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Susceptibility of Whole Cells and Spheroplasts of *Pseudomonas aeruginosa* to Actinomycin D

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Cells of *Pseudomonas aeruginosa* suspended in 0.2 M Mg^{2+}, 20% sucrose, 0.01 M tris(hydroxymethyl)aminomethane, or water partially release lipopolysaccharide. The release of alkaline phosphatase from the periplasmic space and the ability to form spheroplasts on lysozyme treatment is directly related to the lipopolysaccharide released during treatment with 0.2 M Mg^{2+}, 20% sucrose, or other agents. The synthesis of ribonucleic acid (RNA) by intact cells, magnesium-lysozyme spheroplasts, or 20% sucrose-lysozyme spheroplasts is not sensitive to actinomycin D, whereas RNA synthesis by intact cells or spheroplasts in the presence of ethylenediaminetetraacetic acid (EDTA) is sensitive to actinomycin D. EDTA alone has an inhibitory effect on RNA synthesis by whole cell, by magnesium-lysozyme spheroplasts, and by 20% sucrose-lysozyme spheroplasts. The experimental data indicate that, although the cell wall is damaged by 0.2 M Mg^{2+} or 20% sucrose treatment in the presence of lysozyme, the treated cells or spheroplasts are still resistant to actinomycin D. These results suggest that the cytoplasmic membrane should be considered as the final and determinative barrier to this antibiotic in this organism.

It has been found (6, 14, 28) that ethylenediaminetetraacetic acid (EDTA) treatment of gram-negative cells releases a lipopolysaccharide (LPS) component of the molecular sieve layer of the cell wall (11, 17, 23). Treated cells become permeable to antibiotics such as actinomycin D (7, 10, 15, 24, 35) and to enzymes such as lysozyme (7, 35). EDTA-treated, gram-negative cells become more susceptible to osmotic shock, which results in the consequent release of periplasmic enzymes and LPS (8, 18, 19, 39). The barrier to the penetration of actinomycin D into a cell is also destroyed by warm water (26, 27), by infection with bacteriophage (29), by conversion of the cells to spheroplasts by EDTA-lysozyme treatment (7, 35), and by the removal of the outer layers of the cell wall (15). Mutants with defective cell walls have also been shown to be susceptible to actinomycin D (32, 35). The above evidence has been interpreted as indicating that the barrier to the antibiotic lies in the cell wall (11, 30, 31), presumably with a LPS component of the double-track layer.

This deduction is weak because it is not known whether the agents used to render cells permeable to the antibiotic affect the cytoplasmic membrane as well as the cell wall. This is especially true in the case of EDTA, which is known to have a pronounced effect on the cytoplasmic membrane of gram-negative bacteria (38). Thus, the damage caused by EDTA (11) may be at the cell wall level or at the cytoplasmic membrane level. Our previous studies have shown that sucrose-lysozyme spheroplasts of *Escherichia coli* are not penetrated by actinomycin D without EDTA treatment, suggesting that the undisturbed cytoplasmic membrane is the definitive barrier to the antibiotic in this organism (35).

Recently, procedures for the preparation of spheroplasts from *Pseudomonas aeruginosa* by a Mg^{2+}-lysozyme procedure or a sucrose-lysozyme method in the absence of EDTA have provided us with metabolically active spheroplasts (4, 5). Spheroplasts prepared by these methods release neither cytoplasmic membrane nor cytoplasm marker enzymes such as reduced nicotinamide adenine dinucleotide (NADH) oxidase or glucose-6-phosphate dehydrogenase (3-5). This can be taken as evidence of the intact state of the cytoplasmic membrane of these spheroplasts. This present study is concerned with the permeability to actinomycin D of the cytoplasmic membrane of Mg^{2+}-lysozyme or sucrose lysozyme spheroplasts of *P. aeruginosa* prepared in the absence of EDTA. (This work was pre-

sented in part at the 22nd Annual Meeting, Canadian Society of Microbiologists, Quebec City, Quebec, June 13–16, 1972.)

