given. The estimated standard deviations of a single infrared assay of aspirin anhydride are AA, ±0.9%; aspirin ±0.4%; and salicylic acid, ±0.2%; in terms of per cent of the total sample. The 95% confidence limits may be estimated by doubling these values. This is based on a new set of standards run on each day. This information demonstrates the foolishness of reporting apparent values by tenths of per cent. For example, if the obtained numerical value of salicylic acid and aspirin in one assay ranges from 0.0 to 0.4%, the amounts present are actually immeasurable and may be considered absent by the sensitivity of the assay.

The greatest assay variation is among days of assay rather than among replicates on a given day. If a routine control procedure is put into effect, assay duplication on the same day is an unnecessary expense. If the most precise assay estimate is desired at minimum cost, special consideration must be given to assaying the sample for several days.

The use of one set of standards or an average value for standards gives less total variability in the AA assay, ±0.6%, and no apparent variation among days. The variability in the aspirin and salicylic acid assays is not significantly changed by the use of either daily or averaged standards.

A comparative study such as this permits the choice of the better standard. For example, lot A should be preferred to lot E. Lot A, the oldest of the lots but of the highest initial purity, has shown no significant degradation for over a year's time. Less initial purity results in greater degradation. It is also shown that low per cent weight loss (considered as low moisture content) correlates with the higher stability.

The correlation of melting point with purity permits the establishment of a desired m.p. and expected error. The lower limit for good AA should be 81.8 ± 0.2°.

The insignificance of salicylic acid in these AA lots of varied purity show that little definition is lost on ignoring it. In fact, a statistical evaluation of data assayed by a two-component assay, for aspirin and AA only, gave less error of estimation.

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Antibacterial Activity of Mixtures of Quaternary Ammonium Compounds and Hexachlorophene

By G. R. WALTER and W. S. GUMP

Hexachlorophene and several quaternary ammonium compounds in admixture were evaluated for antibacterial activity by *in vitro* techniques commonly employed for the evaluation of lotions, creams, and ointments. It was observed that a maximum decrease in activity occurred as the components of the mixture approached equimolar ratios. The formation of a water-insoluble complex tends to diminish the antibacterial activity of mixtures when tested by broth dilution or agar plate techniques.

Numerous examples of the inactivation of bactericidal cationic substances may be found in the literature and several have been reviewed by Lawrence (1).

The anionic nature of hexachlorophene would also suggest a lesser antibacterial action in the presence of quaternary ammonium compounds. The problem of ascertaining the extent of inactivation, if any, was undertaken because of the possible usage of both hexachlorophene and quaternary ammonium compounds in items such as lotions, powders, and creams. The methods selected for bacteriological evaluation were those commonly associated with the *in vitro* evaluation

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of these materials; namely, the zone of inhibition type of test and the broth tube serial dilution technique.

EXPERIMENTAL

Alcoholic stock solutions of benzalkonium chloride (alkylbenzyldimethylammonium chloride) and of hexachlorophene were mixed to give varying ratios of the active materials. In addition to these mixtures, equimolar amounts of hexachlorophene and benzalkonium chloride were brought to reaction in the following manner: To a solution of 8.1 Gm. of hexachlorophene in 100 ml. of acetone was added 15 Gm. of benzalkonium chloride (50% aqueous solution). The mixture was refluxed for 1 hour, the acetone distilled, and the solid removed by filtration, followed by drying at 50° in a 4-mm. vacuum. A slightly sticky material (15.5 Gm.) was obtained. The alkyl group in benzalkonium chloride represents a mixture of the alkyls C_8H_{17} to $C_{18}H_{37}$, the average being about $C_{13}H_{27}$, therefore the molecular weight of benzalkonium chloride approximates 354. The complex obtained from benzalkonium chloride and hexachlorophene would be about 724 (one mole of hydrogen chloride being removed) and its chlorine content 29.4%. The analysis of the product described above gave 29.5% chlorine.

