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Effect of the Removal of Outer Cell Wall Layers on the Actinomycin Susceptibility of a Gram-Negative Bacterium

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Removal of the outer cell wall layers of a gram-negative marine pseudomonad (B-16) showed that these cells are penetrable by actinomycin D and that, therefore, neither the cytoplasmic membrane nor the peptidoglycan layer constitutes the barrier which excludes this antibiotic from intact cells, but that this barrier is formed by the outer layers of the cell wall which include the lipopolysaccharide component and the double-track layer.

The inability of actinomycin D to affect deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) synthesis in gram-negative bacteria has been explained by postulating the existence of a barrier layer within the cell envelope which excludes the antibiotic (6). The barrier to the penetration of actinomycin into the cell is destroyed by treatment with ethylenediaminetetraacetate (EDTA; reference 7) or warm water (10), by infection with bacteriophage (11), and by conversion of the cells to spheroplasts by EDTA-lysozyme treatment (5). Cell wall-defective mutants have also been shown to be susceptible to actinomycin D (13, 14), and all of this evidence has been interpreted as indicating that the barrier to the antibiotic lies in the cell wall (7).

The weakness in this deduction is that it has not been established that the agents used to render cells permeable to the antibiotic do not affect the cytoplasmic membrane as well as the cell wall, and this is especially critical in the case of EDTA which is known to have a devastating effect on the cytoplasmic membrane of some gram-negative bacteria (15). Thus the transitory damage caused by EDTA (7) could be to a barrier at the level of the cell wall or of the cytoplasmic membrane, and Singh et al. (14) showed that sucrose-lysozyme spheroplasts of *Escherichia coli* are not penetrated by actinomycin D, which indicates that the undisturbed cytoplasmic membrane is a barrier to the antibiotic in that organism. It was suggested (14) that both the cell wall and the cytoplasmic membrane are barriers to the penetration of actinomycin D.

The development of a procedure for the removal of the outer cell wall layers of a gram-negative marine pseudomonad (B-16) by manipulation of ion concentration (1) provides us with metabolically active mureinoplasts (2) which are surrounded only by their cytoplasmic membrane and the peptidoglycan component of their cell wall (3). We have compared the actinomycin D susceptibility of whole cells with that of mureinoplasts to determine whether the barrier to the penetration of this molecule has been eliminated with the removal of the outer layers of the cell wall, so that we can determine both the existence and the location of a barrier layer within this structure.

Whole cells and mureinoplasts were suspended in 5 ml of B-16 medium (1), to a final optical density (OD), at 660 nm, of 1.20 and 1.35, respectively, and actinomycin D (50 μg/ml) and EDTA (0.03 mM) were added to the appropriate flasks. After 5 min (zero time), uracil-*1*-^{14}C (3.5 μCi specific activity, 31 mCi/mmole, 0.29 mM final concentration) was added, the preparations were inoculated at 25 C in a rotary shaker, and 0.2-ml samples were taken and counted as reported by Singh et al. (14).

We found that actinomycin D does not affect the incorporation of ^{14}C-uracil into RNA in whole cells of the marine pseudomonad, but that it does affect ^{14}C-uracil incorporation when the cells have been treated with 0.03 mM EDTA (Fig. 1). This shows that the DNA-dependent RNA synthesis of this organism is susceptible to inhibition by actinomycin D, and that whole cells have a barrier to the penetration of this antibiotic which is damaged by EDTA.

On the other hand, mureinoplasts were very sensitive to inhibition of ^{14}C-uracil uptake by

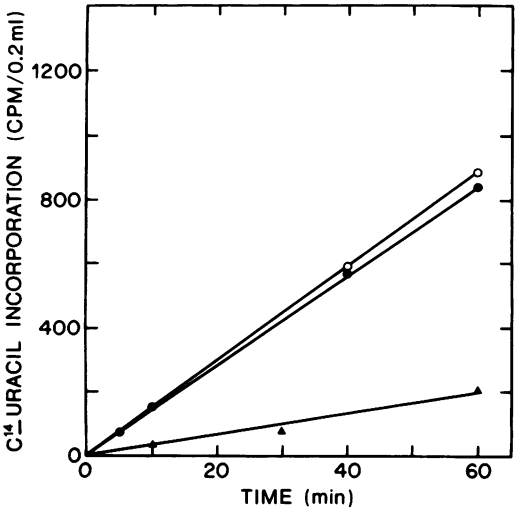

FIG. 1. *Effect of actinomycin D and EDTA on the uptake of ^{14}C-uracil by whole cells of a gram-negative marine pseudomonad (B-16). Logarithmic-phase cells were harvested and resuspended into medium (1) to obtain an OD of 1.20. These cell suspensions were incubated after the addition of 50 μg of actinomycin D per ml or 50 μg of actinomycin D and EDTA (0.03 mM). ^{14}C-uracil was added at zero time, and uptake of uracil was measured. The symbols used are: ○, whole cells; ●, whole cells with actinomycin D; ▲, whole cells with actinomycin D and EDTA.*

