Antipseudomonal Effect of Polymyxin and Phenylethanol

R. M. E. RICHARDS* and R. J. McBRIDE[x]

Abstract □ The effect of subinhibitory concentrations of phenylethanol and polymyxin, alone and in combination, on the growth rate of phenylethanol-sensitive and -resistant or polymyxin-sensitive and -resistant *Pseudomonas aeruginosa* was determined. The combination had an additive effect on sensitive *P. aeruginosa*, while the behavior of the resistant strains indicated that cross-resistance occurs between polymyxin and phenylethanol, which was confirmed by sterilization time measurements. It is suggested that there is no cross-resistance between polymyxin and either benzalkonium chloride or chlorhexidine acetate.

Keyphrases □ *Pseudomonas aeruginosa*, phenylethanol sensitive and resistant or polymyxin sensitive and resistant—effect of subinhibitory concentrations of phenylethanol and polymyxin, evaluation of components and combination □ Polymyxin—effect on *Pseudomonas aeruginosa*, alone and in combination with phenylethanol □ Phenylethanol—effect on *Pseudomonas aeruginosa*, alone and in combination with polymyxin □ Preservatives—evaluation of phenylethanol and polymyxin, alone and in combination, against *Pseudomonas aeruginosa*

Polymyxin B sulfate at a concentration of 1000 units/ml was reported to kill *Pseudomonas aeruginosa* inocula within 30 min (1). Kohn *et al.* (2) reported that polymyxin was unsatisfactory as an ophthalmic preservative since it required 18 hr to sterilize for *P. aeruginosa*; these authors recommended that such preservatives should sterilize a large inoculum of *P. aeruginosa* in less than 1 hr. The difference between the results may be due to differences in resistance between the test organisms. Some ophthalmic formulations contaminated with 10^6 *P. aeruginosa*/ml did not pass this test; but if phenylethanol was included in addition to the preservative, the preparations sterilized such inocula within 1 hr (3, 4). Combinations of phenylethanol with benzalkonium chloride, chlorhexidine acetate, or phenylmercuric nitrate were found to be more effective than the single preservatives against *P. aeruginosa* resistant to either phenylethanol or the other member of the combination (5). Phenylethanol–benzalkonium chloride and phenylethanol–chlorhexidine acetate combinations evaluated at subinhibitory concentrations had an effect against *P. aeruginosa* greater than additive, *i.e.*, greater than the sum of the effects of the single substances used alone (6).

The objectives of this study were to determine if: (*a*) phenylethanol enhances the effect of polymyxin against *P. aeruginosa*, (*b*) the combination has an effect greater than additive against *P. aeruginosa*, and (*c*) the combination is more effective against *P. aeruginosa* resistant to either component in the combination.

EXPERIMENTAL

Materials—Reagent grade phenylethanol[1] and polymyxin B sulfate[2] BP were used. *P. aeruginosa*[3] (NCTC 6750) was the test organism, Oxoid[4] nutrient broth No. 2 was the growth medium, and 37° was the incubation temperature. Maintenance of stock cultures was as previously described (7).

Preparation of Resistant Cells—Phenylethanol-resistant *P. aeruginosa* and log phase cultures of phenylethanol-resistant cells were prepared as described previously (5).

Polymyxin-resistant *P. aeruginosa* was prepared as follows: 0.1 ml of an overnight culture of *P. aeruginosa* was used to inoculate 100 ml of nutrient broth containing 1 unit/ml of polymyxin and incubated for 2 days. An inoculum of 0.1 ml of the resulting culture was used to inoculate 100 ml of broth containing 5 units polymyxin/ml. A similar process was used to inoculate media containing 10, 20, 50, 100, and 200 units/ml in succession. Selected cultures were stored in a refrigerator at 4° until required.

