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Phenylethanol enhancement of preservatives used in ophthalmic preparations

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The growth rates of *P. aeruginosa* in subinhibitory concentrations of antibacterial preservatives alone and in combination with phenylethanol were determined by an extinction method. Phenylethanol enhances the effect of benzalkonium chloride on growing cultures of *P. aeruginosa* and the results have been confirmed by viable counts. A similar effect is shown by phenylethanol in combination with chlorbutol, chlorhexidine diacetate, chlorocresol, merthiolate, methyl and propyl hydroxybenzoate mixture and phenylmercuric nitrate.

Phenylethanol has been shown to enhance the action of phenylmercuric nitrate (PMN) in the preservation of fluorescein solutions against the effects of contamination with *Pseudomonas aeruginosa* (Richards, Suwanprakorn & others, 1969). A permeability effect is indicated (Silver & Wendt, 1967), thus enabling concentrations of PMN, which, alone, could not effect penetration, to pass into the cell and exert an antibacterial effect. The present work was carried out to test the suggestion of Richards & others (1969) that phenylethanol might be used with advantage in combination with other antibacterial agents in preservation of pharmaceuticals.

MATERIALS AND METHODS

The test organism was *P. aeruginosa* NCTC 6750 and the counting procedure and inactivating broth used were described by Richards & others (1969). Stock cultures were maintained and logarithmic phase cultures prepared according to Brown & Richards (1964). Incubation was at 37°. The PMN, chlorbutol, *p*-chloro-*m*-cresol and 2-phenylethanol were all BDH laboratory reagents. The chlorhexidine acetate and propyl *p*-hydroxybenzoate were B.P.C. and the benzalkonium chloride B.P. Merthiolate (a 0·1% w/v thiomersal and 0·1% w/v monoethanolamine solution) was from Eli Lilly Limited and methyl *p*-hydroxybenzoate from MacFarlane Smith Limited. Cell numbers were estimated by colony counts. Growth rates of log phase cultures (shaken at 105 throws min^{-1} in a Mickle incubator) were determined by extinction measurements in 1 cm cells at 420 nm with a Unicam SP600 spectrophotometer.

Comparison of extinction measurements with viable counts

On three separate days, 1 ml quantities of log phase *P. aeruginosa* having an extinction of about 0·35 at 420 nm were used to inoculate 99 ml of prewarmed nutrient broth at 37° and the subsequent growth rate followed by means of viable counts and extinction measurements. The results were expressed in terms of doubling times with the 95% confidence limits. The relation between viable counts and extinction measurements is given in Fig. 1, where log extinction and log viable counts are plotted against time.

Effect of phenylethanol on P. aeruginosa *growing in subinhibitory concentrations of the different preservatives*

1 ml of log phase *P. aeruginosa* (extinction 0·35 at 420 nm) was added to each of four conical flasks, A, B, C and D, containing 99 ml of prewarmed nutrient broth (in A and B) or 99 ml of prewarmed nutrient broth containing benzalkonium chloride 30 μg/ml (in C and D). The flasks were maintained at 37° and samples taken for extinction measurements. When the growth rate had been established, prewarmed phenylethanol to a final concentration of 0·2% v/v was added to flasks B and D. At the same time an equal volume of prewarmed water was added to A and C. Subsequent extinction measurements were made at timed intervals as before. Fig. 2 shows the results obtained as graphs of log extinction against time. The regression coefficient (b) of the log extinction upon time and the 95% confidence limits were calculated for each line and the results expressed as doubling times or mean generation times (0·301/b) in minutes.

The following procedure was used to determine if the effect of the phenylethanol–benzalkonium combination was additive or not. The increase in doubling time, relative to the doubling of the control culture, was determined for phenylethanol and benzalkonium alone. The sum of these relative increases was compared with the relative increase in doubling time for the phenylethanol–benzalkonium combination.

Similar experiments were made using chlorhexidine, PMN, chlorocresol, chlorbutol, merthiolate and a mixture of methyl and propyl hydroxybenzoate, the results being given in Table 1.

Table 1. *The effect of ophthalmic preservatives and phenylethanol–preservative combinations on the growth of* Pseudomonas aeruginosa *NCTC 6750 in nutrient broth.*

Preservative	Concentration (%)	Nutrient broth containing preservative Doubling time (min)	Nutrient broth containing 0·2% v/v phenylethanol and preservative Doubling time (min)	Relative increase[a]
None	—	33·3	106·5 (103·4–109·8)	2·2
Benzalkonium chloride	0·003	37·4	1023 (481–lysis)	26·4
Chlorbutol	0·2	35·0	lysis	lysis
Chlorhexidine diacetate	0·000275	77·7	lysis	lysis
Chlorocresol	0·024	37·9	13 900 (855–lysis)	368
Thiomersal (as merthiolate)	0·0001	65·5	545 (429–750)	7·3
Methyl hydroxybenzoate and propyl hydroxybenzoate	0·023 0·0115	33·4 —	368 (292–496)	10
Phenylmercuric nitrate	0·0004	67·8	1683 (853–61 800)	23·8

[a] The increase in doubling time in comparison with that obtained in nutrient broth containing preservative but not phenylethanol.
The figures in parentheses are the 95% confidence limits.

