SOME OBSERVATIONS ON THE ACTIVITY OF MIXTURES OF ANTIBACTERIAL SUBSTANCES

By E. M. Bavin, E. Kay and D. Simmonite

From Smith and Nephew Research Limited, Hunsdon Laboratories, Hunsdon, Ware, Hertfordshire

Received June 22, 1955

Introduction

During an investigation of the possibility of preparing an improved general antiseptic, the use of mixtures of known antiseptic substances was considered. Little work appeared to have been previously carried out in this direction and it seemed possible that some degree of synergism might exist between certain antibacterials. Attention was first paid to combinations of phenolic substances, such as *o*-phenyl-phenol, octyl-cresol, mono- and di-chlor-*m*-xylenol and hexachlorophene. None of the mixtures had an antibacterial activity significantly greater than existing antiseptics, such as Solution of Chloroxylenol B.P., and consideration was then given to the use of quaternary ammonium compounds. Although the high antibacterial activities of quaternary ammonium compounds have been known for many years[1] and numerous quaternary salts are available as general antiseptics, yet it is surprising to find that no attempt appears to have been made to overcome the main disadvantage of this group—their inactivation by organic matter and soap. The inactivation arises from the very nature of the quaternary salts, and it was because of this fact that attention was turned to the possibility of reinforcing the activity of the salt with substances compatible with quaternary compounds and having high antibacterial powers unaffected by organic matter or by soap. Preliminary work showed that Domiphen Bromide B.P.C. (alkyl dimethyl-2-phenoxy ethyl-ammonium bromides) was the most active of the quaternary ammonium compounds tested and that 5-aminoacridine (Aminacrine Hydrochloride B.P.) was likely to prove a suitable reinforcing agent. It was found that these two substances were compatible over a wide range of concentrations and temperatures, and a solution containing 1·0 per cent. of domiphen bromide and 0·1 per cent. of 5-aminoacridine was finally selected for further investigation. The evaluation of the antiseptic activity of the mixture was attempted under conditions resembling as closely as possible those applying during everyday use. In view of the many conflicting reports in the literature on the antibacterial powers of quaternary ammonium compounds[2–5], it was decided to employ two basic tests, giving bacteriostatic and bactericidal performance. In addition other known antiseptics were compared at the same time.

Experimental Methods

(*a*) *Maintenance of Cultures*

All cultures of micro-organisms used were obtained initially from the National Collection. The stocks were maintained on nutrient agar and

day to day culturing was made in nutrient broth No. 2 (Oxo). The *Vibrio comma* fluid cultures were in peptone water (Oxo) and *Aspergillus niger* and *Candida albicans* were cultured on Sabouraud's agar and in Sabouraud's broth. Only with the last two organisms were the cultures older than 24 hours when used.

(*b*) *Bacteriostatic Tests*

The procedure adopted was that of serial dilution of the stock solution in 5 ml. quantities of broth and to each dilution 0·1 ml. of an undiluted broth culture was added. Growth was recorded after 48 hours incubation.

The effect of soap and blood on the antiseptic mixture was examined using representative organisms. Contact with soap solution (0·1 per cent. in water) or horse blood (20 per cent. in saline) with an equal volume of antiseptic was allowed for 30 minutes before dilution.

(*c*) *Bactericidal Tests*

The procedure adopted was an exposure time of 5, 10 and 15 minutes between the antiseptic solution (in 5 ml. amounts) and 0·2 ml. of broth culture (containing approx. 10 million organisms). 1 loopful was transferred into broth after the exposure times.

The organic matter used was a 5 per cent. killed yeast suspension. A contact time of 15 minutes was allowed between antiseptic and the yeast suspension before the culture was added and exposed to the action of the antiseptic.

To compensate for the alleged strong absorptive properties of the quaternary ammonium salts, a neutralising agent was incorporated in the subculture broth[6]. Lubrol W. (polyethylene oxide condensate, I.C.I.) was used as a 1 per cent. solution in broth.