Log phase cultures of polymyxin-resistant *P. aeruginosa* were prepared by adding 1 ml of refrigerated *P. aeruginosa* resistant to 20 units polymyxin/ml to 100 ml of medium containing 10 units polymyxin/ml, and the product was incubated overnight in a water bath shaken at 105 throws/min. Then 1.0 ml of the resulting culture was added to 100 ml of prewarmed medium containing 3 units polymyxin/ml/ml. When the extinction at 420 nm in a 1-cm cell was approximately 0.35, 1.0 ml of the culture was transferred to each of four flasks containing 100 ml of medium with 3 units polymyxin/ml. Sufficient polymyxin and phenylethanol to give a final concentration of 3 units/ml and 0.2%, respectively, alone and in combination, were added to three flasks when the extinction at 420 nm was approximately 0.12 in each flask; water was added as a control to the fourth flask.

Growth Rate Studies—The effect of phenylethanol concentration on the growth of *P. aeruginosa* was described previously (8). A similar procedure was adopted with polymyxin to determine the growth rates of *P. aeruginosa* in nutrient broth containing 1, 2, 3, 4, 5, 6, 7, or 8 units polymyxin/ml. The growth rate for each concentration of polymyxin is expressed as a percentage of the growth rate of the control culture containing no polymyxin.

The effect of phenylethanol on polymyxin action was observed

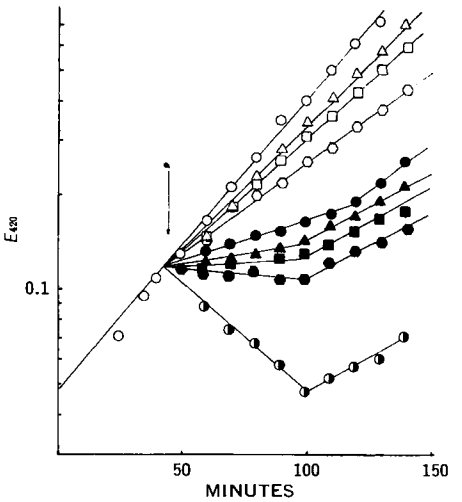

Figure 1—*Effect of various concentrations of polymyxin B sulfate on* P. aeruginosa. *Polymyxin was added at the time indicated by the arrow to give the following concentrations. Key: ○, water added; △, 1 unit polymyxin/ml; □, 2 units polymyxin/ml; ◇, 3 units polymyxin/ml; ●, 4 units polymyxin/ml; ▲, 5 units polymyxin/ml; ■, 6 units polymyxin/ml; ◆, 7 units polymyxin/ml; and ◑, 8 units polymyxin/ml.*

[1] British Drug Houses, Poole, Dorset, England.

[2] Burroughs Wellcome Ltd., Dartford, England.

[3] National Collection of Type Cultures, Colindale, London, England.

[4] Oxo Ltd., London, England.

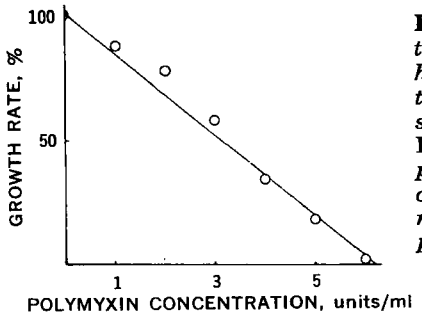

Figure 2 — *Correlation between subinhibitory concentrations of polymyxin B sulfate and growth of* P. aeruginosa *expressed as a percentage of the growth rate in the absence of polymyxin.*

by comparing the growth of *P. aeruginosa* in nutrient broth containing 0.15% phenylethanol after the addition of 3 or 5 units polymyxin/ml with similar cultures in nutrient broth only.

In a test for additivity between polymyxin and phenylethanol, four mixtures, calculated to be equipotent, were added to separate cultures of *P. aeruginosa* and the subsequent growth was followed. The effect of each mixture on *P. aeruginosa* was the difference between the growth rate of the organisms in the control culture and that in the medium containing the mixture. This effect was expressed as a percentage of the control rate and is referred to as the percentage effect.

