2% Casamino Acids (Difco), 0.5% glucose, and 10 μg of cold thymine per ml in the presence of either [^{14}C]thymine (0.025 μCi/ml), [^{14}C]phenylalanine (0.025 μCi/ml), [^{3}H]uracil (0.05 μCi/ml), or [^{3}H]GlcNAc (0.05 μCi/ml). At intervals, aliquots were transferred to cold 5% trichloroacetic acid, and the acid-insoluble material was collected on membrane filters (Millipore Corp.), washed with cold 2% trichloracetic acid, dried, and counted for radioactivity. The arrows indicate the time of antibiotic addition. Symbols: (●) Control; (○) gardimycin, 200 μg/ml.*

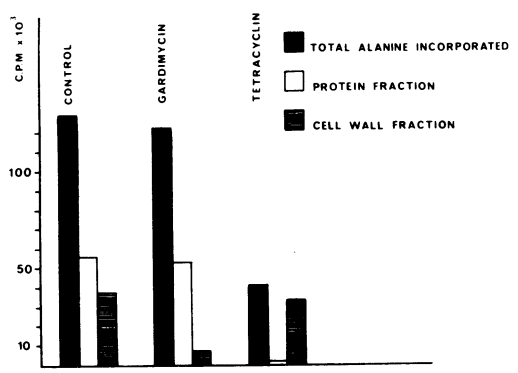

FIG. 4. *Effect of gardimycin and tetracycline on the distribution of [^{14}C]alanine in the cell wall and in the protein fraction of B. subtilis. Exponentially growing cells were incubated for 20 min in Davis minimal medium supplemented with 0.5% glucose, 50 μg of tryptophan per ml, 50 μg of histidine per ml and 0.8 μCi of [^{14}C]alanine per ml. Total radioactivity incorporated and the radioactivity incorporated into cell wall and protein fraction were determined as described in Materials and Methods. Three 10-ml cultures were run: the first was kept as a control, 500 μg of gardimycin per ml was added to the second, and 20 μg of tetracycline per ml was added to the third.*

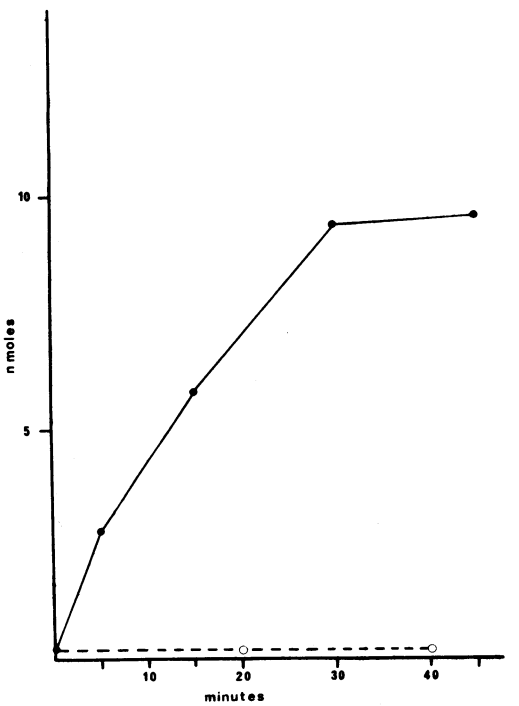

FIG. 5. *Time course of accumulation of cell wall precursors. The amount of N-acetylaminohexoses is expressed as nanomoles per milliliter of culture. Symbols: (○) Control; (●) gardimycin, 50 μg/ml.*

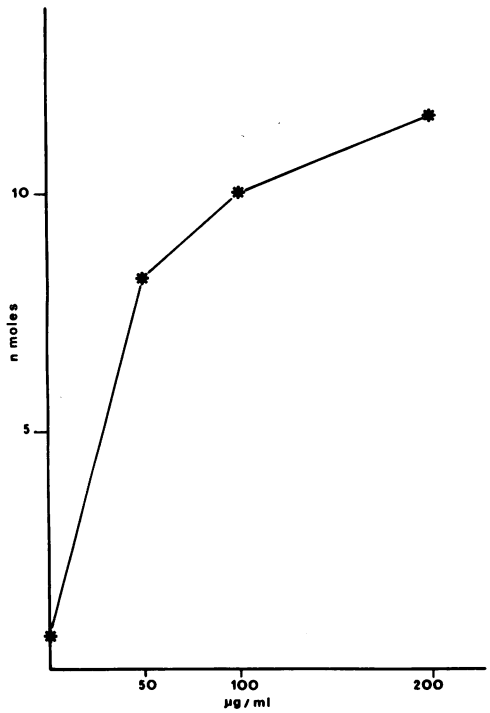

FIG. 6. *Effect of gardimycin concentration on cell wall precursor accumulation. On the ordinate are nanomoles of N-acetylaminohexoses per milliliter of culture, whereas the abscissa shows antibiotic concentrations. Incubation was for 30 min.*

