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The antibacterial activity of chloroxylenol in combination with ethylenediaminetetra-acetic acid

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SUMMARY

The bactericidal activity of RBA 777 has been found to vary with both the cultural and environmental test conditions against *Pseudomonas aeruginosa* and to a lesser extent against *Staphylococcus aureus*. These variations may explain certain anomalies in earlier work regarding the activity of chloroxylenol-based products. The addition of EDTA to RBA 777 has brought about an improvement in the performance against *P. aeruginosa* and this activity is confirmed *in vivo*. Previous reports have already illustrated this potential and the evaluations of the new antibacterial agent DA 136 confirms and extends these results to its performance under adverse conditions, often associated with the hospital environment.

INTRODUCTION

The use of parachlorometaxylenol as an antiseptic was reported by Colebrook & Maxted (1933), who demonstrated that this agent was rapidly lethal to haemolytic streptococci and *Escherichia coli* even in the presence of pus. Since this time a number of conflicting reports have appeared concerning the efficacy of parachlorometaxylenol as an antiseptic. Thus, Colebrook (1941) questioned the ability of chloroxylenol to kill *Staphylococcus aureus* on the skin, whilst on the other hand Beath (1943) demonstrated the efficiency of the same chloroxylenol-based product in controlling wound infection caused by *S. aureus*.

Obviously the major concern about any antiseptic is the efficacy of the in-use concentration, and when Lowbury (1951) found that a *Pseudomonas* sp. could multiply in fairly high concentrations of a chloroxylenol solution, the antiseptic value of this type of preparations was questioned. Subsequently almost all other antiseptics have been shown to be contaminated with *Pseudomonas* spp. (Burdon & Whitby, 1967; Bassett, 1970) and also with other organisms (Boyce & Meddick, 1974).

The weakness of chloroxylenol at working-strength solution against *Pseudomonas aeruginosa* was confirmed by Calman & Murray (1956), but this weakness was not apparent with other Gram-negative bacteria. Ayliffe, Collins & Lowbury (1966), in contrast, reported that chloroxylenol was more effective against *P. aeruginosa* than against *S. aureus*, and Hare, Raik & Gash (1963) showed that chloroxylenol was rapidly lethal to a number of different Gram-positive and Gram-

negative bacteria in a dried state and that seven strains of *P. aeruginosa* were killed within 2·5 min.

Hatch & Cooper (1948) demonstrated that a better result for a chloroxylenol-based product could be obtained against *P. aeruginosa* if 0·5 % of the sequestering agent, sodium hexametaphosphate, was added to the working strength solutions. Similarly, Gray & Wilkinson (1965*a*) showed that the chelating agent ethylenediaminetetra-acetic acid (EDTA) had a similar effect in potentiating the activity of chloroxylenol against *P. aeruginosa*, a finding subsequently confirmed by Reybrouck & Van de Voorde (1969) and Smith (1970).

With the introduction of a new chloroxylenol-based product which incorporates a chelating agent, it seemed opportune to examine the activity of this product against a standard preparation containing only chloroxylenol and against chloroxylenol in combination with EDTA under a variety of controlled conditions likely to affect antibacterial performance. In this way it was hoped that some of the contradictions which have arisen regarding the activity of chloroxylenol could be resolved, and explained.

MATERIALS AND METHODS

Micro-organisms

Pseudomonas aeruginosa pyocine type 6 was isolated from the faeces of a child in the intensive-care room in the Department of Surgery. This strain was used in all experiments. Fifteen other strains of *P. aeruginosa* were isolated from sinks and floors in the hospital and from faeces and sputum of patients. These strains were compared with type 6 for their sensitivity to DA 136.

Staphylococcus aureus phage type 52/81 was isolated from the nose of a member of the laboratory staff of the Laboratory of Medical Microbiology.

Acinetobacter anitratus was isolated from the sink in an isolation room in which a child was treated for leukaemia.

The micro-organisms were identified according to Cowan & Steel (1965). *P. aeruginosa* was typed according to Gillies & Govan (1966).