Sterilization Times—The times required for aqueous solutions of polymyxin and phenylethanol, both alone and in combination, to sterilize large inocula of phenylethanol-sensitive and -resistant or polymyxin-sensitive and -resistant *P. aeruginosa* were determined as described previously (9, 10). The sterilization times for aqueous solutions of benzalkonium chloride and chlorhexidine acetate were also determined.

DISCUSSION

The effect of polymyxin on the growth rate differs from that reported for phenylethanol (8). In subinhibitory concentrations of phenylethanol, *P. aeruginosa* grew at a uniform rate. Figure 1 shows that in the presence of 4 units polymyxin/ml or more, there is an initial slow growth rate or lysis followed by a faster growth rate. Garrett and Brown (7) obtained nonlinear rates with *E. coli* and chloramphenicol and attributed this result to consumption of the antibiotic, development of resistant mutants, or the presence of antibiotic-resistant strains in the original inoculum. The same authors also suggested that the initial growth rate of the culture should be used for evaluation purposes. Figure 2 shows the linear correlation between growth of *P. aeruginosa* and concentration of polymyxin, indicating that the minimum inhibitory concentration of polymyxin against log phase cells is 6 units/ml. This linearity of effect is similar to that of phenylethanol (8) on *P. aeruginosa* and of chloramphenicol, tetracycline (11), or spectinomycin (12) on *E. coli*. Garrett *et al.* (11), in explaining this rectilinear regression as opposed to the more usual curvilinear regression, suggested that only a small fraction of the receptor sites in the biophase need be blocked for total inhibition of bacterial growth.

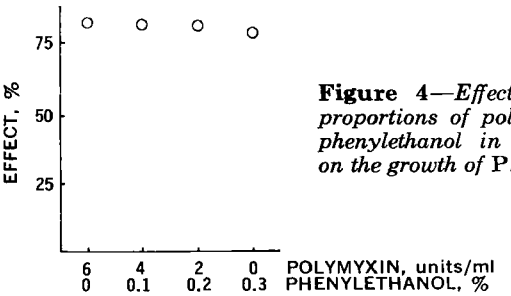

Figure 4—*Effect of relative proportions of polymyxin and phenylethanol in combination on the growth of* P. aeruginosa.

Figure 3 shows the effect of polymyxin on the growth of *P. aeruginosa* in nutrient broth containing 0.15% phenylethanol. A polymyxin concentration of 3 units/ml produces a single response in nutrient broth, but in the phenylethanol medium it produces a bilinear effect similar to that of 6 units polymyxin/ml (Fig. 1). Similarly, a concentration of 5 units polymyxin/ml shows lysis initially in the phenylethanol medium such as occurs with 8 units polymyxin/ml in nutrient broth only. Increasing the concentration of phenylethanol in the medium did not increase the effect of polymyxin but did increase the time during which the initial slow growth rate or lysis occurred.

Figure 3 also shows that 0.15% phenylethanol and 3 units polymyxin/ml have very similar effects on the growth in nutrient broth, suggesting that these concentrations are equipotent. When 3 units polymyxin/ml was added to nutrient broth containing 0.15% phenylethanol, the initial difference in growth rate from the control culture expressed as a percentage of the control growth rate was double that in nutrient broth only. With the minimum inhibitory concentrations of 0.3% phenylethanol and 6 units polymyxin/ml, both drugs have similar concentration effects on growth rate correlations. This implies that 0.1% phenylethanol is equivalent in effect to 2 units polymyxin/ml in its effect on the growth rate of log phase *P. aeruginosa* and that combinations of the substances would have an additive effect. The results in Fig. 4 show that the effects of four mixtures, which should show similar effects if additive, are analogous. This supports the hypothesis that the drugs are additive in their effect on *P. aeruginosa* and have the same mechanism of action.

