Inhibition of Peptidoglycan Synthesis by the Antibiotic Diumycin A

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Diumycin A, a new antibiotic, was found to inhibit cell wall synthesis by Staphylococcus aureus, a phenomenon accompanied by accumulation of uridine-5'-diphosphate-N-acetyl-muramyl-pentapeptide. The antibiotic inhibited in vitro peptidoglycan synthesis by particulate preparations of Bacillus steaerothermophilus and Escherichia coli by preventing the utilization of N-acetyl-glucosamine-N-acetyl-muramyl-pentapeptide. In contrast to vancomycin, the antibiotics diumycin, prasinomycin, moenomycin, 11.837 RP, and enduracidin do not inhibit particulate D-alanine carboxypeptidase.

The biosynthesis of peptidoglycan, a rigid network consisting of amino sugars and amino acids (27), is unique to bacteria, and is, therefore, an ideal target for the action of antibiotics. Phosphoromycin (5) and D-cycloserine (11, 17) inhibit the biosynthesis of uridine diphosphate-N-acetyl-muramyl(UDP-MurNAc)-pentapeptide. A number of antibiotics cause accumulation of UDP-MurNAc-pentapeptide in vivo (15, 21). The development of cell-free systems for peptidoglycan synthesis (1, 2, 22) facilitated studies on the mechanism of action of these antibiotics. Penicillin (12, 26, 29), bacitracin (23, 24), vancomycin, ristocetin (2, 7), enduracidin, moenomycin, prasinomycin, and 11.837 RP (15) all inhibit one of the envelope-bound steps in peptidoglycan synthesis. Although penicillin and vancomycin inhibit peptidoglycan synthesis in a different way, both antibiotics inhibit D-alanine carboxypeptidase I (8, 9), an enzyme that liberates the ultimate D-alanine residue from UDP-MurNAc-pentapeptide in vitro (8).

We recently received a sample of diumycin A, a new antibiotic isolated from Streptomyces umbrinus (16). Like the other phosphorus-containing antibiotics prasinomycin (28), moenomycin (6), and 11.837 RP (D. Mancy et al., Int. Congr. Microbiol., 9th, Moscow, p. 165, 1966), it is especially active against gram-positive bacteria, yeasts, and Mycobacterium bovis, whereas members of the family Enterobacteriaceae are relatively resistant (16). The molecular weight of diumycin in 90% ethanol is 1,600 and in phosphate buffer is 3,200. Similar data have been given for vancomycin (4, 19). Like vancomycin, it adsorbs ultraviolet light (16). Diumycin A differs from prasinomycin and moenomycin. It contains two residues of glucosamine, whereas each of the latter two antibiotics yield one equivalent of both glucosamine and 6-deoxyglucosamine under the same hydrolytic conditions (16).

The present paper describes a study in which the influences of diumycin A and vancomycin on peptidoglycan synthesis were compared.

MATERIALS AND METHODS

Bacterial strains. Bacillus steaerothermophilus NCTC 10339 and Staphylococcus aureus 524/SC were obtained from P. E. Reynolds, Department of Biochemistry, University of Cambridge, Cambridge, England. B. cereus strain T and Escherichia coli K-12 strain KMBL-146 were obtained from K. Izaki, Department of Agriculture, Tokohu University, Sendai, Japan, and A. Rörsch, Medical Biological Laboratory, Rijswijk, Z.H., The Netherlands, respectively.

Growth of bacteria. Bacteria were grown with aeration in the complex CGPY medium, containing 0.5% glucose (12). They were incubated at 37 C, with the exception of B. steaerothermophilus, which was grown at 55 C. Optical density was measured with a Unicam SP-600 spectrophotometer at a wavelength of 660 nm. Exponentially growing cells were used for all experiments.

Buffers. The buffers used had the following compositions (per liter): (A) $5 \times 10^{-3}$ M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride and $10^{-4}$ M MgCl$_2$ (pH 7.8); (B) 1.5 M Tris-hydrochloride and 0.3 M MgCl$_2$ (pH 7.8); (C) 0.5 M Tris-hydrochloride, $10^{-4}$ M MgCl$_2$, and $10^{-3}$ M 2-mercaptoethanol (pH 7.5); and (D) 1 M Tris-hydrochloride and 0.2 M MgCl$_2$ (pH 7.5).
Peptidoglycan synthesis in vivo. Peptidoglycan synthesis in vivo was followed in a modification of the wall medium CWSM-1 (12) containing 10 μg of l-alanine per ml instead of 5 μg per ml. The medium was supplemented with glycine (100 μg/ml) when S. aureus was used. If necessary, antibiotics were added at zero time. For incorporation studies, a sample of the suspension was added to uniformly labeled 14C-L-alanine (2 μCi/ml). The unlabeled suspension was incubated under identical conditions and used to follow the optical density. Incorporation of label was determined (i) by counting the acid-precipitable activity or (ii) by chromatography of heat-inactivated samples as described previously (12, 15). The possibility that radioactive teichoic acid, in addition to peptidoglycan, was counted as acid-precipitable radioactivity or as a nonmoving component on the chromatogram was discussed previously (15). In a number of experiments, the suspension was centrifuged to separate the cells from the medium.