For the bacteriological evaluation, aliquots of the alcoholic mixtures were added to tryptic soy broth (Difco) followed by twofold serial dilution in additional broth. Broth tubes were inoculated with one drop of a 1:100 water dilution of a 24-hour A.O.A.C. broth culture of *Staphylococcus aureus* A.T.C.C. 6538 (2). Tubes were incubated at 34° and growth was recorded by turbidity after 4 days. A slight haze was observed to form when alcoholic aliquots were added to the first tube of the serial dilution series. It was thought that this haze formation might lead to excess variation in the test and obscure any small differences in end points that might occur. For this reason, determinations were replicated seven times, each test being performed on a different day. The log means were analyzed for a significant difference from that of the hexachlorophene mean. It may be noted from Table I that, while both components of the mixture were highly bacteriostatic *per se*, a maximum loss in activity was approached as the ratio of components approached one to one.

An alternate approach for demonstrating the observed loss in activity was performed with the aid of 13-mm. filter paper disks (Schleicher & Schuell No. 740-E) which were impregnated with 0.07 ml. of alcoholic solutions of varying levels of hexachlorophene and benzalkonium chloride. The disks were dried at room temperature to remove solvent and then placed on seeded *S. aureus* plates. Replicate disks were prepared for each solution and placed on assay plates in a random manner. The plates were prepared such that each plate (100 mm. diameter) contained a 20-ml. base layer and a 6-ml. seed layer of agar. Dextrose tryptone extract agar (Difco) was employed for both the base layer and the seed layer. The inoculum consisted of a 1% (v/v) 24-hour A.O.A.C. broth culture of *S. aureus* in agar used for the seed layer. Sharp, well-defined inhibition zones were obtained for both hexachlorophene and benzalkonium chloride by this technique. The loss of zone production by mixtures of the active materials may be noted in Fig. 1. It may be observed that maximum loss of activity was

TABLE I.—BACTERIOSTATIC LEVELS OF MIXTURES OF HEXACHLOROPHENE AND BENZALKONIUM CHLORIDE AGAINST *Staphylococcus aureus*

	Bacteriostatic Levels (mcg./ml.) in Broth of Mixtures of the Following Composition											
% Benzalkonium Chloride	100	90	80	70	60	50	40	30	20	10	0	
% Hexachlorophene	0	10	20	30	40	50	60	70	80	90	100	Complex
Run No. 1	0.39	0.78	0.78	0.78	3.12	3.12	3.12	1.56	1.56	0.78	0.78	3.12
2	0.39	0.78	0.78	1.56	3.12	6.25	6.25	3.12	1.56	0.78	0.39	6.25
3	3.12	0.39	1.56	0.78	1.56	3.12	3.12	0.78	1.56	0.39	0.39	1.56
4	0.78	0.78	0.78	1.56	3.12	6.25	3.12	0.78	1.56	0.78	0.39	6.25
5	3.12	3.12	6.25	12.50	12.50	12.50	1.56	0.78	0.78	0.39	0.78	3.12
6	0.39	0.39	0.39	0.78	1.56	6.25	3.12	3.12	3.12	0.39	1.56	6.25
7	0.39	0.78	1.56	1.56	0.78	1.56	1.56	1.56	1.56	0.78	1.56	12.50
Geometric mean	0.78	0.78	1.16	1.56	2.56	4.60	2.74	1.42	1.56	0.59	0.71	4.65
t^a	...	...	...	1.56	2.75	4.88	4.19	1.85	...	...	...	4.72

[a] Value of t at the 0.05 level of significance for 12 degrees of freedom is 2.18 (3).

TABLE II.—ZONE PRODUCTION BY EQUIMOLAR MIXTURES OF QUATERNARY AMMONIUM COMPOUNDS AND HEXACHLOROPHENE AGAINST *Staphylococcus aureus*

Quaternary Ammonium Compound	Concentration of Components of Mixture, mcg./ml.		Inhibition Zone,[e] mm.		
	Hexachlorophene	Q.A.C.[d]	Hexachlorophene	Q.A.C.	Equimolar Mixture
Hyamine 1622[a]	2500	2750	5.3	1.2	1.7
Hyamine 10-X[b]	2500	2580	5.3	1.1	0.5
Hyamine 2389[c]	2500	2250	5.9	2.0	0.6
Cetylpyridinium chloride	2500	2070	5.8	0.4	0.3

[a] Hyamine 1622, (Rohm & Haas Co.) (diisobutylphenoxyethoxyethyl)benzyldimethylammonium chloride, monohydrate. [b] Hyamine 10-X (Rohm & Haas Co.) (diisobutylcresoxyethoxyethyl)benzyldimethylammonium chloride, monohydrate. [c] Hyamine 2389 (Rohm & Haas Co.) [alkyl(C9–C15)metnylbenzyl]trimethylammonium chloride. [d] Quaternary ammonium compounds. [e] Average zone of three replicates to nearest 0.1 mm.

