Inhibition of Protein Synthesis by Polypeptide Antibiotics

I. Inhibition in Intact Bacteria

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Abstract

Ennis, Herbert L. (St. Jude Children's Research Hospital, Memphis, Tenn.). Inhibition of protein synthesis by polypeptide antibiotics. I. Inhibition in intact bacteria. J. Bacteriol. 90:1102-1108. 1965.—The mechanism of inhibition of growth of cells by the polypeptide antibiotics of the PA 114, vernamycin, and streptogramin complexes was studied. This inhibition apparently was due to the selective inhibition of protein synthesis by these antibiotics. Ribonucleic acid synthesis was unaffected by concentrations of the antibiotics which completely inhibited protein synthesis. Deoxyribonucleic acid synthesis was slightly inhibited. These antibiotics are composed of a number of components. Mixtures of equal amounts of PA 114 A and PA 114 B or vernamycin A and Bα were more active in stopping protein synthesis in intact cells than each of the components of the antibiotic complex alone. Mutants resistant to one of the antibiotics were resistant to all of the group and, in addition, were resistant to erythromycin and oleandomycin.

A large number of polypeptide antibiotics have been isolated from culture filtrates of various Streptomyces species in the past decade. Among these antibiotics are the polypeptides of the group including PA 114, vernamycin, streptogramin, mikamycin, staphylomycin, and osteogrycin. These antibiotics are composed of a number of components. Each component may be active alone in inhibiting bacterial growth, but they are much more potent in combination. (Celmer and Sobin, 1956; Tanaka et al., 1958; Laskin and Chan, 1964). The vernamycin complex has recently been shown to be composed of primarily two different compounds: a nonpeptide macrolide (vernamycin A), and a group of peptide lactones (vernamycin B). Vernamycin B is related in structure to PA 114 B, mikamycin B, staphylomycin S, osteogrycin B, and streptogramin (Vanderhaeghe and Parmentier, 1960; Eastwood, Snell, and Todd, 1960; Watanabe, 1961; Bodansky and Ondetti, 1963).

Some of these antibiotics are potent inhibitors of growth of a variety of gram-positive organisms, but are generally less effective in inhibiting growth of gram-negative bacteria (Charney et al., 1953; English, McBride, and Halsena, 1956; Tanaka et al., 1958; Verwey, West, and Miller, 1958). Mikamycin and streptogramin inhibit protein synthesis in intact bacteria without appreciably interfering with nucleic acid synthesis (Yamaguchi, 1961; Vazquez, 1962). Recently, vernamycin has also been shown to inhibit protein synthesis in cell-free extracts of bacteria (Laskin and Chan, 1964).

The present investigation was initiated primarily to determine the mode of action of the antibiotics of the PA 114, vernamycin, and streptogramin complexes. The work is divided into two sections: the first, in this paper, on the effect of these antibiotics on various aspects of the physiology of intact bacteria; and the second, in the following paper, on the inhibition of protein synthesis in cell-free extracts of bacteria.

In this paper, evidence is presented that the inhibition of growth of gram-positive organisms is probably a result of the selective inhibition of protein synthesis by these antibiotics. All three antibiotics behave similarly. The initial rate of ribonucleic acid (RNA) synthesis is not depressed by concentrations of antibiotics that maximally inhibit protein synthesis. Mixtures of equal amounts of PA 114 A and PA 114 B or vernamycin A and vernamycin Bα are more active in stopping protein synthesis in intact cells than each of the components of the antibiotic complexes alone. Mutants resistant to one of the
antibiotics are resistant to all of the group and, in addition, are resistant to erythromycin and oleandomycin, perhaps indicating the same mode of action for these drugs.

**Materials and Methods**

**Bacterial strains.** Bacillus subtilis ATCC 6051 and Staphylococcus aureus Duncan were used.

**Media and growth of bacteria.** B. subtilis was grown in a mineral salts medium (Davis and Mingioli, 1950) supplemented with 0.25% Casamino Acids (Difco), 0.5% glucose, and 25 μg/ml of uridine or uracil. S. aureus was grown in the same mineral salts medium supplemented with 0.5% Casamino Acids, 1% glucose, BME vitamin mixture (Microbiological Associates, Inc., Bethesda, Md.), and 25 μg/ml of uridine or uracil.