Accumulation of uridine nucleotides. S. aureus cells were grown and transferred to the cell wall medium as described for following the peptidoglycan synthesis. The suspension (0.24 mg, dry weight, per ml) was incubated for 1 hr at 37 C. The cells were harvested, washed, and disrupted by heat treatment. Macromolecular material was precipitated with tri-chloroacetic acid. The supernatant fluid was extracted with ether, neutralized, and decreased in volume under reduced pressure. The amount of N-acetyl-hexosamine was determined by the method of Reissig et al. (20) as modified by Strominger (25).

Details of this procedure have been described previously (15).

Isolation of particulate enzyme. B. stearothermophilus particles were isolated as described previously (15). Small and large envelope fragments were not separated. The preparation was stored at -20 C in buffer A. E. coli particles were isolated in the same way except for the following modifications: (i) the cells were disintegrated for 10 min; and (ii) the particles that were used to assay d-alanine carboxypeptidase I were suspended in buffer A, whereas particles that were used in the complete system were suspended in buffer C.

Cell-free peptidoglycan synthesis. The incubation mixtures for B. stearothermophilus contained: 5 μlirers of UDP-N-acetyl-glucosamine-14C uniformly labeled (specific activity, 270 μCi/mumole); 14C-D-alanine, uniformly labeled (specific activity, 40.9 μCi/mumole); and 14C-L-alanine, uniformly labeled (specific activity, 156 μCi/mumole), were obtained from the Radiochemical Center, Amersham, England. UDP-MurNAc-L-ala-D-glu-meso-diaminopimelic-acid-d-alal(14C)-d-alal(4C) (specific activity, 81.8 μCi/μmole), 5 μlirers of buffer B, 5 μlirers of antibiotic solution or distilled water; and 5 μlirers of particulate enzyme. The different components were mixed in the cold and incubated in a water bath at 37 C. Cell-free peptidoglycan synthesis with E. coli enzyme was performed as described above for B. stearothermophilus except that buffer D was used instead of buffer B, and the mixtures were incubated at 30 C. After incubation, the samples were heat-inactivated for 30 sec at 100 C. After chromatography and autoradiography (usually for 1 week), the radioactive spots were excised and counted (15).

Assay of d-alanine carboxypeptidase I. The incubation mixtures contained: 5 μlirers (28,000 counts/min) of UDP-MurNAc-L-ala-D-glu-meso-diaminopimelic-acid-d-alal(14C)-d-alal(4C) (specific activity, 81.8 μCi/μmole), 5 μlirers of buffer B, 5 μlirers of antibiotic solution or distilled water, and 10 μlirers of E. coli particulate enzyme in buffer A, usually containing 10 mg of protein/ml. Incubation was carried out as described for cell-free peptidoglycan synthesis. The radioactivity in the alanine spot was taken as a measure for d-alanine carboxypeptidase activity. In addition to d-alanine carboxypeptidase I, the preparation also contained an active d-alanine carboxypeptidase II (8), as indicated by the nearly complete absence of UDP-MurNAc-tetrapeptide after incubation. This was shown after isolation of the charcoal-adsorbable material from the supernatant fluid of centrifuged inactivated incubation mixtures. The UDP was split off by mild hydrolysis in 0.05 N HCl for 15 min at 100 C (8). MurNAc-tetrapeptide was separated from MurNAc-pentapeptide in a solvent system composed of phenol-water (4:1) containing 0.04% 8-hydroxyquinoline (3).

Other methods. Radioactive uridine nucleotide precursors were isolated by charcoal adsorption (13). Reference precursors were accumulated (11, 13, 14) and purified (15) as previously described. Methods for the separation and identification of radioactive precursors have been published in a previous paper from this laboratory (13). Protein was determined by the method of Lowry et al. (10).

Chemicals. Diumycin A (ammonium salt) was a gift from F. L. Weisenborn, The Squibb Institute for Medical Research, New Brunswick, N.J. UDP-N-acetyl-glucosamine and vancomycin were obtained from Boehringer Mannheim NV, Amsterdam, The Netherlands, and Eli Lilly & Co., Indianapolis, Ind., respectively. The origin of the other antibiotics has been given in a previous paper (15).