Growth of inocula

All organisms were grown, except where otherwise stated, in nutrient broth No. 2 (Oxoid Ltd) for 18 hr. at 37° C. to give colony counts of 8×10^8 (*P. aeruginosa*), 10^9 (*S. aureus*) and 2×10^9 (*A. anitratus*). Each culture was centrifuged, the pellet washed twice in sterile distilled water and resuspended in quarter-strength Ringer's solution. The influence of glucose on micro-organism resistance was studied by using Oxoid nutrient broth No. 2 enriched with 1 % glucose (Nakamura, 1967). For studies on magnesium depleted and calcium depleted organisms the media described by Brown & Melling (1969*a*) were used. Additional $MgSO_4$ or $CaCl_2$ was added where appropriate. The influence of incubation time on bacterial sensitivity was studied by growing the organisms in nutrient broth no. 2 (Oxoid Ltd) in a shaking incubator for the relevant period of time.

Subculture media

Subcultures from the tests were made on blood agar base (Oxoid Ltd) containing 5% sterile sheep blood, and in the case of the skin tests also on MacConkey agar no. 2.

Antimicrobial products

RBA 777 is a commercial product containing 4·8% (w/v) 4-chloro-3,5-xylenol. DA 136 is a new product containing 12·0% (w/v) 4-chloro-3,5-xylenol supplemented with 21% (w/v) EDTA as the dihydrate of its disodium salt (Reckitt and Colman, Hull, U.K.).

Preparation of material

Antimicrobial products

All dilutions were prepared in sterile tap water with a total hardness of 13·75 German hardness degrees (247·5 p.p.m. $CaCO_3$) except where otherwise stated. In studies involving the influence of divalent cations the antimicrobial products were diluted in demineralized water or in a synthetic hard water (Feisal & Bennett, 1961). DA 136 was always used at a dilution of 10 ml./l., whereas RBA 777 was diluted according to information given in the text. To investigate the influence of EDTA in combination with RBA 777, the chelating agent was added in appropriate concentrations to the solution. Unless stated no control of the final pH of the dilutions of the antimicrobial products was made. Where the influence of pH was evaluated the pH of the dilutions was adjusted with either 0·1 N-HCl or 0·1 N-NaOH. The influence of organic material was determined by adding 10% rabbit serum to the final dilution of the germicides.

Antibacterial evaluation

The activity of RBA 777, DA 136 and combinations of RBA 777 and EDTA was assessed by means of suspensions and surface tests, at 20 °C. unless otherwise stated. The antimicrobial activity was neutralized by the addition of Tween 80 (2%) to the subculture media.

Suspension test

Initially, the suspension test described previously (Kuipers & Dankert, 1970) was compared with a modified method using disposable commercially available serology plates (Thovadec, Nieuwkoop, the Netherlands). The antiseptic solution (1 ml.) was added to each well of the plates. A standard amount of the culture was added to each well by use of a multipoint inoculator. Subcultures were taken at the appropriate time interval onto blood agar using a multipoint transference system. Results are expressed by the average of four tests.

Skin surface test

Areas (10 $cm.^2$) of the forearms of members of the laboratory staff were initially treated with 75% (v/v) ethanol, washed with sterile saline solution and dried in a sterile air flow. These areas were contaminated with one of the test organisms resuspended in quarter strength Ringer's solution so that the contaminating dose

Table 1. *A comparison of the two suspension tests for evaluation of antibacterial performance*

Product	Time to kill (min.)					
	Tube suspension method			Tray suspension method		
	P. aerug.	*S. aureus*	*A. anit.*	*P. aerug.*	*S. aureus*	*A. anit.*
RBA 777, 1·25%	> 30	3	2	> 30	2	2
RBA 777, 2·5%	> 30	2	1	> 30	1	1
RBA 777, 5·0%	30	< 1	< 1	> 30	< 1	< 1
RBA 777, 1·25% + 0·18% EDTA	1	2	1	1	1	< 1
RBA 777, 2·5% + 0·18% EDTA	1	1	1	< 1	1	1
RBA 777, 5·0% + 0·18% EDTA	< 1	< 1	< 1	< 1	< 1	< 1
DA 136	1	1	1	< 1	1	< 1
DA 136 + serum	1	1	1	< 1	1	< 1

was between 1000 and 2000 organisms/cm.^{2}. The skin was allowed to dry and then treated with either a suitable dilution of the product or Ringer's solution (control). After a contact time of either 5, 10 or 15 min., the treated areas were sampled with an agar cylinder (Kuipers, 1968), the agar slices were immediately sprayed with 1 ml. of saline containing the neutralizer and incubated for 18 hr. before the colonies were counted. The number of bacteria recovered from the control sites were used to calculate the reduction in numbers of bacteria recovered from the test site. The reduction is given as a percentage of the control.

In the skin surface tests results are expressed as the average of four tests.