Resistance Emergence

Following a report[7] that organisms rapidly become resistant to quaternary ammonium compounds, experiments were carried out to determine if the mixed antiseptic would delay the emergence of resistance in the Gram-negative organism, *Serratia marcescens*. The method used is that described in the paper quoted.

Results

Comparative values obtained with a wide range of antiseptics and a wide range of micro-organisms are shown in Table I. The effect of incorporating serum in the medium is shown in Table II. It is apparent from these Tables, (*a*) that domiphen bromide has a higher antibacterial activity than the other quaternary ammonium compounds examined, (*b*) that the quaternary compounds are more active bacteriostatically than any other group of compounds examined, and (*c*) 5-aminoacridine is a compound with a high degree of activity which is little affected by the presence of serum.

The outstanding activity of domiphen bromide as seen in the successive dilution test was also apparent in the time exposure test. The results are

TABLE I

MINIMUM INHIBITORY CONCENTRATIONS OF SOME ANTISEPTICS (ORGANIC MATTER ABSENT)

Name of antiseptic	Minimum inhibitory concentration expressed in mg. per 100 ml.						
	Staph. aureus	*P. vulgaris*	*Salm. typhi*	*K. pneumo.*	*B. mycoides*	*Ps. aeruginosa*	*Cl. tetani*
Domiphen bromide B.P.C.	0·097	1·56	1·56	3·12	0·39	12·5	0·78
Benzalkonium chloride U.S.P.	0·097	1·56	3·12	6·25	0·39	25·0	1·56
*Octaphen	0·097	3·12	3·12	3·12	0·39	25·0	1·56
Cetrimide B.P.	0·097	1·56	3·12	12·5	0·78	50·0	1·56
†Dibromopropamidine isethionate	0·097	6·25	1·56	3·12	6·25	25·0	0·39
5-Aminoacridine	3·12	1·56	1·56	1·56	1·56	12·5	0·78
Auramine	6·25	6·25	12·5	3·12	1·56	>50	3·12
Hexyl resorcinol	0·78	1·56	6·25	3·12	1·56	>50	3·12
Di-chloro-*m*-xylenol	6·25	12·5	25·0	12·5	6·25	>50	6·25
p-Chloro-*m*-xylenol	3·12	12·5	25·0	3·12	3·12	>50	3·12
Benzyl cresol	1·56	6·25	12·5	6·25	3·12	>50	6·25
Phenol	>50	50·0	>50	>50	>50	>50	>50

* "Octaphen"—(β-*p*-Tert.-octylphenoxyethyldiethylbenzyl ammonium chloride)—Ward Blenkinsop & Co., Ltd.

† "Brulidine"—May and Baker Limited.

TABLE II

MINIMUM INHIBITORY CONCENTRATIONS OF SOME ANTISEPTICS (10 PER CENT. SERUM PRESENT)

Name of antiseptic	Minimum inhibitory concentration expressed in mg. per 100 ml.						
	Staph. aureus	*P. vulgaris*	*Salm. typhi*	*K. pneumo.*	*B. mycoides*	*Ps. aeruginosa*	*Cl. tetani*
Domiphen bromide	1·56	6·25	12·5	6·25	1·56	25·0	1·56
Benzalkonium chloride	1·56	12·5	25·0	25·0	1·56	50·0	3·12
Octaphen	1·56	12·5	50·0	25·0	3·12	50·0	3·12
Cetrimide	1·56	62·5	50·0	50·0	3·12	>50	6·25
Dibromopropamidine isethionate	0·39	25·0	3·12	12·5	12·5	25·0	1·56
5-Aminoacridine	3·12	3·12	1·56	1·56	1·56	25·0	1·56
Auramine	12·5	12·5	25·0	12·5	6·25	>50	12·5
Hexyl resorcinol	3·12	12·5	25·0	12·5	12·5	>50	12·5
Di-chloro-*m*-xylenol	25·0	25·0	50·0	>50	25·0	>50	50·0
p-Chloro-*m*-xylenol	12·5	12·5	25·0	12·5	12·5	>50	25·0
Benzyl cresol	6·25	12·5	25·0	12·5	6·25	>50	25·0
Phenol	>50	>50	>50	>50	>50	>50	>50

shown in Table III where the effect of an inactivator is seen to have little effect on the result obtained.

