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Teicoplanin, a New Antibiotic from *Actinoplanes teichomyceticus* nov. sp.

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Teicoplanin, a new glycopeptide antibiotic belonging to the same family as vancomycin, inhibits cell wall synthesis in *Bacillus subtilis*; the inhibition is accompanied by an intracellular accumulation of UDP-*N*-acetylmuramyl-pentapetide. A cell-free system from *Bacillus stearothermophilus*, capable of synthesizing peptidoglycan, is 50% inhibited by teicoplanin at 40 μg/ml and 100% inhibited at 100 μg/ml; suppression of peptidoglycan synthesis is accompanied by parallel accumulation of the lipid intermediate. Teicoplanin binds to cell walls and forms a complex with *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine. The association constant of this complex is 2.56 × 10^6 liters mol^{-1}, calculated by spectrophotometric titration. The mechanism of action of teicoplanin is discussed in comparison with those of other inhibitors of cell wall biosynthesis, namely, vancomycin, ristocetin, and gardimycin.

Teicoplanin is a new antibiotic chemically related to the group of glycopeptides which also includes vancomycin and ristocetin. It was isolated from the fermentation broth of *Actinoplanes teichomyceticus* nov. sp. (1, 12) and is endowed with outstanding bactericidal activity against gram-positive pathogenic bacteria (11). The antibiotic is a mixture of five components of very similar polarity (the T-A2 complex, formerly named teichomycin A_2) and a more polar product (T-A3); the components of the T-A2 complex, accounting for 90 to 95% of the product, have the same molecular weight, ca. 1,900 (2).

The main features distinguishing teicoplanin from the other glycopeptide antibiotics are the occurrence of glucosamine as the basic sugar and the presence of aliphatic acid residues. Teicoplanin is not active against the L form of *Staphylococcus aureus* derived from a susceptible strain (12); this is a primary indication that the antibiotic interacts with the bacterial cell wall. We made further investigations on the mechanism of action of teicoplanin, and we present data showing that: (i) teicoplanin inhibits peptidoglycan synthesis in intact cells and in "cell-free" systems; (ii) the inhibition by teicoplanin is reverted by cell wall; (iii) the antibiotic binds to walls of whole cells, rendering them resistant to the action of lysozyme; and (iv) a complex occurs between teicoplanin and the peptide *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine (Ac_2-L-lys-D-ala-D-ala), a synthetic analog of the pentapeptide precursor in the peptidoglycan synthesis.

Part of this work was presented at the 12th International Congress of Chemotherapy, July 1981, Florence, Italy.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bacillus subtilis* SB25 (His^- Try^-) and PB556/1 (Thy^-), derived from strain W168, were grown in Difco Penassay medium (PY) or in Davis minimal medium (4) with the appropriate supplements in flasks at 37°C with reciprocating shaking. *Bacillus stearothermophilus* (*calidolactis*) ATCC 10149 was grown at 55°C in 4-liter tanks with forced aeration in a medium containing (wt/vol): 1% tryptone, 0.5% yeast extract, 0.25% K_2HPO_4, and 0.1% glucose. The pH was aseptically corrected to 7.2 after sterilization. *Micrococcus luteus* (*lysodeikticus*) ATCC 4698 was grown at 37°C on slants of Difco Penassay seed agar. *S. aureus* ATCC 6538P was grown according to the method of Strominger and Threnn (18) for obtaining peptidoglycan precursors.

Incorporation of radioactive macromolecule precursors. *B. subtilis* 556/1 was grown in Davis minimal medium supplemented with 0.2% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.5% (wt/vol) glucose, and uracil, thymidine, and *N*-acetylglucosamine (GlcNac), all at 10 μg/ml. As the culture reached the mid-exponential phase, it was diluted 1:10 in fresh medium also containing [^{14}C]thymidine (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).

Incubation continued for a further 30 min, and then the antibiotic was added to a part of the culture. At intervals portions were transferred to cold 5% trichloroacetic acid, filtered on glass fiber disks (Whatman GF/C), washed in succession with 2% trichloroacetic acid, ethanol, and diethyl ether, and then dried and counted for radioactivity in a scintillation chamber with Instagel (Packard Instrument Co., Inc., Rockville, Md.) as scintillation fluid.

