Enhancement of Benzalkonium Chloride and Chlorhexidine Acetate Activity against *Pseudomonas aeruginosa* by Aromatic Alcohols

R. M. E. RICHARDS* and R. J. McBRIDE▲

Abstract □ The actions of benzalkonium chloride and chlorhexidine acetate in combination with benzyl alcohol, 2-phenylethanol, or 3-phenylpropanol were evaluated at subinhibitory concentrations by measuring the growth rates of *Pseudomonas aeruginosa* NCTC 6750. When tested separately, all three alcohols had an effect on both benzalkonium chloride and chlorhexidine acetate that was more than additive. Phenylpropanol showed the greatest enhancement and benzyl alcohol showed the least enhancement.

Keyphrases □ Benzalkonium chloride—enhanced activity by aromatic alcohols against *Pseudomonas aeruginosa* □ Chlorhexidine acetate—enhanced activity by aromatic alcohols against *Pseudomonas aeruginosa* □ Interactions—enhancement of antibacterial action of benzalkonium chloride and chlorhexidine acetate by aromatic alcohols against *Pseudomonas aeruginosa* □ Preservatives—enhanced activity of benzalkonium chloride and chlorhexidine acetate by aromatic alcohols, tested against *Pseudomonas aeruginosa*

Formulations containing two or more preservatives are increasing in number; 50% of commercial ophthalmic preparations contain such combinations (1), but reports on their evaluation have not appeared. In the past, medicinal antibiotic combinations were evaluated by different *in vitro* techniques which resulted in different definitions to describe the synergistic, additive, antagonistic, or indifferent effects observed between the drugs. The disappointing lack of correlation between such *in vitro* results and *in vivo* effects has been reflected by a lack of reports on *in vitro* evaluation of such combinations. However, pharmaceutical preparations preserved with combinations of bactericides differ from medicinal antibiotic combinations because the preservative capacity of the preparations can be assessed by *in vitro* techniques.

Garrett (2) reviewed the various definitions of antibiotic combination effects and suggested that antibiotic combinations should be evaluated on the basis of: (*a*) additivity, where the combined effect or response is additive with respect to the separate responses of the components; and (*b*) equivalence, in which the antibiotics act in the same manner with the same dose–response curve, separately or in combination, except for a difference in the weight of an arbitrarily defined "unit dose." Garrett (2) also stated that the definition of additivity by Bliss *et al.* (3)—that when one antibiotic is added to another, the effect is improved only to the extent that would occur had an equivalent portion of the same antibiotic been added—was the most restrictive. Thus, the quantity of antibiotic having the same effect or response as the other component of the combination is added to the quantity of the same antibiotic present in the combination, giving the total amount of one antibiotic having the same response as an additive combination. Garrett and coworkers (4, 5) evaluated the additive interaction between chloramphenicol and tetracycline and between erythromycin and lincomycin by measuring the growth rates of *Escherichia coli* in media containing subinhibitory concentrations of the different antibiotics, alone and in combination.

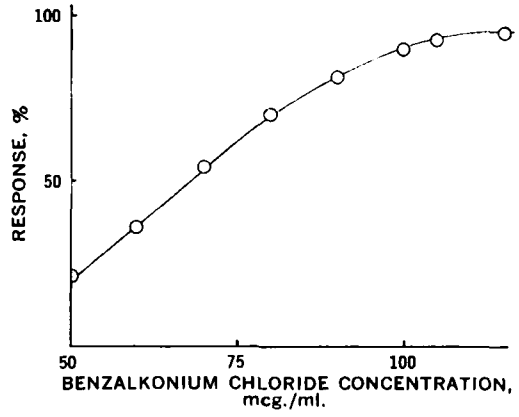

Figure 1—*Dose–response correlation for benzalkonium chloride against* P. aeruginosa.