RESULTS

At first, evaluation of antibacterial activity of the preparations by the plate-test was compared directly with the more conventional tube-method, to determine the accuracy of the newer method. The results have shown that the plate-method gave parallel results to the tube suspension method (Table 1). Because of the ease and the rapidity with which tests could be carried out, the tray-method was adopted for use in the studies reported.

To check whether the cultural or test conditions have any influence on the activity found for RBA 777 and DA 136, a number of factors were studied.

Potentiating effect of EDTA

From Table 2 it can be seen that combinations of RBA 777 and EDTA exert a potentiating effect in killing both *P. aeruginosa* and *A. anitratus*, as may be shown by reduction in the time taken to achieve a total kill. The effect against *P. aeruginosa* was particularly noticeable; the time taken for 2·5% RBA 777 fell from greater than 30 min. to less than 1 min. when 0·18% EDTA was incorporated. It was found that EDTA alone has no noticeable bactericidal activity against either organism. There was no potentiation of activity against *S. aureus*.

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Table 2. *The antibacterial activity of RBA* 777 *supplemented with EDTA against* P. aeruginosa, S. aureus *and* A. anitratus

Organism	Conc. (%); RBA 777	Time to kill (min.) with concentration (%) of EDTA						
		0	0·011	0·022	0·045	0·09	0·18	0·36
P. aeruginosa	0	—	> 30	> 30	> 30	> 30	> 30	> 30
	0·16	> 30	> 30	> 30	> 30	25	20	20
	0·64	> 30	> 30	30	30	20	2·5–5	2·5–5
	1·25	> 30	25	20	10	7·5	< 1	< 1
	2·5	> 30	25	20	10	2·5–5	< 1	< 1
	5	10–15	10	5–10	1–2	< 1	< 1	< 1
S. aureus	0	—	> 30	> 30	> 30	> 30	> 30	> 30
	0·16	20	20	20	20	20	20	20
	0·64	5	5	5	5	5	5	5
	1·25	1–2	1–2	1–2	1	1	1	1
	2·5	< 1	< 1	< 1	< 1	< 1	< 1	< 1
	5	< 1	< 1	< 1	< 1	< 1	< 1	< 1
A. anitratus	0	—	> 30	> 30	> 30	> 30	> 30	> 30
	0·16	25	25	25	20	15	20	20
	0·64	5	5	5	2	2	2	1
	1·25	2	1	1	1	1	< 1	< 1
	2·5	< 1	< 1	< 1	< 1	< 1	< 1	< 1
	5	< 1	< 1	< 1	< 1	< 1	< 1	< 1

Table 3. *The influence of culture incubation time on the resistance of* P. aeruginosa *to RBA* 777 *and DA* 136

Time of incubation (hr.)	Time taken to kill *P. aeruginosa* with various products (min.)			
	RBA 777 1·25%	RBA 777 2·5%	RBA 777 5%	DA 136
8	> 30	> 30	15	< 1
24	> 30	> 30	10	< 1
36	> 30	20–30	5	< 1
48	> 30	20–30	2·5–5	< 1
72	> 30	5–10	2·5	< 1
96	20	5	2·5	< 1

The results in Table 3 show that young cultures of *P. aeruginosa* are more resistant than older cultures to RBA 777. The influence of age of culture on the resistance of *P. aeruginosa* to the combination of chloroxylenol and EDTA (DA 136) could not be determined since the test organisms were killed in less than 1 min. whatever the age of the cultures. In contrast to the results with *P. aeruginosa*, neither *S. aureus* nor *A. anitratus* showed any significant change in sensitivity to either RBA 777 or DA 136 as the age of the culture was increased. The times to kill these test organisms were in agreement with the results previously given in Tables 1 and 2.

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Table 4. *The effect of glucose in the initial culture broth on the resistance of the test organisms to RBA 777 plus EDTA and to DA 136*

Product	Time to kill (min.)					
	Organism grown in Oxoid No. 2 broth			Organism grown in Oxoid No. 2 broth supplemented with 1 % glucose		
	P. aerug.	*S. aureus*	*A. anit.*	*P. aerug.*	*S. aureus*	*A. anit.*
RBA 777, 2·5 %	> 30	0·5	< 1	> 30	1	1
RBA 777, 2·5 % + 0·18 % EDTA	1	0·5	1	1	1	1
RBA 777, 1·25 %	> 30	1·5	2	> 30	3	2
RBA 777, 1·25 % + 0·18 % EDTA	1	1·5	1	< 1	3	1
DA 136	< 1	< 1	1	< 1	1	< 1

Effect of glucose on sensitivity

The incorporation of 1 % glucose in the culture medium resulted in a twofold increase in the resistance of *S. aureus*, which was not influenced by either the concentration of chloroxylenol or by the presence of EDTA (Table 4). Resistance to DA 136 was also increased, compared with the findings for 0·12 % chloroxylenol with or without 0·18 % EDTA.

