$$C_p = \frac{M_C}{M_Q}(Q_a - Q_s) - \frac{M_C}{M_B}\left[xW - \frac{K'_{sp(BQ)}}{Q_s}\right] \quad \text{(Eq. A13)}$$

Combining and rearranging Eqs. A13 and A10 result in Eq. 5 as shown in the text.

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Cross-Resistance in *Pseudomonas aeruginosa* Resistant to Phenylethanol

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Abstract □ The effects of benzalkonium, chlorhexidine, and phenylmercuric nitrate on exponential phase cultures of phenylethanol-sensitive *Pseudomonas aeruginosa* NCTC 6750 growing in nutrient broth and on phenylethanol-resistant cells growing in nutrient broth plus 0.2% phenylethanol v/v were determined. The resistant cultures grown in the presence of phenylethanol were more sensitive to benzalkonium, chlorhexidine, and phenylmercuric nitrate than phenylethanol-sensitive cells grown in nutrient broth. Phenylethanol–antibacterial combinations were active against phenylethanol-resistant and phenylethanol-sensitive cultures. Survival times in solutions of benzalkonium, chlorhexidine, and phenylmercuric nitrate were determined for overnight *P. aeruginosa* cells grown in nutrient broth and for overnight *P. aeruginosa* cells trained to be resistant to phenylethanol and grown in nutrient broth plus 0.5% phenylethanol v/v. The cells grown in the presence of the phenylethanol were more sensitive to the action of the three antibacterials than the cells grown in nutrient broth alone.

Keyphrases □ *Pseudomonas aeruginosa* cultures, phenylethanol resistant and sensitive—effect of benzalkonium, chlorhexidine, phenylmercuric nitrate □ Phenylethanol-resistant and sensitive *Pseudomonas aeruginosa* cultures—effect of benzalkonium, chlorhexidine, phenylmercuric nitrate □ Benzalkonium effect—phenylethanol-resistant and sensitive *Pseudomonas aeruginosa* cultures □ Chlorhexidine effect—phenylethanol-resistant and sensitive *Pseudomonas aeruginosa* cultures □ Phenylmercuric nitrate effect—phenylethanol-resistant and sensitive *Pseudomonas aeruginosa* cultures

Phenylethanol was first recommended for use as a preservative for ophthalmic solutions in 1953 (1) following a report that it was active against Gram-negative organisms (2). Other workers found phenylethanol to have too slow an antibacterial action for use in ophthalmic solutions (3). It has been shown that phenylethanol exerts its antibacterial effects by modifying the permeability properties of the bacterial cell (4, 5).

Richards *et al.* (5) suggested that phenylethanol had a use in combination with other antibacterial agents in the preservation of ophthalmic solutions and other pharmaceutical solutions. Subsequently, the activities of a wide range of preservatives used in the preservation of ophthalmic solutions were shown to be enhanced when used in combination with phenylethanol (5–10).

Although there is widespread use of antibacterial combinations as preservative systems in pharmaceutical solutions (11), there is little or no published support for some of the combinations used. Neither has there been any enumeration of the properties required of an antibacterial combination or of the individual components of the combination. The following properties seem desirable for the combination:

1. The antibacterial combination should have a faster sterilization time against the test organism than the same concentration of either of the antibacterials used individually. For ophthalmic solutions, this sterilization time should be 1 hr. or less for an inoculum of *Pseudomonas aeruginosa* having a final concentration in the test system of not less than 10^6 cells/ml. (3, 9, 10).
2. The antibacterial combination should still be effective when the test organism has acquired a resistance to either one of the antibacterials.
3. The spectrum of activity of the combination should include pathogenic Gram-positive and Gram-negative bacteria and fungi.