It was recommended (6) that quick-acting preservatives should be used in ophthalmic preparations and that the final preparation should sterilize a large inoculum of *Pseudomonas aeruginosa* in less than 1 hr. A number of ophthalmic formulations inoculated with 10^6 *P. aeruginosa*/ml. did not pass this test; but when phenylethanol was present as well as the preservative, the preparations sterilized such inocula within 1 hr. (7, 8). Benzyl alcohol and phenylpropanol also enhanced the bactericidal action of benzalkonium chloride (9).

The present investigation was undertaken to determine whether the enhancement of the antibacterial action of benzalkonium chloride and chlorhexidine acetate by aromatic alcohols was greater or less than additive, using growth rate studies of log phase cultures of *P. aeruginosa* in subinhibitory concentrations of the preservatives.

EXPERIMENTAL

Materials—Benzyl alcohol[1] (reagent grade), phenethyl alcohol[1] (phenylethanol or 2-phenylethanol) (reagent grade), phenylpropanol[1] (3-phenylpropanol) (reagent grade), benzalkonium chloride BP[2], and chlorhexidine acetate BPC[3] were used. *P. aeruginosa* NCTC[4] 6750 was the test organism, Oxoid nutrient broth No. 2 was the growth medium, and incubation was at 37°. Maintenance of stock cultures and experimental details were as previously described (9).

[1] British Drug Houses, Poole, Dorset, England.
[2] McCarthy Limited, Glasgow, Scotland.
[3] ICI, Alderly Park, Macclesfield, Cheshire, England.
[4] National Collection of Type Cultures, Colindale, London, England.

Table I—Effect of Benzalkonium Chloride and Chlorhexidine Acetate, Alone and in Combination with Benzyl Alcohol, Phenethyl Alcohol, or Phenylpropanol on the Growth Rate of *P. aeruginosa*

Bactericide, Percentage Concentration	Percentage Response	Percentage Antibacterial Equivalent to Alcohol	Antibacterial Estimated to be Equivalent to Combination: Percentage Concentration	Antibacterial Estimated to be Equivalent to Combination: Percentage Response		Experimental Percentage Response for Combination
Benzalkonium chloride, 0.003	18.5					
Benzyl alcohol, 0.175	41.9	0.0041	0.0071	53.7		76.7
Phenethyl alcohol, 0.175	51.5	0.0046	0.0076	62.0		91.5
Phenylpropanol, 0.175	45.8	0.0043	0.0073	52.0		Lysis
				Mode of Action: First	Second	
Chlorhexidine acetate, 0.0002	18.5					
Benzyl alcohol, 0.175	41.9	0.0031	0.0051	32.0	Lysis	65.9
Phenethyl alcohol, 0.175	47.6	0.0033	0.0053	32.5	Lysis	86.7
Phenylpropanol, 0.175	44.7	0.0032	0.0052	32.3	Lysis	Lysis

Effect of Single Bactericide—The growth of *P. aeruginosa* in media containing different concentrations of a bactericide was followed by measuring the extinctions at 420 nm. The effect or response of a particular concentration is the difference between the growth rate of the control containing no bactericide and that of the bactericide; the percentage response is the difference expressed as a percentage of the control growth rate. The results for benzalkonium chloride and chlorhexidine acetate are illustrated in Figs. 1 and 2, where the percentage response is plotted against bactericide concentration.

Effect of Alcohol–Bactericide Combinations—The procedure with benzalkonium chloride was described previously (9), and a similar procedure was used with chlorhexidine acetate (Fig. 3). The results for both chemicals are given in Table I.

DISCUSSION

Simple addition of the individual response for each component of a combination assumes that the concentration–response correlation for the antibiotic is rectilinear, when often it is not (10). Figure 4 illustrates the influence of the concentration–response correlation upon the theoretical additive response of a combination. If the percentage response of one component, *M*, is 27%, this response is similar to that of 4 units of Component *N*. In a combination of *M* and 4.5 units of *N* (response of 30%), the theoretical additive response would be the same as that of 8.5 units of *N*. But only when the concentration response correlation is rectilinear, as in Type A, does the percentage response (57%) for the 8.5 units of *N* equal the sum of the individual responses. When the concentration response is of Type B, 8.5 units of *N* has a percentage response of 81%; Type C would indicate a response of 39%. Thus, in measuring concentration–response correlations, the concentration of a single preservative equivalent to that of the combination should be included to avoid extrapolation.