MATERIALS AND METHODS

Organism and culture conditions. *P. aeruginosa* ATCC 9027 was cultivated at 37 C on a rotary shaker in a glucose-ammonium salt-proteose peptone medium as previously described (3). The cells were harvested after incubation for 12 h by centrifugation in a Sorvall RC-2B refrigerated centrifuge for 10 min at 13,000 × *g*.

Optical density of cultures. Because of pigment production in the above medium, the optical density of the culture was measured at 660 nm (3). The optical density of the culture was correlated with the dry weight of cell (constant weight at 90 C), and a standard curve was prepared. An optical density of 1.0 was equivalent to 0.56 mg/ml. Optical densities throughout were determined at 660 nm on a Gilford model 300-N spectrophotometer.

Chemicals. *p*-Nitrophenylphosphate, tris(hydroxymethyl)aminomethane (Tris), EDTA, and lysozyme (muramidase, EC 3.2.1.17) were purchased from the Sigma Chemical Co., St. Louis, Mo. Actinomycin-D was a generous gift from Merck, Sharpe, and Dohme of Canada Ltd., Pointe Claire, Quebec. Uracil-*1*-^{14}C was obtained from New England Nuclear Corp., Boston, Mass. 2-Keto-3-deoxyoctulonic acid (KDO), a standard used in the assay of LPS, was a generous gift from E. C. Heath, Johns Hopkins University, Baltimore, Md. All other reagents and chemicals were the best grade obtainable from local commerical sources.

Enzyme assays. Alkaline phosphatase (EC 3.1.3.1), NADH oxidase, and G-6-P dehydrogenase were assayed as described previously (3). One unit of enzyme activity corresponds to the conversion of 1 μmol of substrate to product per minute.

Chemical assays. LPS was determined by assay of KDO, a unique constituent of LPS (22). The LPS was hydrolyzed in 0.02 N H_2SO_4 in a boiling water bath as described by Osborn et al. (22). The KDO was determined by the thiobarbituric acid procedure of Weissbach (37) after periodate oxidation. Under the conditions of the assay, 1 μmol of KDO gave an optical density of 19.0 at 549 nm (21).

Release of alkaline phosphatase and LPS from cells. Standard quantities of cells obtained from 12-h cultures grown to 1.2 to 1.4 optical density units were washed with 0.2 M $MgCl_2$ in 0.01 M Tris (pH 8.4); 20% sucrose in 0.01 M Tris (pH 8.4); 0.01 M $MgCl_2$ in 0.01 M Tris (pH 8.4); 0.01 M Tris (pH 8.4); or water. The viability of the cells after washing was determined by making serial dilutions in distilled water and plating on *Pseudomonas* agar P (Difco). Growth rates were determined after standard inoculations of appropriately washed cells into 500 ml of glucose-ammonium salts-proteose peptone medium (3). Cell-free extracts were prepared by ultrasonic disruption of cells resuspended in 0.01 M Tris, pH 8.4, as previously described (3).

Preparation of spheroplasts. Sucrose-lysozyme spheroplasts were prepared by centrifuging the cells from 20 ml of a 14-h culture (13,000 × *g*) and resuspending them in 20 ml of 20% sucrose (with 0.01 M Tris, pH 8.4) containing 1.0 mg of lysozyme per ml. The cell suspension was incubated for 1 h at 25 C in a water bath shaker; cells were centrifuged and resuspended in 0.01 M $MgCl_2$ and 0.01 M Tris buffer (pH 8.4) for the formation of spheroplasts (4, 5). Magnesium-lysozyme spheroplasts were prepared similarly, except that the 20% sucrose was replaced by 0.2 M $MgCl_2$ and 0.01 M Tris (pH 8.4). The resultant spheroplasts were centrifuged at 13,000 × *g* and resuspended in a glucose-ammonium salts-proteose peptone medium (3) for actinomycin D sensitivity determinations.