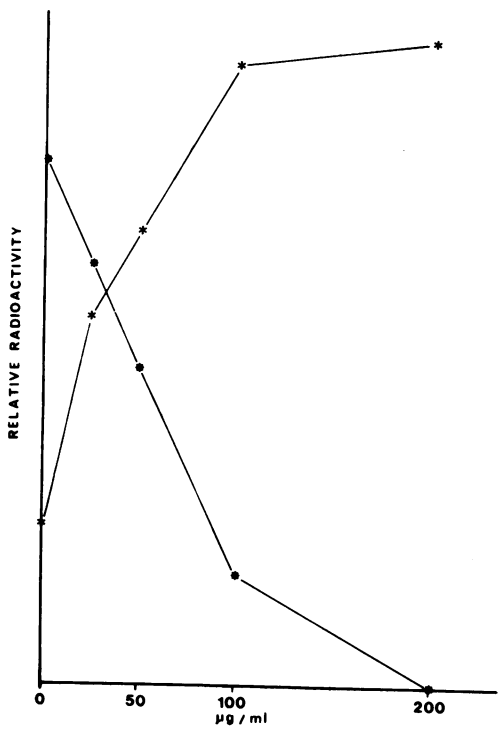

FIG. 7. *Effect of gardimycin on in vitro synthesis of peptidoglycan, catalyzed by a particulate enzyme preparation from B. stearothermophilus. The radioactivity incorporated into the peptidoglycan (●) and into the lipid intermediate (*) is plotted against gardimycin concentration.*

doglycan synthesis with a concomitant accumulation of phospholipid intermediate, bacitracin inhibited the formation of the latter, while allowing some synthesis of peptidoglycan (Table 1). These effects were to be expected from the known mechanism of action of the two antibiotics. Gardimycin behaved in this system exactly like vancomycin, suggesting that its target is the same or similar to that of vancomycin.

TABLE 1. *Comparison of effects of gardimycin, vancomycin, and bacitracin on in vitro synthesis of peptidoglycan*[a]

Antibiotic	% of radioactivity added		
	UDP-MurNAc-pentapeptide	Lipid intermediate	Peptidoglycan
None	0	29.0	71.0
Bacitracin	56.0	21.5	22.5
Vancomycin	51.5	48.5	0
Gardimycin	45.5	54.5	0

[a] Antibiotics were present at a concentration of 240 µg/ml.

DISCUSSION

The results reported in this paper clearly indicate that gardimycin inhibits growth of *B. subtilis* by inhibiting cell wall biosynthesis. This conclusion is supported by the following evidence. First, gardimycin inhibits the incorporation of GlcNAc, a specific precursor, into the cell wall; also, it inhibits incorporation of alanine into the cell wall but not into protein. Second, it induces accumulation of cell wall precursors in growing cells, suggesting that their further utilization is inhibited. Third, the drug has a bactericidal effect on growing cells, but not on resting cells, a phenomenon characteristic of, although not exclusive for, many cell wall inhibitors. As already mentioned, cell wall-lacking bacteria, such as *Mycoplasma* and L-forms of *S. aureus*, are unsusceptible to high concentrations of the drug.

The synthesis of cell wall peptidoglycan may be conveniently divided into four stages, namely: (i) the syntheis of uridino-diphospho-

N-acetylmuramic acid; (ii) the sequential attachment onto it of a pentapeptide side chain; (iii) the synthesis of a disaccharidic pentapeptide unit and its transfer to the growing polysaccharide chains; and (iv) their cross-linking.

Treatment with gardimycin elicits the accumulation of UDP-MurNAc-pentapeptide, indicating that the drug interferes with reactions at stage (iii) or stage (iv) of cell wall biosynthesis. Stage (iii) consists of several discrete reactions: the binding through a pyrophosphate linkage of UDP-MurNAc-pentapeptide to an isoprenyl phosphate carrier called C_{55}, which is associated with cell membrane. UDP-NAc is then attached to this intermediate, with formation of a disaccharidic pentapeptide unit, which is transferred to a cell wall acceptor, with release of C_{55}-pyrophosphate, which is then dephosphorylated and starts a new reaction cycle.