Cultures were grown at 37°C with vigorous aeration and were harvested during exponential growth. The turbidity of the cultures was measured with a Klett-Summerson photoelectric colorimeter (42 filter); 100 Klett units corresponds to 7 X 10^6 B. subtilis cells per milliliter or 3 X 10^6 S. aureus cells per milliliter.

**Antibiotic solutions.** The antibiotics used were PA 114 (the crude mixture of A and B), the components, PA 114 A and PA 114 B, vernamycin A and vernamycin B, and streptogramin. No attempt was made to isolate the components of streptogramin. The antibiotics are very insoluble in water and were therefore used as nonsterile homogenized suspensions. When incorporated into solid medium, the antibiotics were dissolved in ethyl alcohol and then added to the cooled agar just before pouring the plates.

**Measurement of incorporation of radioactive into RNA, deoxyribonucleic acid (DNA), and protein.** 2-C^14-uridine, 2-C^14-uracil, and 1-C^14-L-leucine were purchased from New England Nuclear Corp., Boston, Mass. To measure protein synthesis, cultures were incubated in media containing C^14-leucine and samples were taken at intervals and mixed with equal volumes of cold 10% trichloroacetic acid supplemented with 1 mg/ml of the corresponding nonradioactive precursor. The precipitate was then filtered and washed with cold 5% trichloroacetic acid on a Millipore filter (0.45-μm pore size), and the radioactivity was counted in a Nuclear-Chicago thin-window low-background counter (Nuclear-Chicago Corp., Des Plaines, Ill.) Incorporation of C^14-uridine or C^14-uracil into RNA and DNA was measured by the method of Roodyn and Mandel (1960) by use of their Millipore filter technique. There is little contamination of the DNA fraction with RNA. For S. aureus, 0.1 μc of C^14-uridine, C^14-uracil, or C^14-leucine was added per milliliter of culture. For B. subtilis the amount of each of the radioactive precursors was 0.02 μc/ml of culture.

**Results**

**Effect of antibiotics on growth and on protein, RNA, and DNA synthesis.** Both B. subtilis and S. aureus were very sensitive to growth inhibition by all the antibiotics studied. Except for some small quantitative differences in the response of these bacteria to the antibiotics, in general, the effect of the drugs on the cells was similar. Consequently, except for one figure, only the data for S. aureus are given. The antibiotics inhibited the growth of intact Escherichia coli only at very high concentrations, and therefore the response of this species was not studied in great detail. However, as will be shown in the subsequent paper, in contrast to the relative insensitivity of the intact cell, the cell-free protein-synthesizing system derived from E. coli was extremely sensitive to inhibition by the antibiotics, suggesting that the intact cell was impermeable to the drugs.

Figure 1 shows the inhibition of growth of S. aureus by PA 114 (5 μg/ml) and by PA 114 A (5 μg/ml). Cells were grown for two generations and then divided equally into three flasks. Antibiotics were added as indicated and growth was measured by increase in Klett reading. These antibiotics markedly inhibited growth of these cells. Streptogramin, PA 114 B, and vernamycin A were equally effective in inhibiting growth.

Under the conditions of the experiment, these antibiotics were bacteriostatic, inhibiting growth of either S. aureus or B. subtilis for at least 2 hr, without appreciably killing the cells. On one occasion, 65% of B. subtilis cells were killed after

![Graph showing inhibition of growth of Staphylococcus aureus by antibiotics.](http://jb.asm.org/)
2 hr of incubation with the antibiotics. Vazquez (1962) reported that streptogramin is bactericidal.

The effect of the antibiotics on protein and nucleic acid synthesis was studied by following the incorporation of radioactive precursors into the protein, RNA, and DNA fractions of the cells. C14-leucine incorporation was used as an index of protein synthesis, and C14-uridine or C14-uracil as an index of RNA and DNA synthesis. Cells growing in the appropriate medium for at least two generations were divided into several flasks, and the indicated amount of antibiotic was added. The cultures were shaken at 37°C for 10 min to allow penetration of the drug. The radioactive precursor was added and the incorporation into the appropriate fractions was measured. Chemical measurement of RNA and protein synthesis gave the same results as the incorporation studies.