Determination of sensitivity of whole cells, treated whole cells, and spheroplasts to actinomycin D. The sensitivity of whole cells or spheroplasts to actinomycin D was assayed by determining the sensitivity of ribonucleic acid (RNA) synthesis to the antibiotic (7, 9, 24). Whole cells, 0.2 M Mg^{2+}-washed cells, 20% sucrose-washed cells, 0.01 M Mg^{2+}-washed cells, 0.01 M Tris-washed cells, magnesium-lysozyme spheroplasts, or sucrose-lysozyme spheroplasts were suspended in 5 ml of glucose-ammonium salts-proteose peptone medium (3) to a final optical density of 1.40; actinomycin D (50 μg/ml), and EDTA (0.01 M final concentration) or phenethyl-alcohol, at the concentrations indicated in the particular experiment, were added to the appropriate flasks. After 5 min (zero time) uracil-*1*-^{14}C (7.0 μCi, specific activity 31 mCi/mM, 0.30 mM final concentration) was added, and the preparations were incubated at 25 C on a rotary shaker. At various times, 0.2-ml samples were removed and mixed with 2.5 ml of cold 10% trichloroacetic acid. The trichloroacetic acid-insoluble material was removed by membrane filtration (Millipore Corp.). The filters were dried and placed in scintillation vials to which 10 ml of scintillation fluid (1) was added. The incorporated radioactivity was determined with a Packarb Tricarb liquid spectrometer.

RESULTS

All the detectable alkaline phosphatase and 28.6% of LPS was removed from the cells of *P. aeruginosa* by washing with Mg^{2+} at a concentration of 0.2 M (Table 1). Twenty percent sucrose wash removed 50% of the alkaline phosphatase and 15.5% of LPS from cells of *P. aeruginosa*. In addition, 0.01 M Tris and water released only part of the total enzyme content of the cells and 8% and 6% of the LPS of the cells (Table 1). It is noteworthy that washing

TABLE 1. *Effect of various washes on cells of Pseudomonas aeruginosa*

Treatment	Alkaline[a] phosphatase (units/1 g)		APase released (%)	Viability (cells/ml)	Growth rate	Whole cell KDO released (%)[c]	APase/KDO (%/%)	Spheroplast formation (%)[d]	Plasmolysis of cell
	Wash	CFE							
0.2 M Mg^{2+} + 0.01 M Tris, pH 8.4...	187.0	1.2	99.4	3.8×10^9	0.134	28.6	3.5	100	+
20% sucrose + 0.01 M Tris, pH 8.4...	85.9	79.6	51.9	3.5×10^9	0.133	15.5	3.4	100	+
0.01 M Mg^{2+} + 0.01 M Tris, pH 8.4...	3.7	176.9	2.1	3.9×10^9	0.134	0.6	3.5	5–20	−
Water.....	25.3	149.1	14.5	3.7×10^9	0.134	6.0	2.5	10–30	−
0.01 M Tris, pH 8.4.	35.4	149.1	19.2	3.5×10^9	0.133	8.0	2.4	30–40	−
Control.....	—[e]	183.3	—[e]	3.8×10^9	0.134	—[e]	—[e]	0–5	−

[a] Twenty milliliters of 14-h cells (15.8 mg [dry weight] of a culture grown to 1.3 optical density units) were centrifuged and resuspended into 20 ml of each treatment solution for 20 min at room temperature and recentrifuged. The resulting washes were assayed for alkaline phosphatase and lipopolysaccharide (as KDO). The cell pellet was suspended into 20 ml of Tris buffer and ultrasonically disrupted, and the cell-free extract (CFE) was assayed for alkaline phosphatase or a similar cell pellet was extracted twice with hot phenol to obtain LPS for KDO determination (40).

[b] Growth rate is expressed as the change in optical density (OD) per hour between the 6th and 10th h after treatment.

[c] LPS (22) was determined by assay of KDO (37). The quantity present in the whole cells (485.0 μg of KDO per 158 mg of cells) equals 100%.