As is seen from Table III, 5-aminoacridine has only a weak bactericidal power during the limited 5 minutes contact, but it is seen from Table IV that the activity is greatly increased when a longer contact time is allowed. The organism used was a *Staphylococcus aureus.*

Table IV (*a*) shows that the antibacterial activity of 5-aminoacridine is unaffected in the presence of high concentrations of soap and protein.

In the presence of blood (10 per cent.) and soap (0·05 per cent.) the results shown in Table V indicate that although the antiseptic mixture is to some extent inactivated, yet a high antibacterial activity remains, and that the product, in this respect is not inferior to others such as Solution of

TABLE III

BACTERICIDAL CONCENTRATION OF ANTISEPTIC REQUIRED TO KILL THE TEST ORGANISM IN 10 MINUTES BUT NOT IN 5 MINUTES

Name of antiseptic	Inactivator	5 per cent. yeast suspension	Bactericidal concentration expressed in mg. per 100 ml.					
			Staph. aureus	*P. vulgaris*	*Salm. typhi*	*K. pneumo.*	*S. mycoides*	*Ps. Aeruginosa*
Domiphen bromide B.P.C. ..	Absent	Absent	2·5	50	7·5	7·5	10	5
		Present	75	1000	250	250	250	100
Domiphen bromide B.P.C. ..	Present	Absent	2·5	50	7·5	7·5	10	10
		Present	100	1000	250	250	250	250
Benzalkonium chloride U.S.P.	Absent	Absent	2·5	75	10	75	10	10
		Present	100	1000	250	500	250	100
Dibromopropamidine isethionate	Not required	Absent	50	500	100	250	100	750
		Present	100	1000	1000	1000	750	1000
5-Aminoacridine	Not required	Absent	1000	1000	250	250	1000	250
		Present	1000	1000	750	500	1000	750

Chloroxylenol B.P., the results for which are also shown in Table V. The effect of varying concentrations of soap are shown in Table VI.

As has been previously reported in the literature[8] quaternary ammonium compounds are reduced in activity when in contact with "hard" water.

TABLE IV

EFFECT OF VARYING CONTACT TIMES ON THE BACTERICIDAL ACTIVITY OF 5-AMINOACRIDINE AGAINST *Staph. aureus*

	Contact time			
	5 minutes	10 minutes	30 minutes	60 minutes
Inhibition concentration in mg./100 ml. ..	1000	500	200	100

TABLE IV (*a*)

ANTI-BACTERIAL ACTIVITY OF 5-AMINOACRIDINE

Organism	Minimum inhibitory concentration in mg./100 ml. in presence of:		
	Water	0·05 per cent. soap	10·0 per cent. blood
Staph. aureus	3·12	3·12	3·12
Salm. typhi	1·56	1·56	1·56
B. mycoides	1·56	1·56	1·56
Ps. aeruginosa ..	12·5	25·0	25·0

The results of the investigation with the antiseptic mixture and Solution of Chloroxylenol B.P. when examined by the successive dilution technique diluted with hard water (290 p.p.m. Ca) are shown in Table VII.

The results obtained from the investigation of the emergence of resistance are shown in Table VIII. These are of a preliminary nature, but it seems that 5-aminoacridine delays the emergence of resistance to domiphen bromide.