Figures 2, 3, and 4 give the data for inhibition by the antibiotics of the incorporation of C14-leucine into protein in S. aureus. These data show that all of the antibiotics were very potent inhibitors of protein synthesis, significantly inhibiting C14-leucine incorporation at a concentration as low as 1 μg/ml.

In S. aureus, PA 114 A was a more potent inhibitor than PA 114 B, and likewise vernamycin A was more potent than vernamycin Bα. Symbols: O, control, no antibiotic; Δ, 1 μg/ml; ■, 10 μg/ml.

Figures 4. Inhibition of C14-leucine incorporation into protein of Staphylococcus aureus by mixtures of PA 114 A and PA 114 B, and by streptogramin. For PA 114 A plus PA 114 B experiment: O, control, no antibiotic; Δ, 0.1 μg/ml of PA 114 B; ■, 0.1 μg/ml of PA 114 A; ●, 0.1 μg/ml of PA 114 A plus 0.1 μg/ml of PA 114 B. For streptogramin experiment: O, control, no antibiotic; Δ, 0.2 μg/ml; ■, 0.5 μg/ml; ●, 2.0 μg/ml.

Levels of the antibiotics which maximally inhibited protein synthesis did not appreciably...
effect RNA synthesis. DNA synthesis appeared to be inhibited to some extent (Fig. 6).

Other investigators have shown that the RNA particles that accumulate in the absence of protein synthesis can be differentiated from the normal ribosomes of the cell. They have shown that, in crude extracts of cells which have accumulated RNA without simultaneous protein synthesis, the RNA which accumulates is composed mostly of preribosomal particles and s-RNA (Nomura and Watson, 1959; Sells, 1964). In sucrose density gradients, these preribosomal particles sediment at a slower rate than the normal 50S and 30S ribosomes, but at a faster rate than s-RNA (4S). Purified RNA isolated from these cells appears to possess the normal 23S, 16S, and 4S components.

Two 1-liter batches of cells were grown to about 100 Klett units. C4-uracil was added to one batch, and C4-uracil plus 25 μg/ml of streptogramin to the other. The cells were shaken for 30 min at 37°C, chilled rapidly, and harvested. The cells from each flask were washed once, suspended in 5 ml of 0.01 M tris(hydroxymethyl)amino methane (Tris)-HCl buffer (pH 7.2) containing 10-4 M Mg++, and broken in a French pressure cell. Electrophoretically pure deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.) was added (5 μg/ml), and the extract was centrifuged in the cold once at 10,000 × g for 15 min and once at 30,000 × g for 30 min. The extract was dialyzed overnight against the same buffer, and layered on a linear sucrose gradient (5 to 20%) made up in the above buffer containing 5 × 10-3 M KCl. The gradient was centrifuged and fractions were collected as previously described (Ennis and Lubin, 1965). The optical density profile is an index of RNA in the cell before addition of C4-uracil, and the radioactivity profile is an index of the RNA made during the 30 min of incubation in the presence of C4-uracil. Since actual corrected sedimentation velocities were not obtained, S values have been used only as a convenient label for the main classes of ribosomal subunits and of RNA, as described by others.

Figure 7A shows the profiles of an extract of growing untreated cells. The 50S and 30S ribosomal and 4S s-RNA peaks are evident. The radioactivity curve closely follows the optical density profile.

Figure 7B shows the profile of an extract of cells treated with 25 μg/ml of streptogramin. Little radioactivity was found in the 50S ribosomal peak. A large amount of heterogeneous material which sedimented in a broad area was synthesized during the period of drug treatment. This material obliterated the 30S and 4S regions. Normal ribosomes were not synthesized during drug treatment. The heterogeneous material synthesized during this time was probably ribosomal RNA components which lacked their normal protein complement.

Isolation and properties of mutants resistant to antibiotics. Single-step mutants resistant to 15 μg/ml of PA 114 (B. subtilis) and 5 μg/ml of PA 114 (S. aureus) were isolated by selection of colonies which grew when a large number of cells was plated directly onto antibiotic-containing growth medium. Independent isolates resistant
null
mycin. The parent was resistant to the levels of puromycin used. (iv) Concomitant with the change in resistance was also a change in morphology. The B. subtilis strain resistant to 15 \(\mu g/ml\) of PA 114 grew in long chains, quite unlike the parent which grew individually or in very short chains of two or three cells.