[d] Spheroplasts were prepared by centrifuging the cells from a 14-h culture and then resuspending them into a treatment solution containing 1.0 mg of lysozyme per ml. See Materials and Methods for detailed procedures.

[e] Test not performed.

with 0.01 M Mg^{2+} resulted in almost complete retention of alkaline phosphatase and LPS (Table 1). Furthermore, under conditions in which phosphatase was released, there was a corresponding release of LPS from cells (Table 1).

Our results have shown previously that the alkaline phosphatase is located on the double-track layer (4, 5), and the enzyme is probably linked to an LPS component of this layer and projects inwardly into the periplasm. Hence, any alteration of this layer, which results in the release of LPS and alkaline phosphatase, should also allow the inward passage of large molecules, such as lysozyme, which are normally excluded from intact whole cells. Indeed, we find that Mg^{2+}-washed cells (Fig. 1b), in contrast to untreated cells (Fig. 1a), are converted to spheroplasts (4) by the action of lysozyme as are cells that have been washed in 20% sucrose (Fig. 1c). The inward passage of lysozyme through the outer double-track layer to its substrate, peptidoglycan layer, seems to be related to the amount of LPS released during the treatment in terms of spheroplast formation (Table 1). Cells washed with 0.2 M Mg^{2+} or 20% sucrose were plasmolyzed, and no plasmolysis of cells was observed in 0.01 M Mg^{2+}, water, or 0.01 M Tris wash (Table 1). It also was noted that, under all conditions of washing employed in this study, the cells remained actively motile. In addition, the viability and growth rates for cells from all three treatments were identical (Table 1). No cytoplasmic membrane or cytoplasmic marker enzymes such as NADH oxidase or G-6-P dehydrogenase were released into the cell-free supernatant fluid. This indicates that, although the cell wall may be damaged, the cytoplasmic membrane is still intact.

Although we have shown that the permeability of the cell wall is altered with these spheroplasting treatments, further studies were undertaken to determine if there was a concomitant alteration of the cytoplasmic membrane. We have shown that actinomycin D does not affect the incorporation of uracil-*1*-^{14}C into RNA of whole cells washed with 0.2 M Mg^{2+}, 20% sucrose, 0.01 M Mg^{2+}, 0.01 M Tris, or water (Fig. 2). However, the incorporation of uracil-*1*-^{14}C by Tris-washed cells and 20% sucrose-washed cells was lower than that by Mg^{2+}-washed cells or water-washed cells (Fig. 2). On the other hand, the incorporation of uracil-*1*-^{14}C into RNA in whole cells of *P. aeruginosa* was very sensitive to