Radiochemicals. UDP-N-acetyl-glucosamine-14C uniformly labeled (specific activity, 270 μCi/mumole); 14C-D-alanine, uniformly labeled (specific activity, 40.9 μCi/mumole); and 14C-L-alanine, uniformly labeled (specific activity, 156 μCi/mumole), were obtained from the Radiochemical Center, Amersham, England. UDP-MurNAc-L-ala-D-glu-meso-diaminopimelic-acid-d-alal(14C)-d-alal(4C) (specific activity, 81.8 μCi/mumole) was prepared by addition of d-alanyl(14C)-d-alanine(4C) to UDP-MurNAc-tripeptide with the help of crude enzyme of E. coli strain KMBL-146 as described for the assay of d-alanyl-d-alanine adding enzyme (11), except that excess of enzyme was used. The product was purified by preparative paper chromatography with the solvents isobutyric acid-I M ammonium (5:3, v/v) and ethanol-I M ammonium acetate, pH 7.2 (5:2, v/v). d-alanyl(14C)-d-alanine(4C) was prepared from uniformly labeled 14C-d-alanine as described previously (13).

RESULTS

Influence of diumycin on growth. Diumycin at a concentration of 0.1 μg per ml stopped the increase of the optical density of a culture of S. aureus after some time without causing significant lysis of the cells (Fig. 1). With increased anti-
Biotic concentrations (1 to 100 μg/ml), the rate of initial increase in optical density was lower. Vancomycin, tested at the same concentrations, gave similar results. The influence of diumycin on the growth of *B. cereus* was different. An antibiotic concentration of 0.1 μg per ml decreased the growth rate after 30 min, as a result of lysis of part of the cells; resumption of growth followed, probably caused by growth of the cells that escaped the lysis. Gram-stained preparations showed that these cells were swollen, suggesting that the envelope was impaired by the presence of diumycin. Diumycin concentrations between 1 and 100 μg per ml gave typical lysis curves. Vancomycin also caused lysis of *B. cereus*. It is concluded that the influence of diumycin on the growth of *S. aureus* and *B. cereus* is similar to that of vancomycin, a known cell wall antibiotic.

**Cell wall synthesis in vivo.** These experiments were carried out as described previously. The influence of the presence of diumycin and vancomycin on the incorporation of uniformly labeled 14C-L-alanine from a wall medium into acid-precipitable material of *S. aureus* was measured. Only the results for diumycin are given in Fig. 2. The incorporation was slightly inhibited by diumycin and vancomycin at concentrations of 0.1 and 1.0 μg per ml, respectively. Tenfold higher concentrations of both antibiotics decreased the rate of incorporation dramatically. The optical density remained constant during the course of the experiment.

The influence of diumycin and vancomycin on the synthesis of alanine-containing components was studied under the same conditions. After incubation, samples were cooled and centrifuged. The first supernatant fluid and the resuspended pellet were heat-inactivated and chromatographed. Autoradiography showed that the supernatant fluid contained the majority of alanine ($R_F$, 0.65), a weak spot of alanyl-alanine ($R_F$, 0.79), and two weak spots ($R_F$, 0.42 and 0.50), among which the fast-moving one probably represents pyruvate (11, 13). The pellet contained macromolecular wall material ($R_F$, 0.0), precursors ($R_F$, 0.2), and a small fraction of alanine. At $R_F$ 0.9, the $R_F$ value of lipid intermediates (7), some radioactivity was present, but it is not certain whether this activity represents the lipid intermediates (11). The radioactivities of the main components after 60 min of incubation are given in Table 1. Inhibition of cell wall synthesis by each of the two antibiotics was accompanied by a dramatic accumulation of uridine nucleotide precursors, to amounts that were roughly 50-fold higher than that found in the control. Analysis of the precursors (13) showed that all detectable radioactivity was present in UDP-MurNAc-pentapeptide.

Accumulation of uridine nucleotide precursors was also shown by assay of the hexosamine content. Samples (100 ml) of *S. aureus* in a wall medium without diumycin A and supplemented with diumycin A in final concentrations of 0.1, 1.0, and 10.0 μg/ml contained 0.08, 1.00, 1.12, and 1.12 μmoles of uridine nucleotide precursors, respectively.