The polymyxin-resistant and phenylethanol-resistant strains of *P. aeruginosa* in log phase cultures are not so readily affected as this type culture by polymyxin and phenylethanol, alone and in combination (Fig. 5). This result indicates cross-resistance between polymyxin and phenylethanol and contrasts with the effect of benzalkonium chloride and chlorhexidine acetate on phenylethanol-resistant *P. aeruginosa* (5). Such results suggest that there

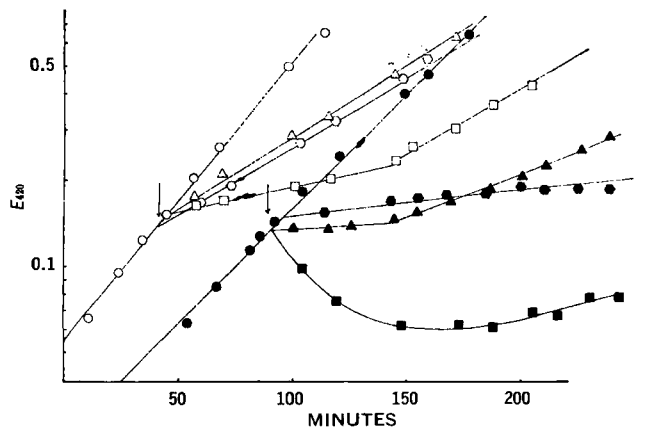

Figure 3—*Effect of 3* (△) *and 5* (□) *units polymyxin B sulfate/ml on* P. aeruginosa *growing in nutrient broth (open symbols) and in nutrient broth containing 0.15% phenylethanol (closed symbols). Sufficient phenylethanol to give 0.15%* (○) *was also added to each medium. Additions were made at the times indicated by the arrows.*

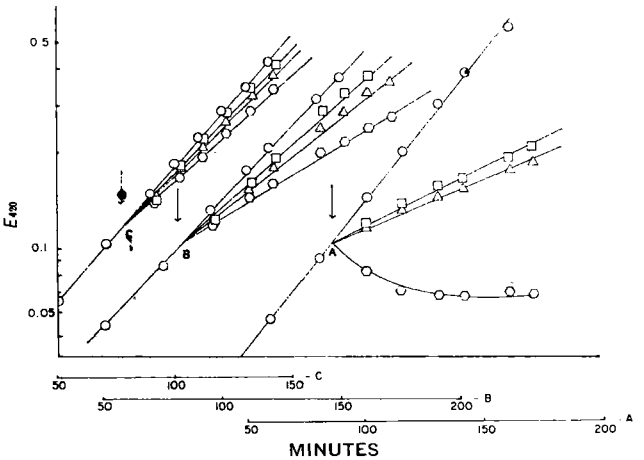

Figure 5—*Effect of 3 units polymyxin/ml* (□), *0.2% phenylethanol* (△), *and a combination of both* (○) *on the growth of* P. aeruginosa (*A*), P. aeruginosa *resistant to phenylethanol* (*B*), *and* P. aeruginosa *resistant to polymyxin* (*C*). *Drugs were added at the times indicated by the arrows.*

Table I—Sterilization Times for *P. aeruginosa*, Sensitive and Resistant to Either Phenylethanol or Polymyxin, in Solutions of Polymyxin and Phenylethanol, Alone and in Combination, Benzalkonium Chloride, and Chlorhexidine Acetate

Antibacterial(s) Concentration(s)	*P. aeruginosa* NCTC 6750		*P. aeruginosa* Resistant to 0.6% Phenylethanol		*P. aeruginosa* Resistant to 200 units Polymyxin/ml	
	Cells × 10^5/ml	Sterilization Time, min	Cells × 10^5/ml	Sterilization Time, min	Cells × 10^5/ml	Sterilization Time, min
Benzalkonium chloride, 0.005%	6.7	30	4.2	<5	6.9	25
Chlorhexidine acetate, 0.005%	6.7	40	4.2	<5	6.9	15
Phenylethanol, 0.4%		24 hr		>24 hr		>24 hr
Polymyxin, 25 units/ml	13.0	15	8.5	>300	5.3	>24 hr
Phenylethanol, 0.4%, and polymyxin, 25 units/ml		15		240		>24 hr
Polymyxin, 100 units/ml				240		>24 hr
Phenylethanol, 0.4%, and polymyxin, 100 units/ml	—	—	12.0	45	5.8	>24 hr
Polymyxin, 300 units/ml						>24 hr
Phenylethanol, 0.4%, and polymyxin, 300 units/ml	—	—	—	—	6.9	>24 hr

is no cross-resistance between polymyxin and benzalkonium chloride or chlorhexidine acetate.