Measurements and identification of accumulated cell wall precursors. Nucleotide cell wall precursors were extracted as described by Garrett (5) and quantitated by the method of Reissig et al. (14) for the estimation of *N*-acetylamino sugars. To identify the precursor accumulated in the presence of teicoplanin, the following procedure was employed: as a growing culture of *B. subtilis* SB25 in PY broth reached the mid-exponential phase, 0.1 μCi of [^{14}C]GlcNAc per ml was added to the medium and 1 min later 40 μg of antibiotic per ml was added. After incubating a further 30 min, the cells were harvested and washed with standard saline citrate solution, and the nucleotides were extracted. Identification was accomplished by paper chromatography with reference nucleotides. Chromatograms were developed with either solvent A or B (see below). Reference nucleotides were localized under UV light, and the radioactivity was detected by a Packard chromatography scanner.

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Isolation of reference cell wall precursor. UDP-*N*-acetylmuramyl-L-ala (UDP-MurNAc-L-Ala) and UDP-MurNAc-L-Ala-D-Glu were accumulated in lysine-deprived *S. aureus* (18). UDP-MurNAc-L-Lys-D-Glu-meso-A_2pm (UDP-MurNAc-tripeptide) and UDP-MurNAc-L-lys-D-glu-meso-A_2pm-D-ala-D-ala (UDP-MurNAc-pentapeptide) were obtained from growing cells of *B. subtilis* in PY medium treated for 30 min with D-cycloserine (200 μg/ml) and vancomycin (40 μg/ml), respectively. Accumulated nucleotides were extracted and applied to a Dowex 1 column (Cl^- form, 100-200 mesh) and eluted with linear NaCl gradient from 0.05 to 0.5 M in 0.01 N HCl; fractions positive for absorbance at 260 nm (A_{260}) and for *N*-acetylamino sugars were pooled, lyophilized, dissolved in 1 mM Tris-hydrochloride (pH 7.2), applied to a Sephadex G-25 column, and eluted with the same buffer. Again, positive fractions were pooled and lyophilized. The purity of the compounds was checked by paper chromatography with solvent systems A and B.

Paper chromatography. Descending paper chromatography was performed on Whatman no. 1 paper strips (6 by 57 cm) for 24 h with the following solvents: solvent A, isobutyric acid–1 M NH_4OH (5:3); and solvent B, 95% ethanol–1 M ammonium acetate (pH 7) (7.5:3).

Cell-free synthesis of peptidoglycan. The preparation of particulate enzyme from *B. stearothermophilus* and the peptidoglycan synthesis assay were done as described previously (17).

Preparation of cell walls and membrane from *B. stearothermophilus*. Cells of *B. stearothermophilus* were harvested in the middle of the exponential growth, washed three times with standard saline citrate solution at 4°C, and frozen in liquid nitrogen; for the wall preparation, the frozen cell paste was ground in a blender with three times the wet weight of Superbrite (3 M Corp., Minn.), under ice-water cooling. After Superbrite was removed through a sintered glass funnel, the sediment between 6,000 × *g* (10 min) and 24,000 × *g* (20 min) was collected, washed twice with distilled water, suspended in 0.05 M potassium phosphate buffer (pH 6.8), and treated with 1 mg of pronase (Boehringer, Mannheim, Federal Republic of Germany) per ml at 37°C. The walls were centrifuged again at 24,000 × *g* (20 min), washed in succession with distilled water, 95% ethanol, absolute ethanol, and diethyl ether, and finally dried in vacuo.

For the membrane preparation, the cell paste was slowly thawed and gently resuspended in a buffer containing 50 mM Tris-hydrochloride (pH 7.8), 20 mM $MgCl_2$, and 22% (wt/vol) sucrose. Lysozyme (Sigma Chemical Co., St. Louis, Mo.) at 0.4 mg/ml was added, and the suspension was incubated at 25°C until complete conversion into protoplast was observed by phase-contrast microscope. The incubation continued for a further 10 min, and then the protoplasts were collected by centrifugation at 3,000 × *g* (20 min). The pellet was gently resuspended in the above buffer, but without sucrose, to rupture the protoplasts.

DNase I (Worthington Diagnostics, Freehold, N.J.) and pancreatic RNase (Sigma) were added at a final concentration of 1 mg/ml, the suspension was incubated for 10 min at 25°C and centrifuged at 3,000 × *g* (10 min), and the pellet was discarded. The supernatant was then centrifuged for 30 min at 38,000 × *g*, and the membrane pellet was washed once, resuspended in buffer at a concentration of ca. 200 mg (dry wt) per ml, and stored in liquid nitrogen.

Spectrophotometric measurements. Absorbance measurements of teicoplanin and of the complex between teicoplanin and Ac_2-L-Lys-D-Ala-D-Ala were made with a Beckman DB GT double-beam spectrophotometer in 4-cm O.P. cells. The compounds were dissolved in 0.05 M potassium phosphate buffer (pH 7.0). The cells contained 10 ml of solution, and the experiments were carried out at room temperature (25 to 27°C).