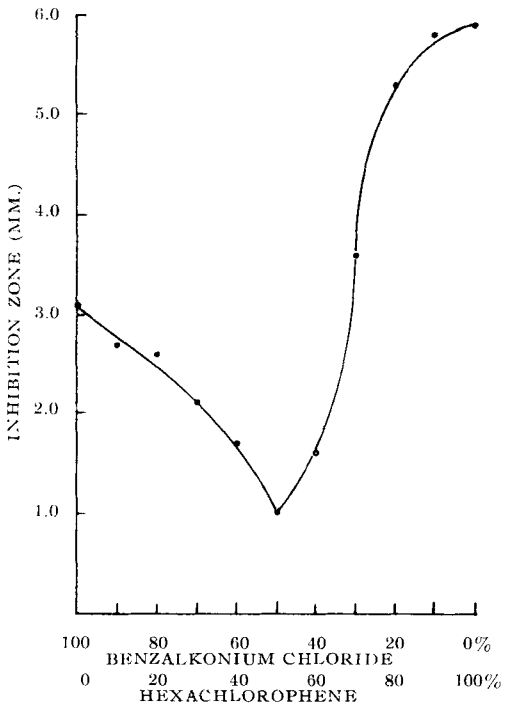

Fig. 1.—Effect of varying ratios of hexachlorophene and benzalkonium chloride on the inhibition zone produced against *S. aureus*. Each point is the mean of three determinations. Solutions were 0.1% with respect to total mixture present.

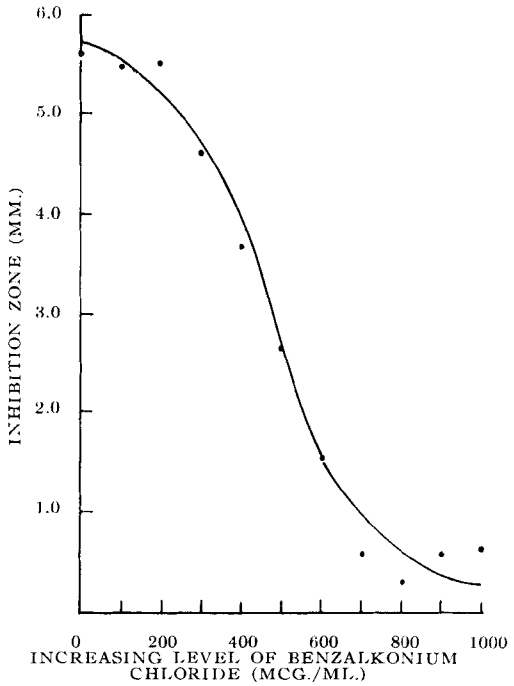

Fig. 2.—Diminishing zone production of a 1000 mcg./ml. hexachlorophene solution with increasing levels of benzalkonium chloride.

found at approximately a 1:1 ratio. Alcoholic mixtures of hexachlorophene and benzalkonium chloride, examined by the plate technique, in which the hexachlorophene level was held constant may be seen in Fig. 2. A loss of activity as the mixture approached a 1:1 ratio was observed.

Several quaternary ammonium compounds structurally dissimilar to benzalkonium chloride were examined in equimolar mixtures with hexachlorophene by the plate technique. As may be seen in Table II, zones produced by these mixtures were also considerably smaller than those of the active materials *per se*.

DISCUSSION AND SUMMARY

Bacteriological examination of mixtures of quaternary ammonium compounds and hexachlorophene demonstrated an antagonistic relationship when evaluated by tube dilution and agar plate techniques. The maximum loss of activity was observed at an approximate equimolar concentration of each component of the mixture. The loss of activity by broth dilution was less dramatic than on agar plates. The haze observed in the broth tubes during the conduct of the tests indicates the formation of an insoluble reaction product. This loss of activity was greatly magnified in the plate technique which relies on the ability of materials to diffuse through agar.

Mixtures of hexachlorophene and quaternary ammonium compounds produce a relatively insoluble complex which is less active than the components, *per se*, of the mixture. The activity loss is greatest at an equimolar ratio, and it must be assumed that one is determining the activity of the uncombined excess of one or the other component when ratios other than equimolar are examined.

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