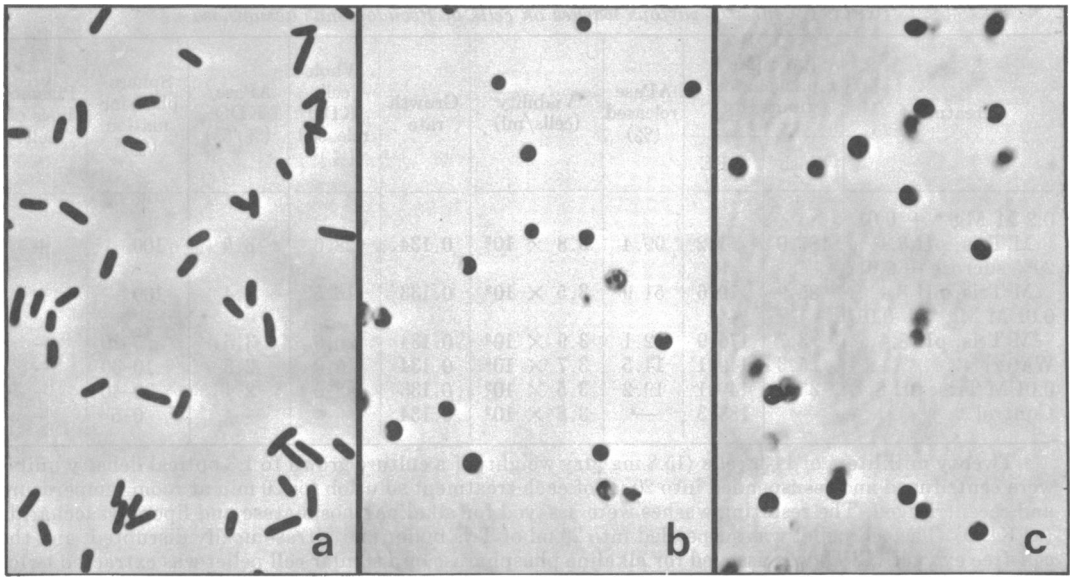

FIG. 1. *Phase contrast micrographs of whole cells (a); spheroplasts prepared in sucrose (b); spheroplasts prepared in 0.2 M Mg^{2+} (c). Magnification: ×2,000.*

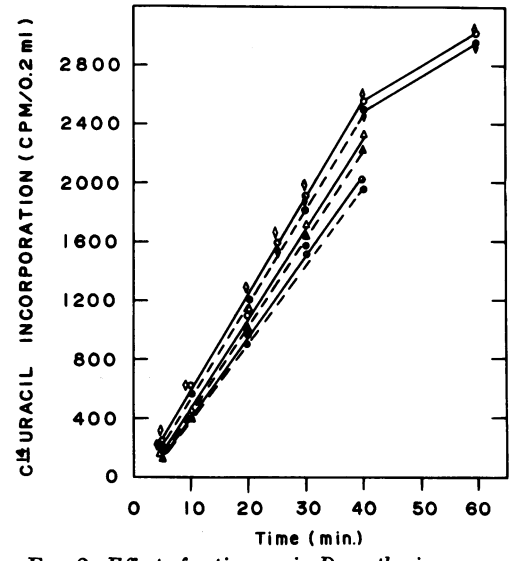

FIG. 2. *Effect of actinomycin D on the incorporation of uracil-1-^{14}C into RNA by whole cells, 0.2 M Mg^{2+}-washed cells, 20% sucrose-washed cells, and 0.01 M Tris-washed cells of P. aeruginosa. The whole cells and the treated cells were resuspended into minimal media (3) to obtain an optical density of 1.40. These cell suspensions were incubated after the addition of 50 μg of actinomycin D per ml. Uracil-1-^{14}C was added at zero time, and the incorporation of uracil into RNA was measured. Symbols: □, whole cells; ■, whole cells with actinomycin D; ○, 0.2 M Mg^{2+}-washed cells; ●, 0.2 M Mg^{2+}-washed cells with actinomycin D; ◑—◑, 20% sucrose-washed cells; ◑---◑, 20% sucrose-washed cells with actinomycin D; △, 0.01 M Tris-washed cells, ▲, 0.01 M Tris-washed cells with actinomycin D.*

actinomycin D when the whole cells were treated with 0.01 M EDTA (Fig. 3). These experiments show that the deoxyribonucleic acid (DNA)-dependent RNA synthesis of this organism is susceptible to inhibition by actinomycin D. EDTA drastically reduced the capacity of whole cells to incorporate uracil-*1*-^{14}C into RNA so that the level of incorporation was only 20 to 35% of that of untreated cells of spheroplasts. In the present studies, however, no inhibition occurred with either Mg^{2+}-lysozyme spheroplasts or sucrose-lysozyme spheroplasts as determined by the incorporation of uracil-*1*-^{14}C into RNA in the presence of actinomycin D (Fig. 4 and 5). The incorporation of uracil-*1*-^{14}C into RNA of both Mg^{2+}-lysozyme spheroplasts and sucrose-lysozyme spheroplasts was totally eliminated in the presence of 0.01 M EDTA and actinomycin D but not in the presence of EDTA alone (Fig. 4 and 5). These results indicate that EDTA damaged the cell membrane and allowed the inward passage of actinomycin D to block DNA-dependent RNA synthesis.

