Influence of Suspending Media upon the Susceptibility of *Pseudomonas aeruginosa* NCTC 6750 and Its Spheroplasts to Polymyxin B

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The lytic and bactericidal actions of polymyxin B on whole cells and spheroplasts of *Pseudomonas aeruginosa* varied markedly with the suspending media, and there was little correlation between them. Relative rates of lysis of these preparations and also of *Bacillus megaterium* protoplasts suggested that polymyxin causes progressive damage to the cytoplasmic membrane, such that membrane permeability towards various ions increased as follows: $K^+ > Na^+ > NO_3^- > Cl^-$, Ca^{2+} , $H_2PO_4^-/HPO_4^{2-}$. Impermeant compounds, such as NaCl and sucrose, protected whole cells against lysis but not against death. It is suggested that lysis of whole cells by polymyxin is a secondary effect, resulting from entry of solutes normally excluded by the cytoplasmic membrane and the fragility of the damaged outer membrane. Because the degree of lysis varies with the external solutes, it should be treated with caution as a descriptor of polymyxin activity.

The polymyxin antibiotics are active against many gram-negative and some gram-positive microorganisms (18). Although their mechanism of action has not been fully defined, there is much evidence to suggest that they disrupt the structure and function of the cytoplasmic (17, 18) and outer membranes (5, 23, 26) of gram-negative bacteria by interaction with both phospholipids and lipopolysaccharide (10, 24, 27), with concomitant leakage of cytoplasmic constituents. It has been proposed that inhibition of growth and respiration is due to a direct interaction between polymyxin and cytoplasmic membrane (6, 22). La Porte et al. (12) demonstrated that this was not necessarily so and that interaction of polymyxin B with the outer membrane alone was sufficient in their system to disrupt normal respiration and growth in *Escherichia coli*.

Leakage of small molecules and cell lysis are associated with low polymyxin concentrations, but neither occur at higher ones, which nevertheless cause rapid death (4). This suggests that autolytic enzymes, inhibited by high concentrations of polymyxin, may be instrumental in drug-induced lysis (20). Teuber (25) observed that primary damage to the cytoplasmic membrane does not kill cells instantaneously but that it

leads to irreversible lytic events and death, which can be circumvented by the use of solid rather than liquid growth media. No explanation was offered for this effect of test media, but Newton (16) noted that 1% saline slows down the rate of leakage of 260-nm-absorbing substances from undamaged cells.

This paper describes the effects of different suspending media on the lytic and bactericidal actions of polymyxin B and their relation to the effects of the drug on membrane permeability. Most of the suspending media tested were salts of monovalent ions. It is well known that divalent ions antagonize the action of polymyxin (15). However, monovalent ions do not interfere with the action of polymyxin in allowing access of *N*-tolyl- α -naphthylamine-8-sulfonic acid to the protein-containing portions of the cell (17). *Pseudomonas aeruginosa* is characteristically lysed by polymyxin, possibly due to special features of its outer membrane (2), and was therefore used as the main test organism.

A preliminary report of some of these results has been published (13).

MATERIALS AND METHODS

Organisms. *P. aeruginosa* NCTC 6750 and an sporogenous strain of *Bacillus megaterium* (KM⁻) were used throughout. Stock cultures were maintained at room temperature on nutrient agar slopes, subcultured at fortnightly intervals.

Chemicals. Polymyxin B was kindly donated by J.

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D. Gurney, Burroughs Wellcome, Ltd., Beckenham, Kent. Lysozyme, tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride and *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffers were obtained from Sigma Chemicals Ltd., Poole, Dorset. All other reagents were of the purest grade available (British Drug Houses Ltd., London).

Growth conditions and preparation of cell suspensions. Carbon-depleted, stationary-phase cells grown in simple salts liquid media were used throughout. For *P. aeruginosa* the medium was composed as described by Finch and Brown (8), and for *B. megaterium* it was composed as described by Brown and Hodges (3). Cells were grown at 37°C in 250-ml Erlenmeyer flasks containing 50 ml of medium in a shaking water bath, harvested by centrifugation ($17,000 \times g$, 10 min) at 37°C, and resuspended in appropriate test menstrua at an optical density (E_{470}) of 0.2, using an SP600 spectrophotometer (Pye Unicam, London).

Lysis of whole-cell suspensions. Lysis of whole-cell suspensions was assessed by measuring the decrease in optical density (E_{470}) after polymyxin addition to cell suspensions prepared to an optical density (E_{470}) of 0.2 ($= 2 \times 10^8$ cells ml^{-1}) in buffer at pH 7.5 (20 mM HEPES plus 5 mM ammonium phosphate). The various test compounds were added to produce concentrations isomolar with 200 mM sodium chloride, and the optical density was read immediately and after 60 min of equilibration at 37°C. Experiments were performed twice in duplicate, and results were expressed as percent decrease in optical density.

