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# Additivity of action between polysorbate 80 and polymyxin B towards spheroplasts of *Pseudomonas aeruginosa* NCTC 6750

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When polymyxin B and polysorbate 80 were used together against spheroplasts of *Pseudomonas aeruginosa*, the activities were found to be additive. These substances have previously been reported to act synergistically against *P. aeruginosa*, but little or no intrinsic activity towards intact cells has been attributed to polysorbate 80. We suggest that in addition to enhancing polymyxin B penetration to the cytoplasmic membrane, polysorbate 80 may also act as an antimicrobial agent when polymyxin-induced damage to the outer membrane facilitates the surfactant's passage through the cell envelope.

Polymyxin B is active against a variety of Gram-negative and some Gram-positive bacteria (Newton 1956) possibly by disrupting the structure and function of the cytoplasmic (Newton 1955, 1956) and outer membranes (Cerny & Teuber 1971; Teuber 1970; Warren et al 1957) of Gram-negative micro-organisms by interaction with phospholipids and lipopolysaccharides (Teuber 1973; Hsu-Chen & Feingold 1973; Weber & Tsang 1976) causing concomitant leakage of cytoplasmic constituents. Recent work (La Porte et al 1977) suggests that in some circumstances the prime mechanism of action is directed towards the outer membrane, and that this alone is sufficient to disrupt growth and respiration. The lytic activity of polymyxin B, however, probably involves interaction at the cytoplasmic membrane. The outer membrane of *Pseudomonas aeruginosa* represents a substantial lipophilic barrier against penetration of drugs to the underlying cytoplasmic membrane (Brown 1975). It is therefore not surprising that there have been reports of synergism between the polymyxins and non-ionic surfactants (Brown 1966; Brown & Richards 1964; Brown & Winsley 1969; 1971). Indeed, Brown & Winsley (1969, 1971) reported that polymyxin B and polyoxyethylene sorbitan mono-oleate (polysorbate 80) act synergistically against *P. aeruginosa* on viability, cellular leakage and lysis, whereas polysorbate 80 alone possessed little intrinsic activity against this organism. They proposed that polysorbate 80 altered outer membrane lipid structure allowing penetration of polymyxin B. Therefore the combined effect of these agents towards cells with damaged or partially removed outer-membranes has been examined using spheroplasts of *P. aeruginosa*.

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## MATERIALS AND METHODS

### *Chemicals*

Polymyxin B was kindly donated by Dr J. D. Gurney of Burroughs Wellcome Ltd., Beckenham, Kent. Carbenicillin ('Pyopen') was obtained from Beecham Research Laboratories, Brentford and polysorbate 80 from Koch-Light Laboratories, London. All other reagents were of the purest grade available (British Drug Houses Ltd., London).

### *Organisms*

*Pseudomonas aeruginosa* NCTC 6750 was used. Stock cultures were maintained at room temperature (20 °C) on nutrient agar slopes (Oxoid, CM3) subcultured at monthly intervals.

### *Growth conditions and preparation of spheroplasts*

Chemically-defined liquid media were used comprising, $(NH_4)_2SO_4$, (1 mM); KCl (5 mM); NaCl (5 mM); $FeSO_4$ (0·1 $\mu$g ml$^{-1}$ $Fe^{2+}$); $MgSO_4$ (2·0 $\mu$g ml$^{-1}$ $Mg^{2+}$); D-glucose (10 mM) and ammonium phosphate buffer (pH 7·8, 20 mM). Cells were cultured for approximately 20 h at 37 °C in 250 ml. Erlenmeyer flasks containing 100 ml medium at 100 throws min$^{-1}$ in a shaking water bath. Cells were harvested in late logarithmic growth phase (optical density, $E_{470}$, 0·5) by centrifugation (5000 *g*, 20 min) at 37 °C. The cells were resuspended to an optical density ($E_{470}$) of 0·1 in 20 ml fresh medium containing sucrose (0·5 M) at 37 °C in 100 ml conical flasks and incubated for 1 h at 50 throws min$^{-1}$ in a

shaking water bath. Carbenicillin (5000 $\mu$g ml$^{-1}$) was added and cells incubated (as above) for 4 h after which time spheroplast formation, as assessed by phase-contrast microscopy, was complete. Spheroplasts were harvested by centrifugation (5000 *g*, 20 min) at 37 °C and resuspended to an optical density ($E_{470}$) of 0·15 in sucrose (0·5 M).

*Lysis of spheroplast suspensions*

Spheroplast suspensions were prepared (as above) to an optical density ($E_{470}$) 0·15 in sucrose (0·5 M) and 4·8 ml amounts were added to tubes containing 0·2 ml solutions of varying concentrations of polymyxin B, polysorbate 80 or both. The tubes were gently agitated, maintained at 37 °C and the optical density ($E_{470}$) measured at appropriate time intervals for 3 h. Experiments were performed twice in duplicate and results expressed as percentage decrease in optical density.