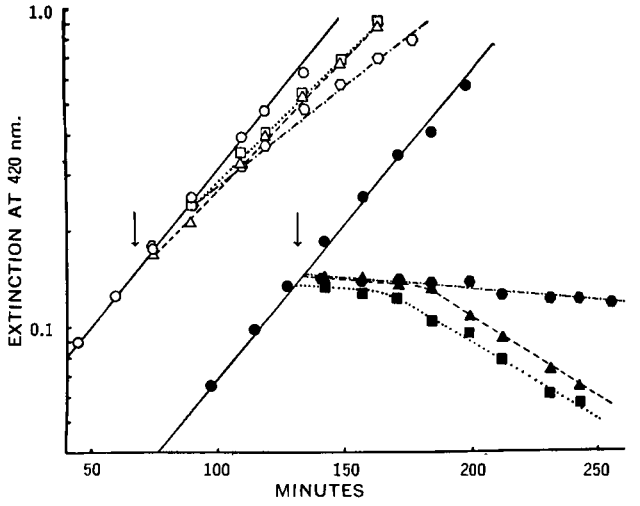

Figure 1—*Effect of benzalkonium, chlorhexidine, and phenylmercuric nitrate on the growth of exponential phase cultures of* P. aeruginosa *grown in nutrient broth (A) and in nutrient broth plus 0.2% phenylethanol v/v (B). Addition of antibacterials was made at the times indicated by the arrows. Key: A—○, 0.6 ml. of water; △, benzalkonium chloride to give a 0.003% solution; □, chlorhexidine acetate to give a 0.0002% solution; and ⬡, phenylmercuric nitrate to give a 0.0004% solution. B—●, 0.6 ml. of water; ▲, benzalkonium chloride to give a 0.003% solution; ■, chlorhexidine acetate to give a 0.0002% solution; and ⬢, phenylmercuric nitrate to give a 0.0004% solution.*

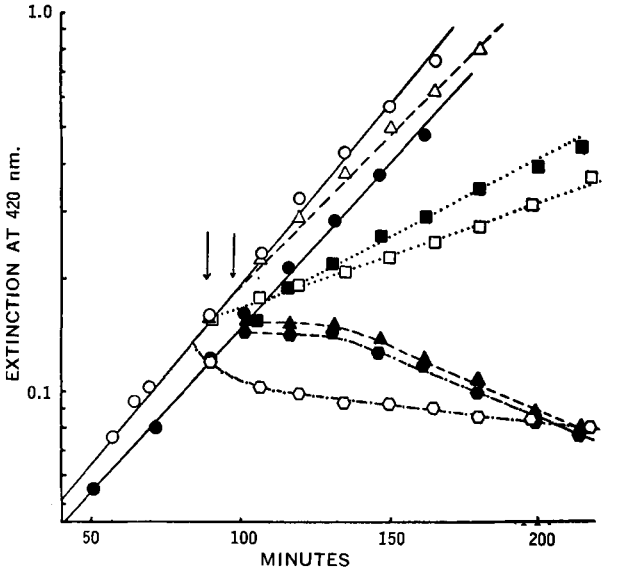

Figure 2—*Effect of benzalkonium and phenylethanol singly and in combination against exponential phase* P. aeruginosa *grown in nutrient broth (A) and in nutrient broth plus 0.2% phenylethanol v/v (B). Addition of antibacterials was made at the times indicated by the arrows. Key: A—○, 0.6 ml. of water; △, benzalkonium chloride to give a 0.003% solution; □, phenylethanol to give a 0.2% solution; and ⬡, benzalkonium to give a 0.003% solution and phenylethanol to give a 0.2% solution. B—●, 0.6 ml. of water; ▲, benzalkonium chloride to give a 0.003% solution; ■, phenylethanol to give a 0.2% solution; and ⬢, benzalkonium to give a 0.003% solution and phenylethanol to give a 0.2% solution.*

The following properties seem desirable for the individual members of the combination:

1. One member of the combination should be chosen for its rapid action against a wide spectrum of microorganisms.
2. The other member of the combination should have properties that enable it to potentiate the action of the first antibacterial, particularly against organisms that have developed a resistance to the first antibacterial.

Phenylethanol–antibacterial preservative systems have already been shown to be effective against *P. aeruginosa* resistant to the "antibacterial" component of the phenylethanol–antibacterial combination (6). Information is also required on the activity of phenylethanol–preservative combinations against *P. aeruginosa* cells resistant to the phenylethanol component of the combination. Therefore, the purpose of this investigation was to evaluate phenylethanol-resistant cells for cross-resistance with benzalkonium, chlorhexidine, and phenylmercuric nitrate. These three chemicals were chosen because of their broad spectrum of antimicrobial activity and their widespread use in preservation.