The dose–response correlation for each aromatic alcohol was rectilinear (9) and is similar to Type A in Fig. 4. Thus, in determining the theoretical additive response based on the quantity of alcohol having the same effect as the combination, the percentage responses for the components of the combination as single substances were added. The sum of either bactericide with any of the three alcohols was always less than the experimental value obtained, indicating that the response was greater than additive and increased with the molecular weight of the alcohol.

The dose–response correlation for the benzalkonium chloride (Fig. 1) was similar to Type C in Fig. 4, *i.e.*, linear at low concentrations and becoming asymptotic at higher concentrations. The concentrations of benzalkonium chloride that have the same response as each alcohol were determined, and the theoretical response based on the quantity of benzalkonium chloride having the same effect as the combination was also determined. The tabulated results again show that the combination has an effect greater than additive, with phenylpropanol having the largest increase and benzyl alcohol having the smallest.

The dose–response for chlorhexidine acetate (Fig. 2) has a bilinear correlation, showing a slow increase in response with increasing concentration to between 2.75 and 3.0 mcg./ml. and higher concentrations having a very rapid response. In all media containing more than 3.75 mcg./ml. chlorhexidine acetate, lysis occurred. A similar result was observed (11) when the growth rate of *Klebsiella cloaca* was measured in media containing different con-

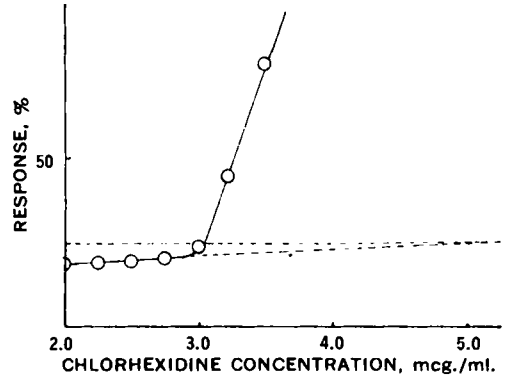

Figure 2—*Dose–response correlation for chlorhexidine acetate against* P. aeruginosa.

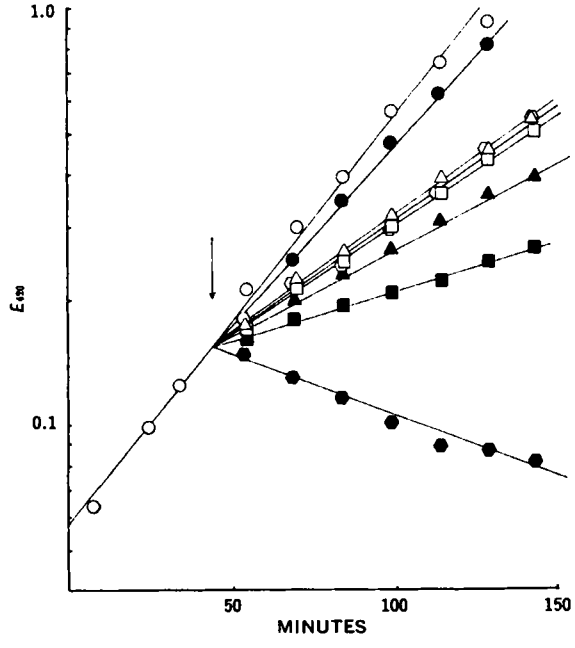

Figure 3—*Effect of 0.175% benzyl alcohol* (△), *0.175% phenethyl alcohol* (□), *and 0.175% phenylpropanol* (○), *alone (open symbols) and in combination with 0.0002% chlorhexidine acetate (closed symbols) on* P. aeruginosa. *The circles indicate alcohol-free control cultures, and additions were made at the time indicated by the arrow.*