Radiochemicals. UDP-[^{14}C]MurNAc-pentapeptide was prepared as described previously (17), except that the eluate from Dowex was subjected to gel filtration through a Sephadex G-25 column, as in the preparation of unlabeled nucleotide precursors. [5-^{3}H]uracil (24.2 Ci/mmol), [^{14}C]phenylalanine (225 mCi/mmol), [2-^{14}C]thymidine (61 mCi/mmol), [1-^{14}C]GlcNAc (548 mCi/mmol), and [1-^{3}H]GlcNAc (4 Ci/mmol) were all obtained from the Radiochemical Centre (Amersham, England).

Other chemicals. Vancomycin and ristocetin were obtained from Sigma, UDP-GlcNAc from Boehringer, and Ac_2-L-Lys-D-Ala-D-Ala from SERVA (Heidelberg, Federal Republic of Germany).

Teicoplanin samples. The teicoplanin samples used in the experiments with whole cells and with cell-free extracts contained 5 to 10% of the T-A3 fraction; in the spectrophotometric studies of the binding of teicoplanin to peptides, a sample of antibiotic free of the T-A3 fraction was used.

RESULTS

Effect on cellular macromolecule synthesis. The action of teicoplanin on the macromolecular metabolism is characterized by the specific inhibition of the synthesis of cell wall polymers. The incorporation of *N*-acetylglucosamine into acid-insoluble material stopped immediately and almost completely after the addition of 10 μg of antibiotic per ml to a growing culture of *B. subtilis* (the antibiotic concentration was 20 times the MIC. (Fig. 1). In contrast, the syntheses of DNA, RNA, and protein, measured as uptake of thymidine, uracil, and phenylalanine, respectively, were practically unaffected for the first 10 min after exposure to teicoplanin.

Accumulation of cell wall precursors. Impairment of cell wall synthesis is usually accompanied with an intracellular accumulation of peptidoglycan precursors in the form of uridine nucleotide. Actually, the addition of teicoplanin to a growing culture of *B. subtilis* resulted in an evident accumulation of these precursors; the time course of the accumulation with different doses of antibiotic is reported in Fig. 2.

The specific composition of the accumulated precursors depends on the step at which inhibition of cell wall synthesis occurs: when radioactively labeled *N*-acetylamino sugar nucleotides were extracted from teicoplanin-treated cells and cochromatographed with authentic samples of peptidoglycan precursors, a main peak of radioactivity was found with an R_f corresponding to that of UDP-MurNAc-pentapeptide in either the A or B solvent systems. This result indicates that the antibiotic does not inhibit the synthesis of the soluble precursors but interferes with a further step in the process of peptidoglycan formation.

Effect on cell-free synthesis of peptidoglycan. To obtain more detailed information about the specific target of teicoplanin, we tested the antibiotic on a cell-free system capable of synthesizing peptidoglycan from the precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide through the formation of a lipid intermediate: a preparation from *B. stearothermophilus* was used, containing both cell wall and membrane fragments (15).

The addition of teicoplanin to the cell-free system blocked the synthesis of peptidoglycan and at the same time stimulated the formation of the phospolipid intermediate (Fig. 3). Antibiotic at a concentration of 40 μg/ml, corresponding to 5