EXPERIMENTAL

P. aeruginosa NCTC[1] 6750 was the test organism. The growth medium was oxoid[2] nutrient broth No. 2, and incubation was at 37°. Phenylmercuric nitrate[3], 2-phenylethanol[3], chlorhexidine acetate BPC[4], and benzalkonium chloride BP[5] were used. Maintenance of stock cultures and experimental details were described previously (5–7, 9, 12).

Preparation of Resistant Cells—Resistance was developed as follows: 0.1 ml. of an overnight culture of *P. aeruginosa* was used to inoculate 100 ml. of nutrient broth containing 0.2% phenylethanol v/v. This culture was incubated for 2 days, and then 0.1 ml. was used to inoculate 100 ml. nutrient broth containing 0.3% phenylethanol v/v. After 2 days of incubation, 0.1 ml. was used to inoculate 100 ml. broth plus 0.4% phenylethanol v/v. The incubation time was then reduced to overnight, and successive cultures were made until a culture was obtained growing in nutrient broth containing 0.7% phenylethanol v/v. This culture was further subcultured to produce resistant cells for growth rate and survival time studies.

Effect on Growth Curves—*Single Chemicals*—The effect of benzalkonium, chlorhexidine, and phenylmercuric nitrate against phenylethanol-sensitive and phenylethanol-resistant, exponentially dividing *P. aeruginosa* was determined.

Phenylethanol-sensitive cells consisted of 1 ml. overnight *P. aeruginosa* culture inoculated into 100 ml. prewarmed nutrient broth and incubated until the extinction at 420 nm. was 0.3–0.35. Then 1-ml. quantities of this culture were inoculated separately into four flasks, each containing 100 ml. prewarmed nutrient broth. The extinction measurements of the resulting cultures were followed; and at an extinction of approximately 0.1, the chemicals were added to the cultures as already described (7, 12).

The exponential phase phenylethanol-resistant cells were prepared as follows: 0.1 ml. of the culture resistant to 0.7% phenylethanol v/v in nutrient broth was inoculated into 100 ml. nutrient broth plus 0.5% phenylethanol v/v and incubated overnight. Then 1 ml. of this culture was inoculated into 100 ml. prewarmed nutrient broth plus 0.2% phenylethanol v/v. This culture was grown to an extinction value between 0.3 and 0.35 as were phenylethanol-sensitive cells. Then 1-ml. quantities of this culture were used to inoculate replicate flasks of prewarmed nutrient broth plus 0.2% phenylethanol v/v. The effect of the chemicals on the growing cultures was determined as before (7, 12), using extinction measurements.

Phenylethanol–Antibacterial Combinations—The effect of combinations of phenylethanol plus each of the other three antibacterials in turn was determined using exactly the same technique as for the single chemicals. Suitable controls were included so that a

[1] National Collection of Type Cultures, Colindale, London, England
[2] Oxo Ltd., London, England.
[3] British Drug House, Poole, Dorest, England.
[4] I.C.I., Alderly Park, Macclesfield, Cheshire, England.
[5] Macarthy Ltd., Glasgow, Scotland.

Table I—Survival Times for *P. aeruginosa*, Sensitive and Resistant to Phenylethanol, in Solutions of Benzalkonium, Chlorhexidine, and Phenylmercuric Nitrate

Antibacterial and Concentration, %	*P. aeruginosa* NCTC 6750		*P. aeruginosa* Resistant to 0.5% Phenylethanol	
	Inoculum, $\times 10^5$ Organisms/ml.	Survival Time, min.	Inoculum, $\times 10^5$ Organisms/ml.	Survival Time, min.
Benzalkonium chloride, 0.005	6.7	25–30	4.2	<5
Chlorhexidine acetate, 0.005	6.7	30–40	4.2	<5
Phenylmercuric nitrate, 0.002	10.9	450–480	6.1	390–420

particular experiment provided the following information: the effect of the antibacterial alone and in combination with phenylethanol, and the effect of phenylethanol alone, all against both phenylethanol-sensitive and phenylethanol-resistant cultures.