Bactericidal activity. Bactericidal activity was assessed against whole-cell preparations of *P. aeruginosa*, prepared and equilibrated as above. Polymyxin (20 IU ml^{-1}) was added, and the mixtures were shaken at 37°C for 75 min. Samples were withdrawn at suitable intervals into lecithin broth to stop the reaction. Viable counts were made by spreading 0.25-ml samples of appropriate culture dilutions in nutrient broth on predried nutrient agar plates. The colonies were counted after overnight incubation at 37°C. Experiments were performed twice. Results were averaged and were expressed as the percentage of the surviving population. The confidence limits for each experiment lay between ± 6 and 14% of the numbers counted.

Preparation of *B. megaterium* protoplasts. Protoplasts were prepared by a method based on that of Repaske (19). Cells were harvested by centrifugation ($17,000 \times g$, 10 min), washed three times at 37°C in distilled water and resuspended to an optical density (E_{470}) of 1.0 in Tris-hydrochloride buffer (pH 7.5, 10 mM) containing sucrose (15%, wt/vol) and lysozyme (33 $\mu\text{g ml}^{-1}$). The mixture was incubated without shaking at 37°C for 15 min, after which time protoplast formation, as assessed by phase-contrast microscopy, was complete. The protoplasts were harvested by centrifugation ($6,000 \times g$, 15 min) and resuspended to an optical density (E_{470}) of 0.3 in solutions of the various test menstrua (700 mM).

Preparation of *P. aeruginosa* spheroplasts. Spheroplasts were prepared by a method based on that of Repaske (19). Cells were harvested by centrifugation ($6,000 \times g$, 20 min) at 4°C, washed twice at 4°C in Tris-hydrochloride buffer (pH 8.0, 30 mM), and resuspended in 16 ml of Tris-hydrochloride buffer (pH 8.0, 3.0 mM) containing sucrose (20%, wt/vol) at 4°C.

The suspension was held on ice for 5 min. One milliliter of a precooled lysozyme solution (7 mg ml^{-1}) and 3 ml of ethylenediaminetetraacetate (100 mM in Tris-hydrochloride buffer, 30 mM, pH 8.0) were added simultaneously. The suspension was mixed by gentle agitation and allowed to stand on ice for 10 min. The suspension was then gradually diluted with cold distilled water until spheroplast formation, as determined by phase-contrast microscopy, was complete. The spheroplasts were then harvested and resuspended (as above for protoplasts).

Lysis of protoplast and spheroplast suspensions. Samples (3 ml) of the spheroplast or protoplast suspensions (optical density, 0.3 [E_{470}]) in 700 mM solutions of various salts were transferred to 1-cm² cuvettes at room temperature. Lysis rates were assessed after the addition of varying concentrations of polymyxin B by monitoring rates of change in optical density (transmittance, E_{470}) with an SP600 spectrophotometer (Pye-Unicam, London) and chart recorder. This produced linear traces from which rates of lysis could be calculated. Determinations were performed twice in duplicate.

RESULTS

Susceptibility of whole-cell preparations to polymyxin B. Figure 1 shows that the degree of lysis varied with the test menstrua, using solutes at concentrations isomolar with 200 mM sodium chloride. Those suspensions with added water, ammonium acetate, or potassium acetate were most susceptible, whereas sodium acetate gave moderate protection. Suspensions with added sucrose or sodium chloride showed little lysis. The protective action of sodium chloride was dependent upon its concentration over a narrow range. Some effects were detectable with

FIG. 1. Effect of various test menstrua isomolar with 200 mM NaCl upon the lysis of *P. aeruginosa* whole cells, suspended in buffer, by polymyxin B (20 IU ml^{-1}). Symbols: ●, NH_4 acetate; ○, K acetate; ▲, KCl; △, NH_4Cl ; ■, water; □, Na acetate; ◆, $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$; ▼, NaCl; ▽, sucrose. Control: No added polymyxin gave a 7% decrease in optical density after 60 min.

100 mM and reached a maximum at 150 to 200 mM (Table 1).

The relative protective capability of the solutes changed when drug susceptibility was assessed by viability rather than lysis (Fig. 2). Sucrose did not significantly alter the survival of cells from the controls. However, cells suspended in sodium chloride appeared to be more susceptible. Potassium phosphate and, to a lesser extent, potassium chloride were moderately protective.