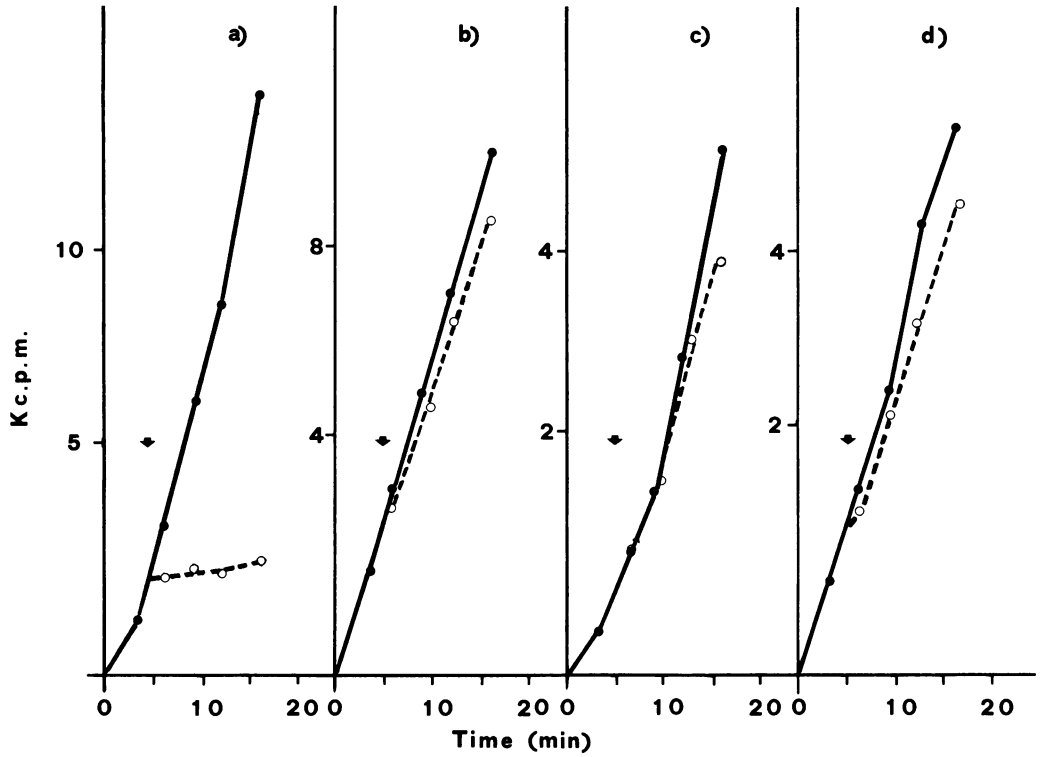
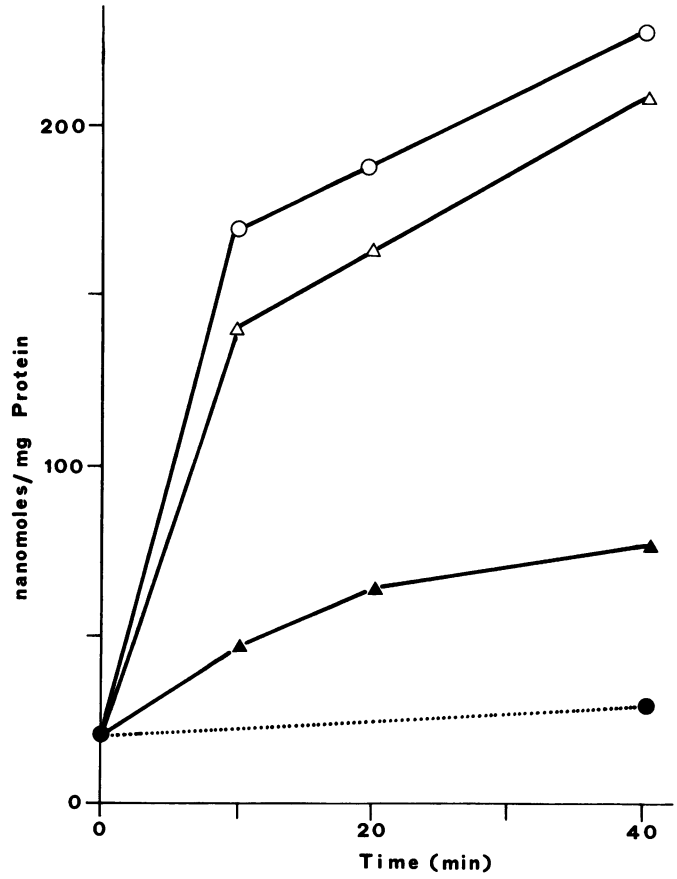


FIG. 1. Effect of teicoplanin on the incorporation of macromolecule precursors into growing cells of *B. subtilis* PB556/1. Uptake of radioactivity was determined as described in the text. The arrows indicate the time of antibiotic addition (final concentration, 10 μg/ml). (a) [^{3}H]GlcNAc; (b) [^{3}H]uracil; (c) [^{14}C]phenylalanine; (d) [^{14}C]thymidine. Symbols: ●, no antibiotic; ○, teicoplanin.



μg of teicoplanin per mg of enzyme protein, inhibited the synthesis of peptidoglycan by 50%, and at 100 μg/ml the inhibition was complete. At the highest antibiotic concentration the amount of phospholipid intermediate rose 1.8 times.

Reversal of peptidoglycan inhibition by cell wall. In the following experiments we tested the influence of cell wall or membrane components of the cell-free system on the inhibition of peptidoglycan synthesis by teicoplanin; for this purpose pure membrane and cell wall fragments were prepared and added separately to the reaction mixture. Figure 4 shows the results of these experiments: increasing the cell wall content of the cell-free system resulted in a complete reversal of the inhibition of peptidoglycan synthesis by teicoplanin, whereas increasing the membrane concentration had only a limited effect on the system. However, when the peptidoglycan synthesis was prevented by the antibiotic gardimycin, the addition of wall fragments to the cell-free system did not have any effect, whereas the addition of membranes completely reversed the inhibition.