Gardimycin, added to a cell-free system that is able to carry out all these reactions, results in accumulation of disaccharide-C_{55} intermediate, thus indicating that it is active at the site of the further reaction, namely, its transfer to the cell wall acceptor. In fact, gardimycin behaves like vancomycin, a known inhibitor of such a transfer reaction (1, 14).

Ristocetin (1, 5), macarbomycin (14), diumycin (6, 7), enduracidin (6), meonomycin, prasinomycin, 11.837 RP (8), janemycin, and subtilin (6) have also been reported to interfere with this reaction. The mechanism of action of gardimycin differs from that of bacitracin (12), which prevents the dephosphorylation of C_{55}-pyrophosphate, from that of nisin (6), which does not induce accumulation of the phospholipid intermediate, and from that of the penicillins and cephalosporins, which interfere with transpeptidase and carboxypeptidase activity involved in the cross-linking (5).

LITERATURE CITED

1. **Anderson J. S., M. Matsuhashi, M. A. Naskin, and J. L. Strominger.** 1967. Biosynthesis of the peptidoglycan of bacterial cell wall. II. Phospholipid carrier in the reaction sequence. J. Biol. Chem. 242:3180–3190.
2. **Arioli, V., M. Berti, and L. G. Silvestri.** 1976. Gardimycin, a new antibiotic from Actinoplanes. III. Biological properties. J. Antibiot. 39:511–515.
3. **Coronelli, C., G. Tamoni, and G. C. Lancini.** 1976. Gardimycin, a new antibiotic from Actinoplanes. II. Isolation and preliminary characterization. J. Antibiot. 39:507–510.
4. **Garret, A. J.** 1969. The effect of magnesium ion deprivation on the synthesis of mucopeptide and its precursors in *Bacillus subtilis*. Biochem. J. 115:419–430.
5. **Izaki K., M. Matsuhashi, and J. L. Strominger.** 1968. Biosynthesis of peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions in strains of *Escherichia coli*. J. Biol. Chem. 243:3180–3192.
6. **Linnet, P. E., and J. L. Strominger.** 1973. Additional antibiotic inhibitors of peptidoglycan synthesis. Antimicrob. Agents Chemother. 4:231–236.
7. **Lugtenberg E. J. J., J. A. Hellings, and G. J. Van De Berg.** 1972. Inhibition of peptidoglycan synthesis by the antibiotic diumycin A. Antimicrob. Agents Chemother. 2:485–491.
8. **Lugtenberg E. J. J., A. van Schijndel-van Dam, and T. M. H. van Bellegem.** 1971. In vivo and in vitro action of new antibiotics interfering with the utilization of *N*-acetyl-glucosamine-*N*-acetyl-muramyl-pentapeptide. J. Bacteriol. 108:20–29.
9. **Parenti, F., H. Pagani, and G. Beretta.** 1976. Gardimycin, a new antibiotic from Actinoplanes. I. Description of the producer strain and fermentation studies. J. Antibiot. 39:501–506.
10. **Reissig, J. L., J. L. Strominger, and F. Leloir.** 1955. A modified colorimetric method for the extimation of N-acetyl-amino sugars. J. Biol. Chem. 217:959–966.
11. **Reynolds, P. E.** 1971. Peptidoglycan synthesis in Bacilli. I. Effect of temperature on the in vitro system from *Bacillus megaterium* and *Bacillus stearothermophilus*. Biochim. Biophys. Acta 237:239–254.
12. **Siewert, G., and J. L. Strominger.** 1967. Bacitracin, an inhibitor of the dephosphorylation of lipidpyrophosphate, an intermediate in biosynthesis of the peptidoglycan of bacterial cell walls. Proc. Natl. Acad. Sci. U.S.A. 57:767–773.
13. **Strominger, J. L., M. Matsuhashi, J. S. Anderson, C. P. Dietrich, G. M. Sievert, and J. M. Gilbert.** 1966. Glycopeptide synthesis in *Staphylococcus aureus* and *Micrococcus lysodeikticus*, p. 473–486. *In* E. N. Neufeld and V. Ginsbury (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
14. **Suzuki J., M. Hori, T. Saeki, and H. Umezawa.** 1972. Macarbomycin, an inhibitor of peptidoglycan synthesis. J. Antibiot. 25:94–104.