TABLE V

COMPARATIVE INACTIVATION OF BACTERIOSTATIC ACTIVITY BY SOAP AND BLOOD
MINIMUM INHIBITORY DILUTIONS

Organism	Domiphen bromide 1 per cent.			Antiseptic mixture			Solution of Chloroxylenol B.P.		
	Water	0·05 per cent. soap	10 per cent. blood	Water	0·05 per cent. soap	10 per cent. blood	Water	0·05 per cent. soap	10 per cent. blood
Staph. aureus ..	1:32,000	1:2000	1:4000	1:33,000	1:8250	1:8250	1:2000	1:2000	1:1000
Strep. pyogenes ..	1:32,000	—	—	1:33,000	—	—	1:1000	—	—
B. mycoides ..	1:32,000	1:2000	1:2000	1:33,000	1:8250	1:8250	1:4000	1:2000	1:2000
Ps. aeruginosa ..	1:125	1:32	1:32	1:200	1:100	1:100	1:64	1:64	1:32
C. diphtheriæ ..	1:8000	—	—	1:8000	1:2000	1:4000	1:2000	1:2000	1:1000
Cl. welchii	1:16,000	—	—	1:16,000	—	—	1:4000	—	—
P. vulgaris	1:2000	1:250	1:500	1:2000	1:500	1:1000	1:500	1:500	1:250
E. coli	1:1000	1:125	1:125	1:1000	1:250	1:250	1:250	1:250	1:125
Salm. typhi	1:1000	1:125	1:250	1:1000	1:250	1:250	1:250	1:250	1:125
Sh. shigae	1:1000	1:125	1:250	1:2000	1:500	1:1000	1:500	1:500	1:250
V. comma	1:2000	—	—	1:2000	—	—	1:500	—	—
Asp. niger	1:8000	—	—	1:8000	1:2000	1:2000	1:2000	1:2000	1:1000
Cand. albicans ..	1:16,000	—	—	1:16,000	1:4000	1:4000	1:2000	1:2000	1:1000

TABLE VI

EFFECT OF VARYING CONCENTRATIONS OF SOAP ON BACTERIOSTATIC ACTIVITY

Antiseptic mixture control	Minimum inhibitory dilution = 1:33,000 (*Staph. aureus*)
Antiseptic mixture plus 0·05 per cent. soap	,, ,, ,, 1:8250 (*Staph. aureus*)
Antiseptic mixture plus 0·1 per cent. soap ..	,, ,, ,, 1:8250 (*Staph. aureus*)
Antiseptic mixture plus 0·5 per cent. soap ..	,, ,, ,, 1:4125 (*Staph. aureus*)

TABLE VII

EFFECT OF HARD WATER ON BACTERIOSTATIC ACTIVITY
MINIMUM INHIBITORY DILUTION

Organism	Antiseptic mixture		Solution of Chloroxylenol B.P.	
	Control	Hard water	Control	Hard water
Staph. aureus	1:33000	1:8250	1:2000	1:1000
Salm. typhi	1:1000	1:250	1:250	1:125

TABLE VIII

EMERGENCE OF RESISTANCE IN *S. marcescens* TO SINGLE AND MIXED ANTISEPTICS

	No. of transfers										
	1	2	3	4	5	6	7	8	9	10	
Domiphen bromide mg./100 ml.	1	1	2	32	128	1000	—	—	—	—	Minimum inhibitory dilution
5-Aminoacridine mg./100 ml.	3	6	6	6	6	24	48	—	—	—	
Antiseptic mixture mg./100 ml.	1	1	2	2	4	4	16	—	—	—	
Solution of chloroxylenol mg./100 ml.	25	25	50	50	50	100	200	—	—	—	

DISCUSSION

The results of the investigation on a mixture of domiphen bromide and 5-aminoacridine have confirmed preliminary deductions about the complementary nature of these two antiseptics.

Experiments with the two substances separately have shown firstly,

that of a number of quaternary ammonium compounds and other antiseptics examined, domiphen bromide is the most antibacterial, and secondly, they have confirmed the stability of 5-aminoacridine in the presence of soap and protein.