Although diumycin caused lysis of growing *B. cereus* cells (Fig. 1) and inhibited cell wall syn

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**Fig. 1.** Influence of the addition of diumycin A on the growth curves of exponentially growing cells of *S. aureus* and *B. cereus*. The optical density measured at 660 nm in the absence (○) and in the presence of 0.1 (●) and 1.0 (Δ) μg of diumycin per ml is plotted against time.
which inhibited the incorporation nearly completely. Such a difference of effect on cell wall synthesis of *S. aureus* and *B. cereus* has been observed for prasinomycin (15), an antibiotic related to diumycin (16).

The results with *S. aureus* show that diumycin inhibits peptidoglycan synthesis, accompanied by accumulation of UDP-MurNAc-pentapeptide. This inhibition thus must be due to an action on one of the membrane-bound steps.

**Peptidoglycan synthesis in vitro.** Particulate preparations of *B. stearothermophilus* (22) and *E. coli* (7) contain all enzymes that are required for the synthesis of cross-linked peptidoglycan. Preparations of the former bacterium imitate the in vivo situation better than those of *E. coli* (15).

The effect of various diumycin concentrations on peptidoglycan synthesis by an extremely active particulate preparation of *B. stearothermophilus* is shown in Fig. 3. Lipid intermediate and peptidoglycan were the only detectable products. Low concentrations of diumycin inhibit peptidoglycan synthesis, a phenomenon accompanied by accumulation of the lipid intermediate. When 1 µg of diumycin per ml was present, 50% inhibition of peptidoglycan synthesis was observed. The radioactivity in the lipid intermediate gradually decreased with increasing diumycin concentrations. The solubility in water of peptidoglycan synthesized in the absence and presence of diumycin was the same (14 to 18%). Uncross-linked peptidoglycan, synthesized by particulate preparations of *E. coli*, is soluble in water (7). It was therefore concluded that diumycin has no significant influence on the transpeptidation reaction.

**TABLE 1. Influence of diumycin and vancomycin on cell wall synthesis by *S. aureus***

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fraction</th>
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<td>Cell wall,</td>
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<td></td>
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<td>$K_F 0.0$</td>
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<tr>
<td>None</td>
<td>SN</td>
<td>85</td>
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<td></td>
<td>P</td>
<td>12,000</td>
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<tr>
<td>Diumycin, 1 µg/ml</td>
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<td>72</td>
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<tr>
<td></td>
<td>P</td>
<td>11,700</td>
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<tr>
<td>Diumycin, 10 µg/ml</td>
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</tr>
<tr>
<td></td>
<td>P</td>
<td>1,290</td>
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<tr>
<td>Vancomycin, 10 µg/ml</td>
<td>SN</td>
<td>56</td>
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<td></td>
<td>P</td>
<td>830</td>
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* Exponentially growing cells of *S. aureus* were centrifuged, washed with, and resuspended in wall medium. After incubation for 10 min at 37 C, 14C-L-alanine (2 µCi/ml) and, if indicated, antibiotic were added. After 30, 60, and 120 min, samples (0.5 ml) were centrifuged in the cold. The pellet was resuspended in the same medium except that it did not contain labeled alanine. Samples (0.1 ml) of the first supernatant fluid (SN) and the resuspended pellet (P) were heat-inactivated and chromatographed. After autoradiography, radioactive compounds were excised and counted. This table gives the results after incubation for 60 min. Details of the method are described in Materials and Methods.
**DIUMYCIN A INHIBITION OF PEPTIDOGLYCAN SYNTHESIS**

**E. coli** preparations are very active in the synthesis of the labeled lipid intermediate, but peptidoglycan synthesis clearly is the limiting step in the system. Fifty percent inhibition of peptidoglycan synthesis by particulate preparations of E. coli K-12 strain KMBL-146 (2 mg of protein per ml) was obtained at diumycin concentrations of 1 to 3 mg per ml. With all diumycin concentrations tested (0.1 to 30 mg/ml), the radioactivity in the lipid intermediate was higher than in the control. The differences with the control were less pronounced than with preparations of B. stearothermophilus, owing to the high activity of lipid intermediate in the incubation mixture without antibiotic.