Survival Times in Antibacterial Solutions—The survival times of high inocula of overnight phenylethanol-sensitive cells were determined using duplicate tubes of one concentration each of benzalkonium, chlorhexidine, and phenylmercuric nitrate, as previously described (5, 9).

The phenylethanol-resistant culture was prepared using 0.1 ml. of the culture growing in nutrient broth plus 0.7% phenylethanol v/v to inoculate 100 ml. nutrient broth containing 0.5% phenylethanol v/v. The resultant culture was incubated overnight, and the survival time was determined on samples from this culture against the three chemicals concurrently with the survival time determinations on the phenylethanol-sensitive cells.

RESULTS AND DISCUSSION

The effect of benzalkonium, chlorhexidine, and phenylmercuric nitrate on the growth rate of phenylethanol-resistant cells in nutrient broth plus 0.2% phenylethanol v/v was much greater than the effect of the same concentration of each chemical against exponentially dividing phenylethanol-sensitive cells in nutrient broth (Fig. 1). This indicates that there is no cross-resistance between phenylethanol and any one of these three chemicals. In fact, phenylethanol-resistant cells were more sensitive to the action of benzalkonium, chlorhexidine, and phenylmercuric nitrate than phenylethanol-sensitive cells. It, therefore, appears that phenylethanol can have an effect on the permeability of *P. aeruginosa* without having any effect on its viability.

It can be seen from Fig. 2 that the combination of phenylethanol with benzalkonium is still effective against phenylethanol-resistant cells. Similar results were obtained with phenylethanol–chlorhexidine and phenylethanol–phenylmercuric nitrate combinations.

Phenylethanol alone is less effective against phenylethanol-resistant cells than against phenylethanol-sensitive cells, as would be expected, but benzalkonium alone is much more effective against phenylethanol-resistant cells. (This effect is the same as in Fig. 1.) It is seen from Fig. 2, however, that benzalkonium alone is as effective as the phenylethanol–benzalkonium combination against phenylethanol-resistant cells. This shows that the activity of the combination against phenylethanol-resistant cells is due to the activity of the benzalkonium component of the combination.

The survival time results (Table I) show that there is no cross-resistance between phenylethanol and any of the three antibacterials tested but that the phenylethanol-resistant cells are more sensitive to these antibacterials than the phenylethanol-sensitive cells. This further confirms that where the phenylethanol resistance is due to training the *P. aeruginosa* cells in the presence of the phenylethanol, these phenylethanol-resistant cells are affected in such a way by the phenylethanol that they are made sensitive to the action of benzalkonium, chlorhexidine, and phenylmercuric nitrate.

Phenylethanol is again shown to have properties that make it well suited for use in combination with other antibacterials for the preservation of pharmaceutical solutions against contamination with *P. aeruginosa*. It is also effective in combination with other antibacterials against *Escherichia coli* and *Proteus vulgaris*[6]. By combining these results with those previously published (5–10), it is seen that phenylethanol possesses the properties enumerated in the introduction for use as one component of an antibacterial combination suitable for use in preservation. It needs to be used in combination with an antibacterial having a wide spectrum of antibacterial activity, because phenylethanol is more active against Gram-negative than Gram-positive organisms (2). Therefore, it is very suited for use in combination with either benzalkonium or chlorhexidine, because both of these antibacterials are rapidly lethal to Gram-positive organisms. Benzalkonium and chlorhexidine may both be ineffective against certain resistant Gram-negative organisms, however, especially *Pseudomonas*. In these situations the phenylethanol should be active itself and also reverse the resistance of the resistant cells to both benzalkonium and chlorhexidine.

From Table I, it can be seen that phenylmercuric nitrate was slow-acting against both phenylethanol-sensitive and phenylethanol-resistant cells. Phenylmercuric nitrate is known to be slow acting (3, 13), but phenylethanol–phenylmercuric nitrate combinations in alkaline solutions had a more rapid action (5).

The effect of phenylethanol on fungi is being investigated.

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