We have also demonstrated that the incorporation of uracil-*1*-^{14}C into RNA by magnesium-lysozyme spheroplasts in the presence of actinomycin D is not affected by treatment with 0.1% phenethyl alcohol, but that it is partially inhibited by 0.2% phenethyl alcohol (Fig. 6).

DISCUSSION

In previous papers (3–5) we reported that all of the cell-bound alkaline phosphatase is removed from *P. aeruginosa* by 0.2 M Mg^{2+}. In the present study we have shown that the LPS

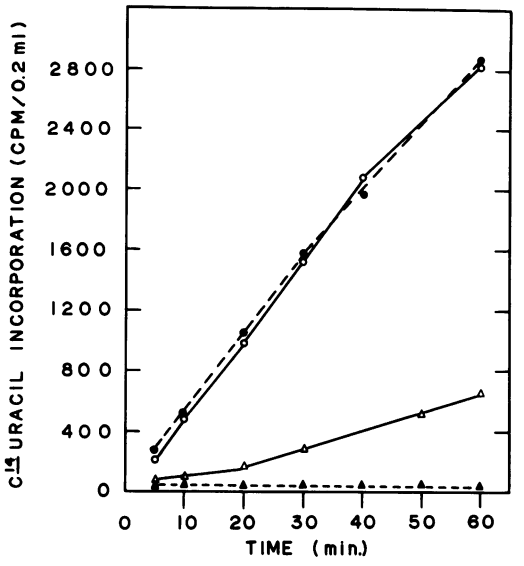

FIG. 3. *Effect of actinomycin D and EDTA on the incorporation of uracil-1-^{14}C into RNA by whole cells of P. aeruginosa. Cells were harvested and resuspended into minimal medium (3) to obtain an optical density of 1.40. These cell suspensions were incubated after the addition of 50 μg of actinomycin D per ml or 50 μg of actinomycin D per ml and EDTA (0.01 M). Uracil-1-^{14}C was added at zero time, and the incorporation of uracil into RNA was measured. Symbols: ○, whole cells; ●, whole cells with actinomycin D; △, whole cells with EDTA; ▲, whole cells with actinomycin D and EDTA.*

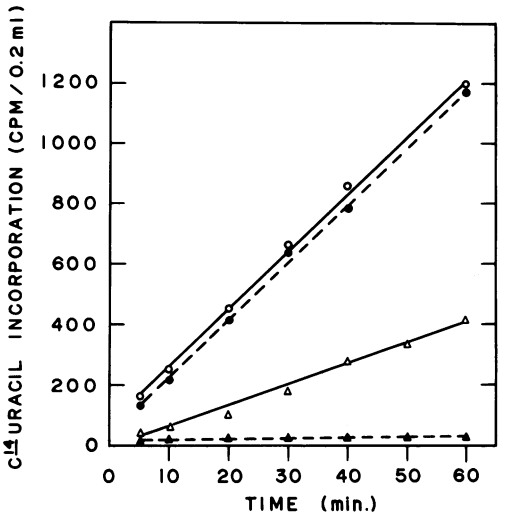

FIG. 4. *Effect of actinomycin D and EDTA on the incorporation of uracil-1-^{14}C into RNA by magnesium-lysozyme spheroplasts of P. aeruginosa. Spheroplasts were harvested and resuspended into minimal media (3) to obtain an optical density of 1.40. These spheroplasts were incubated after the addition of 50 μg of actinomycin D per ml or 50 μg of actinomycin D per ml and EDTA (0.01 M). Uracil-1-^{14}C was added at zero time, and the incorporation of uracil into RNA was measured. Symbols: ○, spheroplasts; ●, spheroplasts with actinomycin D; △, spheroplasts with EDTA; ▲, spheroplasts with actinomycin D and EDTA.*