The mixture of the two substances is stable over a wide range of temperatures and possesses the high bacteriostatic and bactericidal activity which is characteristic of domiphen bromide. In normal circumstances, the small amount of 5-aminoacridine present does not contribute much to the high antibacterial activity of the domiphen bromide in the mixture, but in the presence of high concentrations of soap and protein, 5-aminoacridine plays an additive role, in which it maintains the activity of the mixture at a high level by superimposing its own unimpaired activity on the reduced activity of the quaternary compound.

Many recent reports have stressed the clinical value of a mixture of two or more chemotherapeutic drugs in delaying the emergence of bacterial resistance and it is interesting that a similar action is seen with the present mixture of antiseptic substances *in vitro*. Investigation, at present has been confined to one organism, *S. marcescens*, which has permitted tests to be carried out in both acid and alkaline *p*H, but further experiments are in progress to determine whether a similar result is obtained with the common pathogens. The development of bacterial resistance is probably rare during the normal use of an antiseptic but any general delaying effect on the emergence of bacterial resistance possessed by the present mixture of antiseptics would be of value in its protracted use against wound infection.

Summary

Of a number of antiseptics examined, domiphen bromide was found to be the most active in normal media, but partial inactivation occurred in the presence of soap or protein. It was confirmed that 5-aminoacridine was not affected by either of these substances. A mixture of 1·0 per cent. of domiphen bromide and 0·1 per cent. of 5-aminoacridine possessed high antiseptic activity which was largely retained in the presence of large concentrations of soap or protein.

Preliminary results suggest that the mixture of antiseptics delays the development of bacterial resistance *in vitro* to a much greater extent than does domiphen bromide alone.

The authors wish to thank their colleagues who have assisted in many ways with the experimental work.

We would also like to thank Mr. A. R. G. Chamings for much help and advice during the progress of this work.

References

1. Domagk, *Dtsch. med. Wschr.*, 1935, **61**, 829.
2. Dubois and Dibblee, *Science*, 1946, **103**, 734.
3. Quinso and Foter, *J. Bact.*, 1946, **52**, 111.
4. Klarmann and Wright, *Soap (N.Y.)*, 1946, **22**, 125.
5. Klarmann and Wright, *Amer. J. Pharm.*, 1948, **120**, 146.
6. Davies, *J. Hyg.*, 1949, **47**, 271.
7. Chaplin, *J. Bact.*, 1952, **63**, 453.
8. Ridenour and Armbruster, *Amer. J. Publ. Hlth.*, 1948, **38**, 504.

DISCUSSION

The paper was presented by MR. E. KAY.

MR. G. SYKES said he found some difficulty in understanding the Tables. Some were expressed as minimum inhibitory concentration in mg. per 100 ml., others were in terms of one in so many, so that without careful arithmetic it was difficult to relate the two. Although the effect of the domiphen bromide might not be reduced by the addition of 5-aminoacridine, it would seem that the effect of 5-aminoacridine was diminished by domiphen bromide. Hence the mixture would seem to be no better than the simple acridine solution. With regard to the emergence of resistance, there seemed to be little difference. In Table VIII 5-aminoacridine started with an inhibitory concentration of 3 mg./100 ml. and rose to 48 at the seventh generation. The mixture achieved exactly the same, namely, a relative increase from 1 to 16.

MR. R. LEVIN (Liverpool) said that with the advent in recent years of several potent agents having a wide spectrum of antibacterial activity, he agreed that it was opportune to subject the commonly used antiseptic solutions to a reappraisal.

MR. E. KAY, in reply, emphasised that the work was of a preliminary nature. In regard to the emergence of resistance, it was pointed out that one did not obtain a thousandfold increase in the mixture whereas it was obtained with domiphen bromide. It was difficult to express the concentration of the mixture itself in terms of mg./ml.