This experiment was repeated to check whether extinction measurements were proportional to the viable counts when the preservatives were present in the culture

separately and in combination. The doubling times for each culture were determined by extinction measurements and concurrent viable counts.

Effect of different preservatives on P. aeruginosa *growing in subinhibitory concentration of phenylethanol*

1 ml of log phase *P. aeruginosa* (extinction 0·35 at 420 nm) was added to each of four conical flasks, E, F, G and H, containing 99 ml of prewarmed nutrient broth (in E and F) or 99 ml of prewarmed nutrient broth containing 0·15% v/v phenylethanol (in G and H). When the growth rate had been established by a sequence of extinction measurements, 0·5 ml of prewarmed benzalkonium chloride solution was added to flasks F and H to produce a final concentration of 35 μg/ml when the cultures had similar extinctions. At approximately the same time, 0·5 ml of prewarmed water was added to flasks E and G. The extinctions were then measured at intervals for a further period of time. Similar experiments were made with chlorhexidine and PMN. The results expressed as doubling times for each culture (Table 2).

Table 2. *The effect of the addition of benzalkonium chloride, chlorhexidine diacetate and phenylmercuric nitrate on the growth of* P. aeruginosa *NCTC 6750 in nutrient broth containing phenylethanol.*

Preservative	Concentration (%)	Nutrient broth containing 0·15% phenylethanol Doubling time (min)	Relative increase
None	—	42·7	—
Benzalkonium chloride	0·0035	94·9 (90·3–99·9)	1·22
Chlorhexidine diacetate	0·000275	52·5 (50·1–54·6)	0·22
Phenylmercuric nitrate	0·00075	824·6 (416–40 600)	18·3

The figures in parentheses are the 95% confidence limits.

RESULTS AND DISCUSSION

Extinction measurements compared with viable counts

The doubling times were 30·1 (26·6–34·5), 29·4 (23·7–38·7) and 30·6 (28·0–33·7) min as determined by the viable count and 27·6 (25·11–30·7), 28·3 (27·3–29·5) and 30·6 (28·7–32·7) were the corresponding times for the extinction method. These results indicate that there is no significant difference at the 95% level (the figures in parentheses are the 95% confidence limits) between the doubling times determined by extinction measurements and those by viable counts, but the extinction measurements gave the closer limits of error.

Fig. 1 shows that the growth rate of *P. aeruginosa* NCTC 6750 can be followed by extinction measurements over the concentration range 2×10^7 to 4×10^8 cells/ml. The difficulties of determining growth rates of *P. aeruginosa* NCTC 8203 by means of extinction methods (Brown & Richards, 1964) were not experienced with strain NCTC 6750.

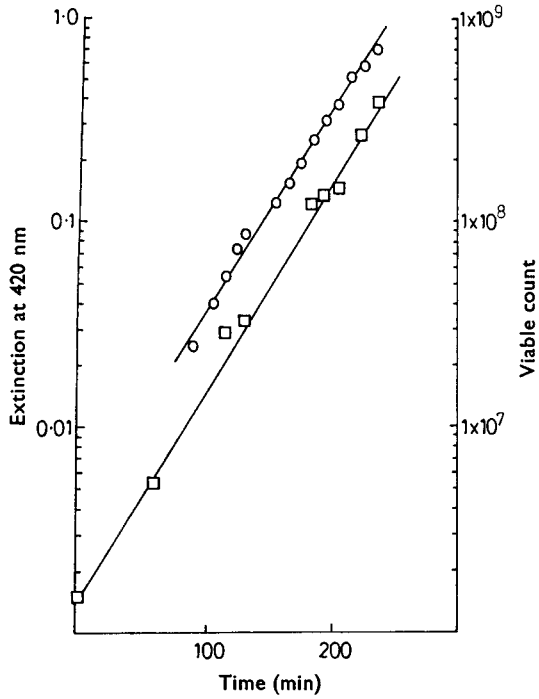

FIG. 1. Growth of *P. aeruginosa* NCTC 6750 in nutrient broth determined by measurement of extinction (○) and viable counts (□).

Effect of phenylethanol on P. aeruginosa *growing in subinhibitory concentrations of the different preservatives*

The growth rate of *P. aeruginosa* NCTC 6750 in nutrient broth containing 30 μg/ml of benzalkonium chloride does not differ markedly from that in nutrient broth alone (Fig. 2). Addition to similar media of sufficient phenylethanol to give 0·2% v/v concentration, however, gave different results. The growth rate in nutrient broth alone was reduced by phenylethanol, but that of the medium containing 30 μg/ml of benzalkonium chloride was almost stopped (Fig. 2). The sums of the relative increase in doubling time for each preservative and for 0·2% v/v phenylethanol were always less than the relative increase in doubling time for similar concentrations used in combination. Thus the combination appears to have a greater effect than addition.