Experiments with protoplasts derived from both PA 114-sensitive and -resistant B. subtilis have shown that the resistance is not due to a change in the permeability of the cell wall. Incorporation of C\(^\text{14}\)-leucine into protoplasts of the resistant cell line was not inhibited by 10 \(\mu g/ml\) of PA 114, whereas the incorporation into protoplasts of sensitive cells was inhibited to the same extent as in intact sensitive cells.

**Effect of antibiotics on respiration.** To rule out other sites of action of the antibiotics, the effect of the drugs on respiration of S. aureus was studied. Neither PA 114 (5 \(\mu g/ml\)) nor streptomycin (5 \(\mu g/ml\)) depressed \(O_2\) uptake or \(CO_2\) evolution, when glucose was used as the energy source. At these levels of antibiotics, protein synthesis was completely inhibited.

**Discussion**

The results presented in this paper implicate protein synthesis as being the primary site of growth inhibition by the antibiotics of the PA 114, vernamycin, and streptomycin complexes. The following paper will show that the cell-free protein-synthesizing system is indeed highly sensitive to inhibition by these antibiotics. The rate of DNA synthesis is slightly inhibited at concentrations of antibiotics which inhibit protein synthesis completely.

The individual components of the antibiotic complexes can inhibit protein synthesis. However, certain combinations of these components are much more potent in stopping both growth and protein synthesis than the individual compounds themselves. Work on the cell-free protein-synthesizing system indicates that the role of the B component is to potentiate the activity of the A component. However, the mechanism of this synergism is difficult to understand because the results of inhibition studies in intact cells depend on the bacterial species used. PA 114 A and vernamycin A are more potent inhibitors of protein synthesis in S. aureus than in B. subtilis, and conversely the B components are more potent against B. subtilis than S. aureus. English et al. (1956) have shown this to be true also of the inhibition of the growth of these species by the individual components. Tanaka et al. (1958) have also shown the same effect by the components of mikamycin.

The inhibition of protein synthesis without the concomitant cessation of RNA synthesis results in an imbalance in the macromolecular composition of the cell. Bacterial cells treated with chloramphenicol or puromycin pile up ribosomal precursors lacking the full protein complement (Nomura and Watson, 1959; Sells, 1964). Soluble RNA is also made. The results of this investigation are in agreement with the previous work in which other antibiotics were used to stop protein synthesis. Sucrose density gradients of bacterial extracts have shown that very few stable ribosomes are synthesized when growth of B. subtilis is inhibited by streptomycin. The RNA formed under these conditions appears heterogenous and sediments in a broad area extending from 30S to 45S. These results point to the generality of this phenomenon of RNA synthesis in the absence of protein synthesis.

The isolation of mutants resistant to relatively high concentrations of PA 114 and streptomycin indicates the similarity among the antibiotics of this group. Mutants resistant to one of the antibiotics are resistant to them all. Thus, the PA 114-resistant mutant is also resistant to streptomycin and vernamycin A and Bz. These results, and the results of work to be presented in the accompanying paper indicate that the antibiotics are very similar in mode of action, and may be the same or similar in chemical structure. Studies on the chemical composition of these antibiotics have also led to the same conclusion (Vandenhauwe and Parmentier, 1960; Eastwood et al., 1960; Watanabe, 1961; Bodansky and Ondetti, 1963).

The interesting observation of the cross-resistance of a PA 114-resistant mutant with erythromycin and oleandomycin points, perhaps, to a similarity in mode of action of these drugs. Both erythromycin and oleandomycin inhibit protein synthesis. Garrod and Waterworth (1956) showed cross-resistance between oleomycin and erythromycin. The fact that the PA 114-resistant mutant is not cross-resistant with a large number of other inhibitors of protein synthesis may indicate different modes of action. As will be shown in the following investigation, PA 114 does not inhibit protein synthesis the same way as neomycin, kanamycin, streptomycin, the tetracyclines, chloramphenicol, and puromycin.

The mechanism of resistance to the polypeptide antibiotics is not known, but perhaps the resistance lies in an altered protein-synthesizing system no longer sensitive to inhibition by the antibiotics, as is the case in streptomycin resistance. This possibility is currently being studied.
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LITERATURE CITED