**D-Alanine carboxypeptidase I.** Partly purified soluble D-alanine carboxypeptidase I of E. coli strain Y-10 is susceptible to penicillins and vancomycin (8). Because diumycin, as well as 11.837 RP, moenomycin, prasinomycin, and enduracidin (15), inhibits the same reaction in peptidoglycan synthesis as vancomycin, we decided to test the sensitivity of D-alanine carboxypeptidase to these antibiotics. Particulate preparations of E. coli strain KMBL-146 were used as described previously. At 30 C, the liberation of alanine was linear with time for at least 2 hr. The presence of D-alanine (0.1 mM), the product of carboxypeptidase I, had no influence on the enzyme activity. Penicillin G and methicillin, added to the assay in concentrations of 300 mg/ml, reduced the liberation of alanine to less than 5%. As carboxypeptidase II activity was present, this result shows that the labeled nucleotide-substrate did not contain a considerable amount of UDP-MurNAc-tetrapeptide. Penicillin G and vancomycin (Fig. 4) inhibited the liberation of D-alanine by 50% in concentrations of 0.004 and 45 mg per ml, respectively, which is in good agreement with the data of Izaki and Strominger (8) for the soluble enzyme. Diumycin A, 11.837 RP, moenomycin, prasinomycin, and enduracidin, tested in concentrations between 10 and 300 mg per ml, did not inhibit the carboxypeptidase activity.

**DISCUSSION**

Like vancomycin, diumycin A inhibits the growth of S. aureus (Fig. 1) and inhibits cell wall synthesis by this organism (Fig. 2), events which are accompanied by accumulation of UDP-MurNAc-pentapeptide. Although a diumycin concentration of 0.1 mg/ml inhibited growth of S. aureus (Fig. 1), this antibiotic concentration had no significant influence on the rate of cell wall synthesis by the same bacterium during incubation in the wall medium (Fig. 2). This lack of correlation is probably due to the different composition of the media.

Growing B. cereus cells lysed in the presence of...
1.0 μg of diumycin per ml (Fig. 1). In contrast to vancomycin, but like prasinomycin (15), diumycin had no detectable influence on cell wall synthesis by nongrowing *B. cereus* cells. We have no good explanation for this phenomenon. One can assume that, in *B. cereus*, protein synthesis is required for penetration of diumycin and prasinomycin to their target(s).

When the *B. cereus* cells that escaped lysis in the presence of 0.1 μg of diumycin per ml (Fig. 1) were subcultured and grown under identical conditions, the same growth curve was obtained, showing that this typical curve is not due to two populations of cells in the original culture. The same type of growth curve has been found for *E. coli* cells grown in the presence of certain concentrations of penicillin or vancomycin (Lugtenberg, unpublished data). We do not have a clear-cut explanation of this growth curve. As stated earlier (Lugtenberg, Ph.D. thesis, State Univ. of Utrecht, Utrecht, The Netherlands, 1971), one can speculate that peptidoglycan synthesis occurs in a particular stage of the cell cycle. Cells that reach this stage may bind or accumulate relatively more of the antibiotic than cells that reach this stage later. The latter cells then escape lysis since they grew in a relatively lower antibiotic concentration. Another explanation, also based on the assumption that peptidoglycan biosynthesis occurs in a certain step in the cell cycle, is that after lysis of part of the bacteria the lysate of these cells causes partial inactivation of the antibiotic. Inactivation of the well-known antibiotics penicillin and vancomycin may, for instance, be caused by penicillinase and UDP-MurNAc-pentapeptide (18, 19), respectively. However, as conditions or agents that may inactivate diumycin A are not known at the moment, we are unable to answer the question of whether such an explanation would be reasonable for diumycin A.

Experiments with particulate preparations showed that diumycin in low concentrations interferes with the utilization of *N*-acyl-glucosamine- *N*-acyl-muramyl-pentapeptide for peptidoglycan synthesis. Higher concentrations of the antibiotic also inhibit the synthesis of the disaccharide-lipid intermediate (Fig. 3). Diumycin has no influence on the degree of solubility of peptidoglycan, and therefore does not interfere with the transpeptidation reaction. Diumycin, prasinomycin, 11.837 RP, enduracidin, and moenomycin do not inhibit d-alanine carboxypeptidase, in contrast to vancomycin (Fig. 4). This observation indicates that the mechanism of action of vancomycin differs from that of the other five antibiotics. The inhibition of d-alanine carboxypeptidase by vancomycin can be explained by the observed complex formation of the antibiotic with acyl-d-alad-alal (18). The other five antibiotics probably do not bind to acyl-d-alad-alal. They may inhibit peptidoglycan synthetase (i) by direct interaction with the enzyme, (ii) by causing degradation of the acceptor, although no degradation products of peptidoglycan could be detected, or (iii) by distortion of the membrane structure. The latter explanation is probably true for the ionic detergents sodium dodecyl sulfate, deoxycholate, and Triton X-100, which inhibit the in vitro utilization of the disaccharide for peptidoglycan synthesis by the *E. coli* particulate system, in contrast to the nonionic detergent Brij 58, which inhibits peptidoglycan synthesis earlier in the pathway (Lugtenberg, unpublished data).

ACKNOWLEDGMENTS

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