## RESULTS AND DISCUSSION

The activities of polymyxin B and polysorbate 80 towards spheroplasts of *P. aeruginosa* stabilized in sucrose (0·5 M) were assessed by following the decrease in optical density (Fig. 1). Incubation of the mixtures beyond 180 min did not significantly increase the degree of lysis observed. The decrease in optical density ($E_{470}$) at 180 min was therefore used as an indicator of drug sensitivity and plotted with drug concentration (Fig. 2). Similar plots for the two agents acting in combination are shown in Fig. 3. There was no significant difference between the observed response for the combined use of these agents, and the summation of their individual activities at similar concentrations (Fig. 3). Their combined activity was therefore considered to be additive rather than synergistic.

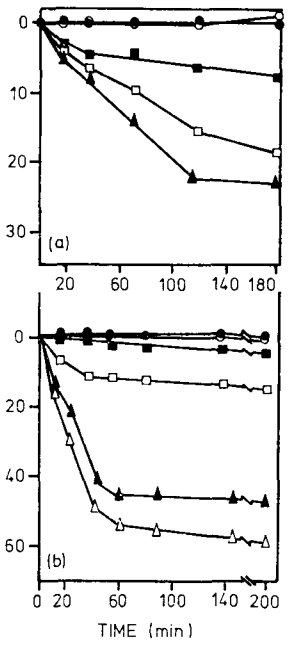


FIG. 1. Lysis of *P. aeruginosa* spheroplasts by polymyxin B and polysorbate 80 (a) polysorbate 80 concentration (% w/v), ●, 0·0; ○, 0·002; ■, 0·008; □, 0·014; ▲, 0·02. (b) polymyxin B concentration (i.u. ml$^{-1}$), ●, 0·0; ○, 3·0; ■, 9·0; □, 15·0; ▲, 21·0; Δ, 27·0. Ordinate: % decrease in optical density [$E_{470}$].

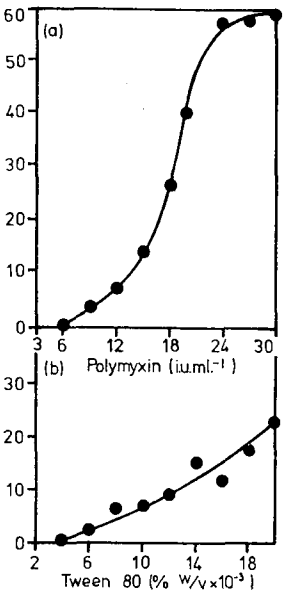


FIG. 2. Lysis of *P. aeruginosa* spheroplasts by (a) polymyxin B and (b) polysorbate 80. Ordinate: decrease in optical density [$E_{470}$] after 180 min (%).

Polysorbate 80 enhances the activity of several antimicrobial agents against a variety of microorganisms (Kirby & Dubos 1947; Fisher 1948; Bliss & Warth 1950). Brown & Winsley (1969, 1971) report that although polysorbate 80 possesses little activity against *P. aeruginosa* it acts synergistically with polymyxin B towards this organism. They suggested that subtle perturbations of the outer membrane by polysorbate 80 might enhance the activity of polymyxin B by increasing penetration to the underlying cytoplasmic membrane, its prime lytic site (Newton 1956). The possibility that polysorbate 80 and polymyxin B act in separate ways upon the cytoplasmic membrane was not excluded.

It has been suggested that both polysorbate 80 and polymyxin B directly affect outer membrane per-

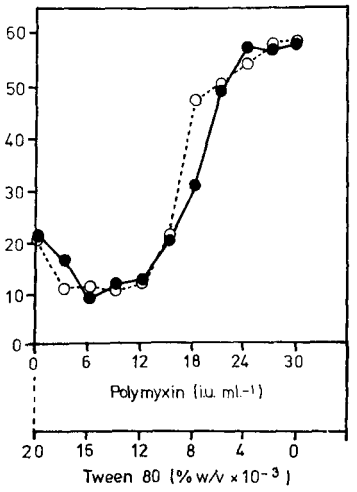


FIG. 3. Additivity of lytic action against *P. aeruginosa* spheroplasts by polysorbate 80 and polymyxin B in combination. ○ - - - - ○, Empirical value for combined activity; ●——●, value for combined activity derived by summation of individual activities. Ordinate: decrease in optical density [$E_{470}$] after 180 min (%).

meability. Polysorbate 80 renders the Gram-negative microorganisms permeable to the fluorescent dye 2-anilinonaphthylamine-8-sulphonic acid and more sensitive to changes in pH, temperature and tonicity (Brown & Winsley 1969). Disruption of outer membrane permeability alone by polymyxin B has been demonstrated to inhibit growth and respiration of *P. aeruginosa* (La Porte et al 1977).

This paper reports additivity of activity for these compounds towards spheroplasts of *P. aeruginosa*. Polysorbate 80 acts lytically against spheroplasts of *P. aeruginosa*, therefore lack of intrinsic activity towards intact cells suggests failure to reach the cytoplasmic membrane. Indeed, concentrations of polysorbate 80 > 10% w/v are required to reduce the growth rate of *P. aeruginosa* cultures (Brown & Richards 1964) whilst < 0·01% will lyse the corresponding spheroplast. Synergism of action, previously reported, might therefore be primarily due to penetration of polysorbate 80 to the cytoplasmic membrane, facilitated by polymyxin-damage to the outer membrane, and secondly to the combined action of these agents upon outer membrane structure and function. Possibly the non-ionic polysorbate 80 selectively disrupts proteins in the cytoplasmic membrane but not in the outer membrane (Schnaitman 1971).

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