Both the phenylethanol–chlorbutol and the phenylethanol–chlorhexidine combinations caused lysis of *P. aeruginosa*, while the phenylethanol–chlorocresol and phenylethanol–benzalkonium combinations were so effective that one of the 95% confidence limits indicated lysis.

The doubling times of both merthiolate and PMN alone are comparable. Merthiolate has a lower mercury content than PMN, the ratio being 1 to 2·6. Phenylethanol–merthiolate combination has a doubling time 3·2 times faster than phenylethanol–PMN combination, the ratio is similar to the mercury content of the two compounds. Thus the monoethanolamine in merthiolate appears either to enhance the activity of thiomersal or to modify the effect of the phenylethanol.

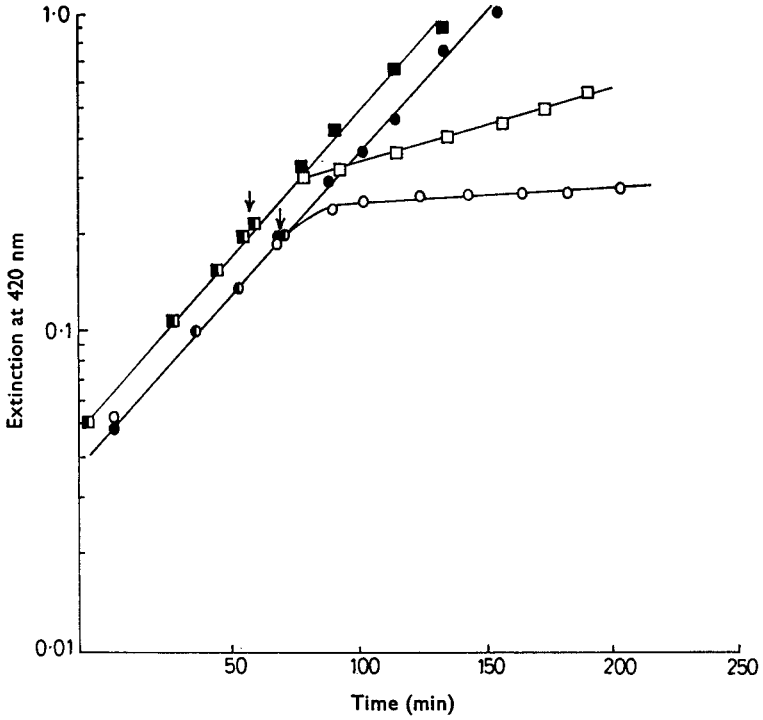

FIG. 2. The effect of 0·2% v/v phenylethanol, 0·003% w/v benzalkonium chloride, both alone and in combination on the growth of *P. aeruginosa* NCTC 6750. ■ *P. aeruginosa* grown in nutrient broth to which water was added after 58 min. □ *P. aeruginosa* grown in nutrient broth to which phenylethanol was added after 58 min. ● *P. aeruginosa* grown in nutrient broth containing 35 μg/ml of benzalkonium chloride to which water was added after 69 min. ○ *P. aeruginosa* grown in nutrient broth containing 35 μg/ml of benzalkonium chloride to which phenylethanol was added after 69 min.

Methyl and propyl hydroxybenzoates at the concentrations recommended by the B.P.C. 1959 in Solution for Eye-drops had no significant effect on the growth of *P. aeruginosa*. This is in agreement with the results of Hugo & Foster (1964).

In all of the experiments phenylethanol enhanced the effect of the preservative; no antagonism was observed. Richards (1971) has investigated the effect of phenylethanol–preservative combination against resistant *P. aeruginosa* by an end-point technique and obtained similar results. This supports the hypothesis that phenylethanol affects the permeability of the pseudomonas cell, allowing more effective penetration of the antibacterial agent (Richards & others, 1969).

The doubling times determined by means of viable counts were similar to those obtained from extinction measurements. Thus the presence of phenylethanol or benzalkonium chloride alone or in combination did not affect the extinction.

Effect of different preservatives on P. aeruginosa *growing in subinhibitory concentrations of phenylethanol*

Potentiation of the antibacterial effect of 0·15% v/v phenylethanol occurred with the three preservatives tested (Table 2), but the degree of enhancement was much reduced compared with the results for these preservatives, especially chlorhexidine, in Table 1. However, the preservatives were added to suspensions already containing

thirty times the number of viable organisms used in the previous test. The likelihood of a drop in effect is supported by the observation that the doubling time of *P. aeruginosa* in nutrient broth initially containing 0·15% phenylethanol is only slightly greater than in nutrient broth alone. *P. aeruginosa* cells selected by 0·2% phenylethanol being present in the broth before inoculation grew more than twice as fast as the cells in the log phase after addition of phenylethanol (Richards & McBride, unpublished observation). The phenylethanol–preservative combination is, however, more effective in reducing the growth rate than either phenylethanol or preservative alone.

In conjunction with previous work (Richards & others, 1969; Richards, 1971) the present results support the use of phenylethanol–preservative combinations because they are more effective in inactivating resistant contaminants than either preservative alone. The combinations also permit the use of lower individual preservative concentrations.

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