components of the outer double-track layer are also released by 0.2 M Mg^{2+}. Physiological studies have established that a selectively permeable layer does exist in the cell wall of *E. coli* (23). This molecular sieve layer is presumed to be responsible for the retention of the enzymes in the periplasmic space (17) and for the exclusion of such molecules as lysozyme. We have shown that washing in 0.2 M Mg^{2+} causes the release of a periplasmic enzyme (alkaline phosphatase) and LPS and that it renders the cell wall sufficiently permeable to allow lysozyme access to the peptidoglycan layer in the inner region of the cell wall so that spheroplasts are formed (Fig. 1b). Furthermore, the results indicate (Table 1) that the inward passage of lysozyme to attack the peptidoglycan layer and thus form spheroplasts is proportional to the amount of LPS released during the treatment.

Winshell and Neu (39) also reported that the release of LPS following EDTA or osmotic shock treatment is required for the release of periplasmic enzymes. The release of LPS by EDTA treatment of whole cells is required also for a permeability change within the cells (36, 39).

We also found that washing in 20% sucrose released LPS and about 50% of alkaline phosphatase and allowed the inward passage of lysozyme to form 100% spheroplasts (Fig. 1c). The observation that washing in 20% sucrose causes the release of LPS and plasmolysis of cells of *P. aeruginosa* and renders the cell wall permeable to lysozyme without releasing all of the alkaline phosphatase in the cell can be explained by our observation that alkaline phosphatase is linked to a cell wall LPS component (3–5; Cheng, Costerton, and Ingram, submitted for publication). The formation of very small numbers of spheroplasts when untreated cells were incubated with lysozyme may be explained by partial leakage of alkaline phosphatase and partial release of LPS into the culture medium during growth (2; Ingram, Cheng, and Costerton, submitted for publication).

The observation that washing with 0.01 M Mg^{2+} results in almost complete retention of alkaline phosphatase and LPS is in agreement with the observation by Rogers (27) on the release of a LPS-protein complex from *E. coli* A by warm water treatment.

The presence of actinomycin D did not inhibit

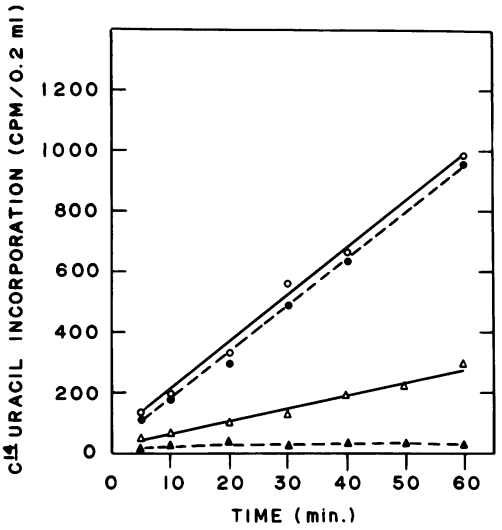

FIG. 5. *Effect of actinomycin D and EDTA on the incorporation of uracil-1-^{14}C into RNA by 20% sucrose-lysozyme spheroplasts of P. aeruginosa. Spheroplasts were harvested and resuspended into minimal media (3) to obtain an optical density of 1.40. These spheroplasts were incubated after the addition of 50 μg of actinomycin D per ml or 50 μg of actinomycin D per ml and EDTA (0.01 M). Uracil-1-^{14}C was added at zero time, and the incorporation of uracil into RNA was measured. Symbols: ○, spheroplasts; ●, spheroplasts with actinomycin D; △, spheroplasts with EDTA; ▲, spheroplasts with actinomycin D and EDTA.*