Prevention of bacteriolytic activity of lysozyme. Since the above experiments indicated that teicoplanin interacted in some way with cell walls, at least in a cell-free system, we determined the capability of the antibiotic to bind to the walls of intact cells. As an indication of a functional binding, we assessed the inhibition of the bacteriolytic activity of lysozyme. Therefore, samples from a suspension of *M.*

FIG. 2. Intracellular accumulation of peptidoglycan precursors induced by teicoplanin. The amount of precursor accumulated is expressed as nanomoles of GlcNAc per milligram of total cell protein. Symbols: ●, no antibiotic; ▲, teicoplanin at 1 μg/ml; △, teicoplanin at 5 μg/ml; ○, teicoplanin at 25 μg/ml.

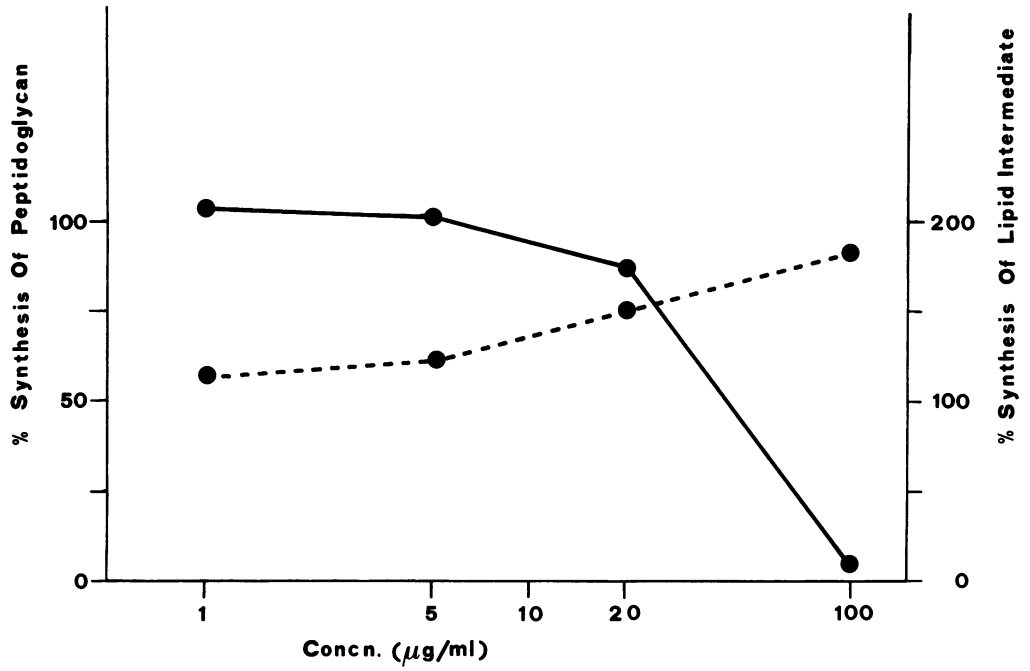


FIG. 3. Effect of teicoplanin on the cell-free synthesis of peptidoglycan catalyzed by a particulate enzyme system from *B. stearothermophilus*. Incubation was done for 40 min at 30°C. In the absence of antibiotic, [^{14}C]MurNAc-pentapeptide was incorporated into the peptidoglycan at a rate of 90 nmol/min per mg of enzyme protein. The concentrations of teicoplanin are given in the abscissa. Symbols: ●——●, peptidoglycan; ●– –●, lipid intermediate.