Lacey (13) divided combinations of drugs which are additive or synergistic (greater than additive) into six classes according to the presumed site of action of the drug, the presumed route by which the drug arrives at the site, and the presumed biochemical sequence blocked. The characteristics used to decide into which class a combination of two drugs falls are the effect of the combination on a homogeneous population, the pattern of cross-resistance, and a comparison of the drug antagonists. Polymyxin and phenylethanol combinations are additive in effect and show cross-resistance, so they would be in one of two possible classes, as defined by Lacey, that have the same mode of action. The factor that decides whether the combination is Class 1 (same route) or Class 2 (different routes) is the route by which the drugs arrive at the site. The factor that decides the class is whether the effect of one drug can be stopped by an antagonist having no effect on the other; no potent antagonists have been reported for either drug. The phenylethanol–chlorhexidine and phenylethanol–benzalkonium chloride combinations do not fit readily into Lacey's classification. The greater-than-additive effect of the combinations indicates that the drugs have different sites of action. The lack of cross-resistance or one-way resistance suggests that the drugs have different routes, *i.e.*, Class 6 (13). Although cross-resistance is not found in Class 6, an increased sensitivity to one drug may accompany resistance to the other. This is known as "collateral sensitivity" (14) and was exhibited by phenylethanol with benzalkonium chloride and chlorhexidine acetate. Barber (15) stated that combinations used for their synergistic effect in antibacterial chemotherapy belong to Class 6.

Sterilization times were determined to confirm that the results obtained with subinhibitory concentrations also pertain when inhibitory concentrations are used. Table I gives the results of attempts to determine suitable polymyxin–phenylethanol concentrations which would sterilize large inocula of sensitive and resistant *P. aeruginosa* within 1 hr. If a culture was sterilized in less than 1 hr by a combination, that strain was not tested with the higher concentrations of polymyxin. The results corroborated the findings of the growth studies in that: (*a*) the type culture was more affected than the resistant strains, and (*b*) the combination of phenylethanol and polymyxin was more effective than either preservative alone against the type culture and phenylethanol-resistant culture. The polymyxin-resistant strain was the most resistant tested and was not sterilized by any combination. An attempt was made to determine the minimum inhibitory concentration of the polymyxin-resistant strain, but growth occurred in a medium containing 3000 units polymyxin/ml, which was the highest concentration tested. The effect of benzalkonium chloride and chlorhexidine acetate on the three strains shows that there is no cross-resistance between polymyxin and these bactericides against *P. aeruginosa*. The lack of cross-resistance between polymyxin and benzalkonium chloride is in agreement with previous results (16).

It was suggested (5) that, among the desirable properties of an antibacterial combination, the spectrum of activity should include pathogenic Gram-positive and Gram-negative organisms and should also be effective against organisms resistant to one component of the combination. The present results show that the additive combination of polymyxin and phenylethanol would not be a suitable combination because of cross-resistance. Furthermore, both substances have weak activity against Gram-positive organisms. Polymyxin does not show cross-resistance with benzalkonium chloride or chlorhexidine acetate, but collateral sensitivity was not shown because the sterilization times for benzalkonium-resistant and chlorhexidine-resistant *P. aeruginosa* were not determined against polymyxin.

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* Present address: Pharmacy Department, University of Rhodesia, P.O. Box MP 167, Salisbury, Rhodesia.

ˣ To whom inquiries should be directed.