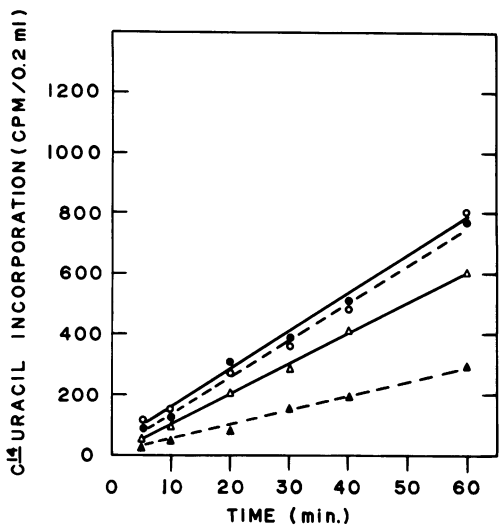

FIG. 6. *Effect of actinomycin D and phenethyl alcohol on the incorporation of uracil-1-^{14}C into RNA by magnesium-lysozyme spheroplasts of P. aeruginosa. Spheroplasts were harvested and resuspended into minimal media (3) to obtain an optical density of 1.20. These spheroplasts were incubated after the addition of 50 μg of actinomycin D per ml and 0.1% or 0.2% phenethyl alcohol. Uracil-1-^{14}C was added at zero time, and incorporation of uracil into RNA was measured. Symbols: ○, spheroplasts with 0.1% phenethyl alcohol; ●, spheroplasts with actinomycin D and 0.1% phenethyl alcohol; △, spheroplasts with 0.2% phenethyl alcohol; ▲, spheroplasts with actinomycin D and 0.2% phenethyl alcohol.*

the incorporation of uracil-*1*-^{14}C in whole cells of *P. aeruginosa*. The incorporation of uracil-*1*-^{14}C into RNA also was not inhibited in the presence of actinomycin D in 0.2 M Mg^{2+}-treated cells, 20% sucrose-treated cells, 0.01 M Mg^{2+}-treated cells, 0.01 M Tris-treated cells, H_2O-treated cells, Mg^{2+}-lysozyme spheroplasts, or 20% sucrose-lysozyme spheroplasts, even though LPS had been released and the cell walls of these cells had been damaged. In the presence of 0.01 M EDTA, whole cells, treated cells, magnesium-lysozyme spheroplasts, and sucrose-lysozyme spheroplasts are all sensitive to actinomycin D, and the incorporation of uracil-*1*-^{14}C into DNA-dependent RNA was totally eliminated. The EDTA itself has a drastic effect on the incorporation of uracil-*1*-^{14}C into RNA, and, in the complete absence of actinomycin D, it caused a fourfold reduction in the capacity for uracil-*1*-^{14}C incorporation of whole cells or spheroplasts. Roberts, Gray, and Wilkinson (25) reported that treatment of *P. aeruginosa* with EDTA released LPS from the cell wall and that the leakage of intracellular solutes is proportional to the release of LPS from the cell wall. This suggests that the cell membrane and transport system have also been damaged. We also have found that phenethyl alcohol at 0.2% does partially inhibit the incorporation of uracil-*1*-^{14}C into RNA by magnesium-lysozyme spheroplasts in the presence of actinomycin D. Phenethyl alcohol has been reported to affect the permeability of the cell membrane (34). These results contrast with previous proposals, which suggested that the effect of EDTA on the cell wall was responsible for the change in permeability or sensitivity of gram-negative bacteria to actinomycin D (10, 11, 36). Our results suggest that it is the effect of EDTA on the cytoplasmic membrane, which is responsible for the actinomycin D sensitivity of these organisms.

In conclusion, the evidence presented here strongly suggests that, although the intact cell walls may serve as a primary molecular sieve or barrier to large molecules such as lysozyme or actinomycin D (7, 9, 10, 11, 15, 20, 35, 36), they

should not be considered the only barrier. The cytoplasmic membrane was also found to be an effective barrier in systems where the cells were not treated with EDTA. It is our opinion, therefore, that previous conclusions—entry of actinomycin D prevented by cell wall only—drawn from experiments in which EDTA was used to treat whole cells (10–13, 15) or in the preparation of spheroplasts (7, 16, 24, 33) should be reconsidered in the light of these studies.

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