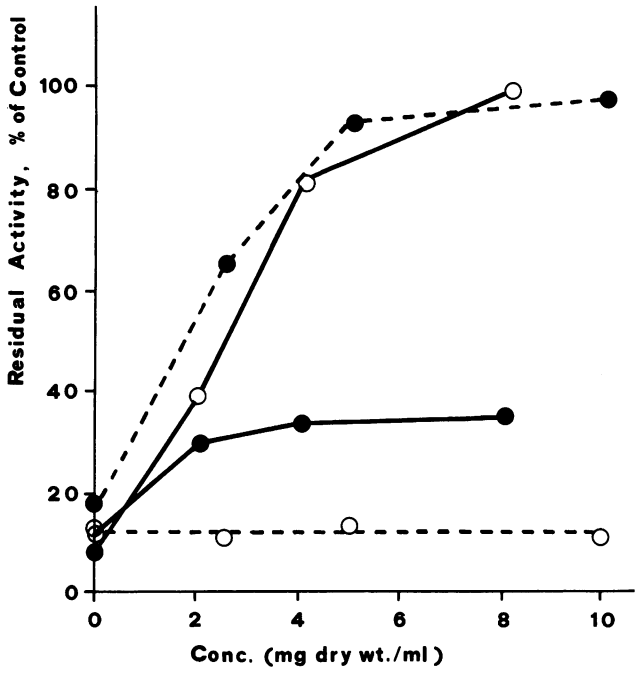


FIG. 4. Influence of the concentration of cell wall or membrane on the cell-free synthesis of peptidoglycan in the presence of either teicoplanin or gardimycin. Membrane and cell wall fragments were prepared as described in the text and added separately to the assay tubes. The concentrations of either the membrane or cell wall are reported in the abscissa; the amounts of peptidoglycan synthesized in the presence of antibiotic are reported in the ordinate, expressed as a percentage of the amounts synthesized in the absence of antibiotic. Teicoplanin was present at 80 μg/ml, and gardimycin was present at 100 μg/ml. Symbols: ●——●, teicoplanin plus membrane; ○——○, teicoplanin plus cell wall; ●– –●, gardimycin plus membrane; ○– –○, gardimycin plus cell wall.

luteus were incubated with teicoplanin, or vancomycin, or ristocetin, which are known to bind strongly to bacterial walls.

After the unbound antibiotic was washed out, the suspensions were exposed to 1 mg of lysozyme per ml, and the cell lysis was followed turbidimetrically; a cell suspension not pretreated with antibiotic was run as a control. Antibiotic-treated cells appeared to be more resistant to bacteriolysis than the nontreated ones (Fig. 5).

Interaction of teicoplanin with Ac_2-L-Lys-D-Ala-D-Ala. Perkins (13) first observed that the association between vancomycin and some peptidoglycan precursors or other suitable peptides resulted in a shift of the UV spectrum of the antibiotic; because of this phenomenon it was possible to utilize a relatively simple and reliable method based on the difference spectra to determine the parameters of the complex formation. In this work the interaction between teicoplanin and the synthetic peptide Ac_2-L-lys-D-ala-D-ala was studied by difference spectroscopy. Figure 6 shows a difference spectrum induced on teicoplanin by the peptide; a relative minimum at 283 nm and a sharp decrease at wavelengths shorter than 272 nm are relevant.

The extent of the differential absorbance (ΔA) at a suitable wavelength can be used as a measure of the association between the antibiotic and the peptide. In fact, to improve the accuracy of the determinations the difference ($\Delta A_{250} - \Delta A_{283}$) between ΔA_{250} and ΔA_{283} was employed. In Fig. 7 the results of the spectrophotometric titration of teicoplanin with the tripeptide are reported. The increase of the differential absorbance was linear with respect to the peptide concentration at a low peptide-antibiotic ratio; when this

ratio exceeded 1, a plateau was rapidly reached, suggesting that a one-to-one complex occurred. From these data a Scatchard plot (8) was drawn (Fig. 7b), and a association constant was calculated; the value obtained was 3.56×10^6 liters mol^{-1}.

DISCUSSION

The antibiotic teicoplanin specifically inhibits the cell wall biosynthesis in susceptible organisms: bacteria treated with the drug failed to incorporate GlcNAc, a peptidoglycan precursor, whereas they continued to synthesize DNA, RNA, and protein. The cell wall inhibition was accompanied by an accumulation of UDP-MurNAc-pentapeptide, thus indicating that the antibiotic interferes with the polymerization of the peptidoglycan but not with the synthesis of soluble precursors. Experiments with a cell-free system confirmed this observation: synthesis of peptidoglycan was completely abolished in the presence of 100 μg of teicoplanin per ml. At the same antibiotic concentration the formation of the disaccharide-pentapeptide-lipid intermediate was unaffected, since an accumulation of the compound was observed.

Teicoplanin firmly binds to bacterial walls, as revealed by the fact that the inhibition of peptidoglycan cell-free synthesis was completely reversed by the addition of appropriate amounts of the cell wall fragments in the assay mixture; a similar effect has been reported by Sinha and Neuhaus (16) for vancomycin.

However, the activity of the antibiotic gardimycin, an inhibitor of peptidoglycan polymerization (17), was indifferent to cell wall concentration and was reversed by the addition of membranes. Thus, it appears to have a mode of action different from that of teicoplanin or vancomycin. As a consequence of teicoplanin binding to cell wall, cultures of *M. luteus* pretreated with the drug became more resistant to bacteriolysis by lysozyme, the same effect was observed with vancomycin and ristocetin. Vancomycin is already known to protect other bacterial species, such as *S. aureus*, *Bacillus megatherium*, and *B. stearothermophilus*, from the action of muralytic enzymes (6).