Effects of solutes on the optical densities of whole-cell suspensions of *P. aeruginosa*. Using the same concentrations as in Fig. 1 and 2, ammonium acetate had no effect upon the optical density of cell suspensions, but there was an immediate rise of about 25% after addition of sodium or potassium acetate or sodium, potassium, or ammonium chloride. Cells suspended in sodium chloride retained this increase during 1 h of equilibration at 37°C, but those suspended in potassium acetate reverted almost to the con-

trol optical density. Suspensions in other solutes showed partial reversion (Table 2). The compounds which afforded the highest protection against the lytic action of polymyxin were associated with increases in optical densities stable throughout the period of equilibration.

Susceptibility of spheroplast and protoplast preparations to polymyxin B. Spheroplasts suspended in ammonium acetate lysed immediately. In a number of other salt solutions they were completely stable; the rates of polymyxin B-induced lysis were assessed in these at various drug concentrations (Fig. 3). The relative susceptibilities of the suspensions to polymyxin B varied with the test solutions in the order: potassium acetate > sodium acetate > ammonium nitrate > ammonium chloride, ammonium hydrogen phosphate, and calcium acetate. Similar results were obtained with *B. megaterium* protoplasts (Fig. 4), but much higher concentrations of polymyxin B were required to effect their lysis than for the *Pseudomonas* spheroplasts or whole-cell suspensions.

TABLE 1. Effect of sodium chloride concentration upon lysis of *P. aeruginosa* whole-cell suspensions by polymyxin B

NaCl concn ^a (mM)	% Lysis ^b after 60 min
0	47
10	49
50	52
100	31
150	17
200	15

^a Cells were suspended in 20 mM HEPES-5 mM NH₄ phosphate buffer (pH 7.5).
^b Percent decrease in optical density (E₄₇₀) after addition of polymyxin B (20 IU ml⁻¹).

FIG. 2. Effect of different test menstrea isosmolar with 200 mM NaCl upon the bactericidal activity of polymyxin B towards *P. aeruginosa*. Symbols: ○, KH₂PO₄/K₂HPO₄; □, KCl; ●, water; ■, sucrose; △, NaCl; ↓ (solid lines), addition of polymyxin B (20 IU ml⁻¹); dotted lines, no polymyxin B added.

TABLE 2. Effect of solutes on the optical density of *P. aeruginosa* whole-cell suspensions and on susceptibility to lysis by polymyxin B

Added solute ^a	% Change in optical density		% Lysis ^b by polymyxin
	1 min	60 min	
None	0	-3.0	48
NH ₄ acetate	-1.0	-3.0	50
K acetate	21.5	4.5	49
Na acetate	26.5	13.5	44
KCl	24.5	10.0	31
NH ₄ Cl	25.0	15.0	29
NaCl	24.0	21.5	16

^a Cells were suspended in 20 mM HEPES-5 mM NH₄ phosphate buffer, pH 7.5. All solutes were added to give concentrations isosmolar with 200 mM NaCl.
^b Percent decrease in optical density, 60 min after addition of polymyxin B (20 IU ml⁻¹).

FIG. 3. Effect of various test menstrea upon the rates of lysis of *P. aeruginosa* spheroplasts by polymyxin B. Symbols: ○, K acetate; ●, Na acetate; □, NH₄NO₃; ■, NH₄Cl; △, Ca acetate; ▲, (NH₄)₂HPO₄. O.D., Optical density.

FIG. 4. Effect of various test menstrea upon the rate of lysis of *B. megaterium* protoplasts by polymyxin B. Symbols: ○, K acetate; ●, Na acetate; □, NH_4NO_3 ; ■, NH_4Cl ; △, Ca acetate; ▲, $(\text{NH}_4)_2\text{HPO}_4$. O.D., Optical density.

DISCUSSION

Suspensions of spheroplasts are stable in isotonic solutions of salts, which comprise a membrane-permeant and -impermeant ion pair. The ready movement of the permeant ion into the cells is retarded by the resulting electrical gradient produced by its nonpermeant ion partner (9). If suspensions are treated in such a way as to destroy this specific impermeability, then the ion pair is able to equilibrate into the spheroplasts, which swell rapidly and lyse due to the concomitant osmotic entry of water. Comparable effects may be seen with whole cells. Permeant compounds may enter the cells together with water, but lysis may be prevented by the cell walls unless these are also damaged. Impermeant ions or compounds cause loss of water, shrinkage of the cell, and rises in optical density (14).