actinomycin D (Fig. 2). EDTA pretreatment eliminated the very small amount of ^{14}C-uracil incorporation remaining in the presence of the antibiotic, and also exerted some effect on incorporation in the absence of actinomycin D in later stages of the incubation. This shows that the barrier to the penetration of the antibiotic has been eliminated by the removal of the outer layers of the cell wall of this gram-negative bacterium. Because this barrier has been removed, EDTA has only a slight effect in enhancing the penetration of actinomycin D, and it also exerts a somewhat enigmatic effect on DNA-dependent RNA synthesis.

The physiological state of the mureinoplasts used in this study is of vital importance in the interpretation of these results in relation to the location of the barrier to actinomycin D within the cell envelope. DeVoe et al. (2) have shown that some mureinoplasts leak ^{14}C-α-aminoisobutyrate (^{14}C-AIB) when they are retained in sucrose and when sucrose is added to the suspending menstruum, and they have deduced that some cells have sustained membrane damage. On the other hand, the 50% of mureinoplasts which were later successfully converted to protoplasts retained all of their accumulated ^{14}C-AIB and showed no decrease in their ability to take up this amino acid analogue. Thus we may conclude that approximately 50% of mureinoplasts sustain membrane damage during their formation. The complete inhibition of DNA-dependent RNA synthesis in mureinoplasts by the action of actinomycin D (Fig. 2) shows that membrane damage alone cannot account for the increased penetration of these cells by the antibiotic and that cells whose membranes are intact are affected by its presence.

These data indicate, therefore, that the barrier to the antibiotic in this organism exists at some level of the cell wall, as has been suggested by many previous workers (5–7, 11, 13), and it is based on the specific removal of the outermost layers of the cell wall by a manipulation of ion concentration which leaves the cytoplasmic membrane of half of the cells still capable of transporting and retaining ^{14}C-AIB. The cell wall component which has been most often invoked in the formation of the barrier layer is lipopolysaccharide (7–9), but recent studies (12; Forge, Costerton, and Kerr, *manuscript in preparation*) indicate that the double-track layer of this organism, which is composed of phospholipids and proteins, has a membrane-like molecular archi-

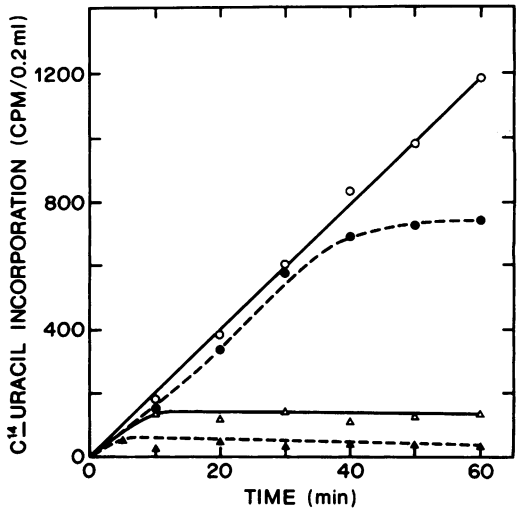

FIG. 2. *Effect of actinomycin D and EDTA on the uptake of ^{14}C-uracil by mureinoplasts of a gram-negative marine pseudomonad (B-16). Logarithmic-phase cells of B-16 were transformed into mureinoplasts (2), and these mureinoplasts were diluted to an OD of 1.35 in medium (1). At zero time, 50 μg of actinomycin D/ml (final concentration) or 50 μg of actinomycin D/ml and 0.03 mM of EDTA were added, and uptake of uracil was measured. The symbols used are: ○, without actinomycin D and EDTA; ●, without actinomycin D and with EDTA; △, with actinomycin D and without EDTA; ▲, with actinomycin D and EDTA.*

tecture which would allow it to perform this function. Chemical studies of the cell envelope of mureinoplasts (4) have shown that the only cell wall component remaining on these cells is the peptidoglycan, and that the double-track layer and the lipopolysaccharide have been removed. The fact that these cells are penetrable by actinomycin D establishes, therefore, that neither the cytoplasmic membrane nor the peptidoglycan layer constitutes the barrier which excludes this antibiotic from intact cells, but that this barrier is formed by the outer layers of the cell wall which include the lipopolysaccharide component and the double-track layer.

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