The relationship between the ability to bind to cell wall and the bactericidal effect of these antibiotics is, however, still unclear. Chatterjee and Perkins (3) first demonstrated the formation of a complex between vancomycin and peptidoglycan precursors, namely, those terminating in D-alanyl-D-alanine. Then vancomycin and ristocetin were found to combine more generally with peptides having the two last amino acids of the carboxy terminus in the D-configuration (9, 10). Teicoplanin shows a high affinity for the peptide Ac_2-L-Lys-D-Ala-D-Ala ($K_A = 2.56 \times 10^6$ liters mol^{-1}); this value is in the same order of magnitude of those reported for the vancomycin complex ($K_A = 1.5 \times 10^6$ liters mol^{-1}) (9) and the ristocetin complex ($K_A = 5.9 \times 10^5$ liters mol^{-1}) (10) with the same peptide.

Although the specificity of the binding with peptides is not yet known, all the data presented here enable us to presume that the mechanism of action of teicoplanin is essentially similar to that of vancomycin and ristocetin. This mechanism is almost certainly related to the binding of the antibi-

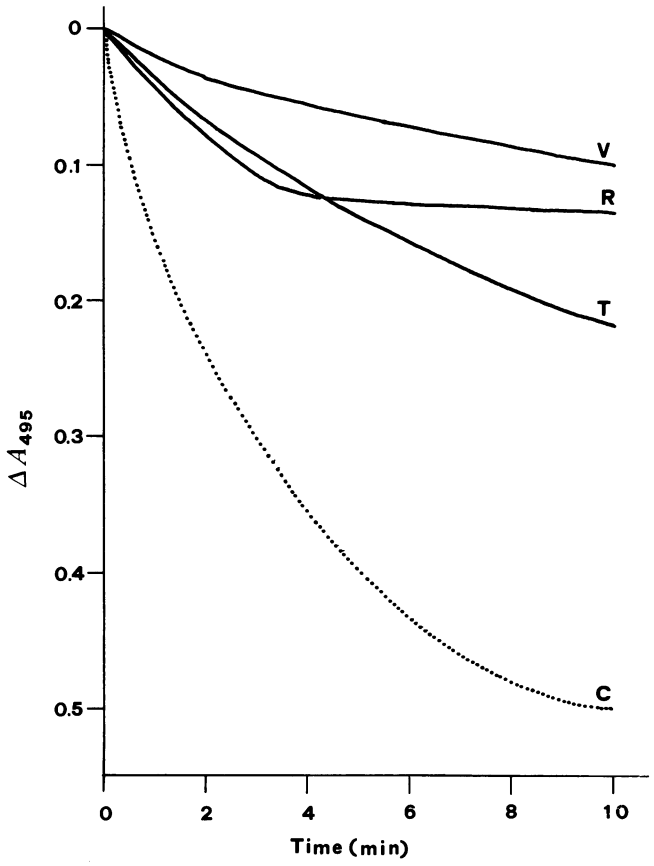


FIG. 5. Effect of preincubation with antibiotics on the lysis of *M. luteus* caused by lysozyme. An overnight culture was suspended in 0.05 M potassium phosphate buffer (pH 6.0), washed by centrifugation, and resuspended in the same buffer at an optical density of 0.6 at 495 nm. The suspension was distributed in four tubes. Teicoplanin, vancomycin, and ristocetin, respectively, were added at 200 μg/ml to three of them; the fourth tube was kept as a control. After 10 min at 4°C, the suspensions were centrifuged for 10 min at 6,000 × *g*, washed once, and resuspended in the above buffer at room temperature. The content of each tube was distributed in two spectrophotometer cells; lysozyme at a final concentration of 1 mg/ml was added to the sample cell, and the decrease in optical density at 495 nm was recorded. T, Teicoplanin; R, ristocetin; V, vancomycin; C, control.