As observed by Hamilton (9), spheroplasts of *P. aeruginosa* rapidly lysed in ammonium acetate. These ions had no effect upon the optical density of whole-cell suspensions. It was concluded that they freely permeate the membrane and that this may be correlated with their absence of effect upon whole-cell lysis by polymyxin (Fig. 1). Cells suspended in potassium acetate were equally sensitive to the lytic action of this drug; those in sodium acetate were less so. These results suggest that an early action of polymyxin is to destroy the relative impermeability of the cytoplasmic membrane, first to K^+ and then to Na^+ ions. Increased K^+ permeability of the cytoplasmic membrane has been commonly observed as an early indicator of drug action for many membrane-active antimicrobial agents (11; W. A. Hamilton, Symposium of Microbial Cell Surfaces and Membranes Group, Meet. Soc. Gen. Microbiol., April 1978, Southampton). Following from this, the slower lysis of cells suspended in potassium or ammonium

chloride indicates the onset of drug-induced membrane permeability to Cl^- ions. As expected, the results for these salts were similar. On the same rationale, permeability towards phosphate ions occurs at about the same time as that towards chloride.

Suspending cells in sodium chloride or sucrose afforded them a high degree of protection against lysis by polymyxin. Optical density changes indicated that *P. aeruginosa* cells are almost impermeable to sodium chloride (Table 1). Similar results were obtained by Bernheim (1) for both sodium chloride and sucrose.

Thus, the results with whole cells suggest that polymyxin B, rather than causing generalized damage to the cytoplasmic membrane, progressively increases its permeability to a number of molecular species in the sequence $\text{K}^+ > \text{Na}^+ > \text{Cl}^-$, $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^- > \text{sucrose}$. Water enters the cell with the permeant ion and lysis follows, owing to polymyxin-mediated damage to the restraining outer membrane. The release of periplasmic enzymes from *E. coli* by polymyxin (5), for example, argues for a considerable disruption of the outer membrane. Furthermore, the concentration of sodium chloride critical for preventing polymyxin-mediated lysis (Table 2) suggests that its role is osmotic protection.

To test this hypothesis further, *P. aeruginosa* spheroplasts and *B. megaterium* protoplasts were suspended in solutions containing combinations of one permeant ion with one nonpermeant ion species and exposed to varying concentrations of polymyxin B. Spheroplasts and protoplasts were stable in potassium or sodium acetate, whereas the data in Table 1 for whole-cell preparations suggest that these ions may be moderately permeant. This anomaly is possibly a function of pH in these systems, which were unbuffered. Mitchell and Moyle (14), found that potassium or sodium acetate failed to penetrate *E. coli* at pH 9 but entered rapidly at pH 7.

The results with polymyxin (Fig. 3 and 4) confirmed the observations on whole-cell preparations. The susceptibilities of spheroplasts and protoplasts suspended in $\text{K}^+ > \text{Na}^+ > \text{NO}_3^- > \text{Cl}^-$, HPO_4^{2-} , Ca^{2+} . It is probable that lysis of cells in the presence of Cl^- , HPO_4^{2-} , or Ca^{2+} was the result of additional damage, since these ions possess widely varying ionic radii and are unlikely to become membrane permeant simultaneously in the absence of generalized nonspecific damage.

The marked difference in susceptibility between *P. aeruginosa* spheroplasts and *B. megaterium* protoplasts to polymyxin B probably reflects dissimilarities in the phospholipid compositions of their respective cytoplasmic membranes (7). This observation is in contrast to the suggestion of Hamilton (9) that gram-negative

spheroplasts and gram-positive protoplasts might have similar susceptibilities to membrane-active agents.

The relative susceptibility of whole-cell *P. aeruginosa* suspensions in the presence of different test menstria changed when the measure of drug susceptibility was bactericidal activity rather than lysis (Fig. 2). Sodium chloride appeared to increase the rate of cell death, although it protected against lysis. Sucrose, although it was equally effective in circumventing lysis, had no effect on the death rate. The potassium salts tested, however, conferred some protection in both situations. Increased permeability to the K^+ ions, an essential cell component with a major role in osmoregulation, seems to be an early result of polymyxin action. Possibly high external K^+ concentrations may slow down the K^+ loss from damaged cells. The K^+ ion pool concentration is related to the medium sodium chloride content (21). High external sodium chloride concentrations may therefore magnify the physiological consequences of K^+ loss from the osmoregulatory pool.

We suggest, therefore, that at the concentrations of polymyxin used, an early event leading to cell death is a progressive and specific increase in membrane permeability to ions but that this process may be distinct from lysis, a secondary action due to loss of osmotic protection after outer membrane damage. On a cautionary note, our results emphasize that care must be exercised when relating drug-induced cell lysis with death of cells and that the influence of test menstria upon the magnitude of such responses may be great.

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