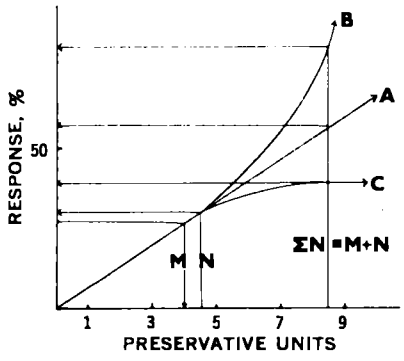

Figure 4—*Effect of the concentration–response correlation on the theoretical additive response of a combination.*

centrations of phenol. It was postulated that if an inhibitor combined with cellular enzymes, the velocity of the enzyme-catalyzed reaction will depend on the concentration of uncombined enzyme, and where an inhibitor affects more than one enzyme in different concentration ranges of inhibitor, each range will display different concentration effects or modes of action. The dose–response correlation indicates that chlorhexidine acetate has two modes of action according to the enzyme model of Harris and Morrison (11). The quantities of chlorhexidine acetate having the same responses as the alcohols would affect the growth of *P. aeruginosa* by the second mode of action; calculation of the theoretical additive response based on the equivalent quantity of chlorhexidine acetate indicates that lysis would occur (Table I). Since the alcohol has a different mode of action from chlorhexidine acetate, sufficient chlorhexidine may not be present to enable the second mode of action to occur, even though the quantity of chlorhexidine acetate in the biophase may increase due to the permeability effect of the alcohol. The amount of chlorhexidine acetate in the combination only affects the initial mode of action when used as a single substance; if this controls the growth rate, the theoretical additive response can be obtained by extrapolation of the initial dose–response correlation (Fig. 2). As shown in Table I, the theoretical percentage response was approximately 32%, which is less than that with the alcohol alone. The experimental results indicate a response greater than additive for chlorhexidine acetate in combination with the aromatic alcohols, with phenylpropanol showing the greatest enhancement and benzyl alcohol showing the least.

In a description of the desirable properties for a combination of preservatives, it has been stated that the combination should act more rapidly against the test organism than do the same concentrations of each agent used separately (12). This does not differentiate between agents that combine to give a response greater or less than additive while still exceeding that of any one member of a combination. While such differentiation may not be important, there are obvious advantages in using combinations whose response is greater than additive.

REFERENCES

(1) "Remington's Pharmaceutical Sciences," 14th ed., Mack Publishing Co., Easton, Pa., 1970, p. 1570.

(2) E. R. Garrett, *Antibiot. Chemother.*, **8**, 8(1958).

(3) E. A. Bliss, P. T. Worth, and P. H. Long, *Bull. Johns Hopkins Hosp.*, **90**, 149(1952).

(4) E. R. Garrett and M. R. W. Brown, *J. Pharm. Pharmacol.*, **15**, 185(1963).

(5) E. R. Garrett, S. M. Hernan-Ackah, and G. L. Perry, *J. Pharm. Sci.*, **59**, 1448(1970).

(6) S. R. Kohn, L. Gershenfeld, and M. Barr, *ibid.*, **52**, 967 (1963).

(7) R. M. E. Richards and R. J. McBride, *J. Pharm. Pharmacol.*, **24**, 145(1972).

(8) R. M. E. Richards and R. J. McBride, *Pharm. J. (London)*, **210**, 118(1973).

(9) R. M. E. Richards and R. J. McBride, *J. Pharm. Sci.*, **62**, 585 (1973).

(10) E. Jawetz and J. B. Gunnison, *J. Amer. Med. Ass.*, **150**, 693(1952).

(11) M. Harris and G. A. Morrison, *Nature (London)*, **191**, 1276 (1961).

(12) R. M. E. Richards and R. J. McBride, *J. Pharm. Sci.*, **61**, 1075(1972).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 14, 1973, from the *Microbiology Group, Pharmacy Department, Heriot-Watt University, Edinburgh EH1 2HJ, Scotland.*

Accepted for publication July 31, 1973.

* Present address: Pharmacy Department, University of Rhodesia, P.O. Box MP167, Salisbury, Rhodesia.

▲ To whom inquiries should be directed.