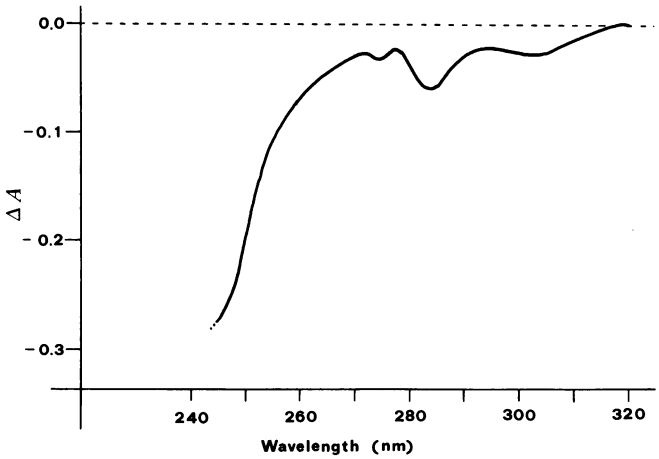


FIG. 6. UV difference spectrum induced on teicoplanin by Ac_2-L-Lys-D-Ala-D-Ala. The reference cell contained 80 μM teicoplanin; the sample cell contained 80 μM teicoplanin plus the tripeptide at 160 μM. At this concentration the peptide did not absorb significantly at wavelengths above 240 nm.

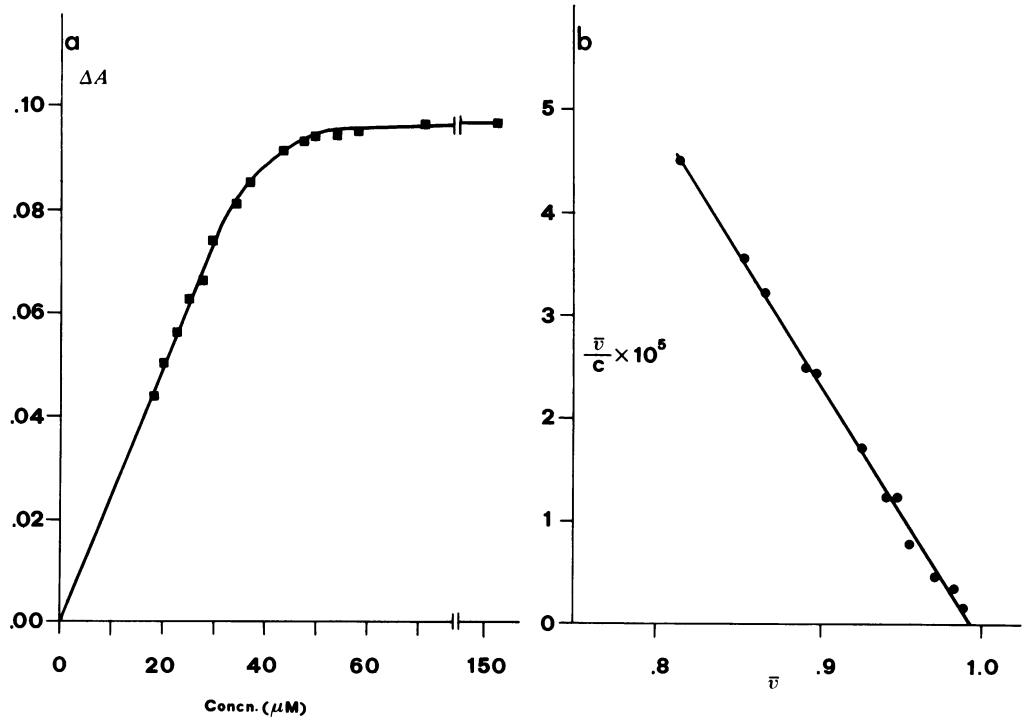


FIG. 7. (a) Spectrophotometeric titration of teicoplanin with Ac_2-L-Lys-D-Ala-D-Ala. The reference cell contained a 40 μM antibiotic solution; the sample cell contained the antibiotic at the same concentration and the peptide at different concentrations. The A_{250} and A_{283} values were recorded at every peptide concentration, and the difference between the absorbances at the two wavelength ($\Delta A_{250} - \Delta A_{283}$) was plotted against the peptide concentration. Absorbance values are reported to 1 cm O.P. (b) Scatchard plot of the titration results of (a). $\bar{v}$ is the spectral difference ($\Delta A_{250} - \Delta A_{283}$) at every point divided by the value of the difference ($\Delta A_{250} - \Delta A_{283}$) at the saturating peptide concentration; c is the molar concentration of the free peptide. The curve is a mean-squares fit with a correlation coefficient of 0.9964.

otic with the D-Ala-D-Ala termini of peptidoglycan or peptidoglycan precursors, but the exact step involved is still conversial.

The transfer of disaccharide-pentapeptide units from the lipid intermediate to a nascent peptidoglycan chain will be the most obvious target if the reaction sites are accessible to the drug. The inhibition of transpeptidase and carboxypeptidase reactions involving D-Ala-D-Ala termini and taking place outside of the cell membrane could also account for the bactericidal activity of these antibiotics, as suggested by Leyh-Bouille et al. (7) and Jordan and Reynolds (6).

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