Effect of cations on sensitivity

(*a*) *In growth medium.* Brown & Melling (1969*a*) have shown that *P. aeruginosa* grown in a simple inorganic salt solution under conditions of divalent cation limitation became more resistant to lysis by EDTA. Our experiments, based upon their paper, showed that *P. aeruginosa* grown in the absence of divalent cations gave a culture more resistant to the action of RBA 777 plus EDTA. *S. aureus* and *A. anitratus* grown under identical conditions did not show this increase in resistance (Table 5). Supplementation of the growth media with Mg^{2+} and Ca^{2+} led to return of the sensitivity towards EDTA which was dependent on the concentration of divalent cations incorporated. Replacement of magnesium on a weight for volume basis led to a greater sensitivity to EDTA than did calcium ions.

The antibacterial activity of the products diluted in tap water alone or with additional concentrations of Ca^{2+} and Mg^{2+} was evaluated.

(*b*) *In diluent.* RBA 777 and EDTA combinations diluted in tap water containing 0·01 % $MgSO_4$ or 0·01 % $CaCl_2$ were as effective as those in tap water alone. An increase in the salt concentration to 0·1 % adversely affected activity against *P. aeruginosa* (Table 6), but not against the other two organisms. A similar effect against *P. aeruginosa* was observed with DA 136 when tested under identical conditions, but not on the other two test organisms (Table 7).

Temperature, organic matter, pH and hardness of water used to prepare the antiseptic solutions affect the performance of RBA 777 against both *P. aeruginosa* and *S. aureus* but in particular against the former (Table 8). In contrast, DA 136 remains active against *P. aeruginosa* irrespective of changes in test conditions,

Table 5. *The effect of Mg^{2+} and Ca^{2+} limitation in the growth media on the resistance of the test organisms to RBA 777, to RBA 777 plus EDTA and to DA 136*

	Time to kill (min.)								
	RBA 777, 2·5%			RBA 777, 2·5% + EDTA, 0·18%			DA 136		
Culture medium	*P. aerug.*	*S. aureus*	*A. anit.*	*P. aerug.*	*S. aureus.*	*A. anit.*	*P. aerug.*	*S. aureus.*	*A. anit.*
Oxoid Broth No. 2	> 30	1	1	0·5–1	1	1	< 0·5	1	< 1
B & M (Brown and Melling)	> 30	1	1	10	1	1	2·5–5	1	1
B & M + $MgSO_4$ 0·01%	> 30	1	1	2·5–5	1	1	1	1	< 1
B & M + $MgSO_4$ 0·1%	> 30	1	< 1	1	1	< 1	< 0·5	1	< 1
B & M + $CaCl_2$ 0·01%	—	1	1	10	1	1	10	1	1
B & M + $CaCl_2$ 0·1%	—	1	< 1	5	1	< 1	7·5	1	< 1

Table 6. *The influence of divalent cations in the diluent on the antimicrobial activity of RBA 777, and of RBA 777 supplemented with varying concentrations of EDTA against* P. aeruginosa

	Time to kill (min.) when product is diluted in		
Product	Tap water	Tap water + $MgSO_4$ 0·1%	Tap water + $CaCl_2$ 0·1%
RBA 777, 5%	25	> 30	> 30
RBA 777 5%			
+ EDTA 0·36%	< 1	25	> 30
+ EDTA 0·18%	< 1	> 30	> 30
+ EDTA 0·09%	1	> 30	> 30
+ EDTA 0·045%	2	> 30	> 30
RBA 777, 2·5%	> 30	> 30	> 30
RBA 777 2·5%			
+ EDTA 0·36%	< 1	> 30	> 30
+ EDTA 0·18%	< 1	> 30	> 30
+ EDTA 0·09%	5	> 30	> 30
+ EDTA 0·045%	10	> 30	> 30

except in artificial hard water at pH 7 at 20° and 30° C. At pH 9 and 10 the preparation was fully active at both temperatures. Minor changes occurred in the performance of DA 136 against *S. aureus*, and no obvious changes were noted against *A. anitratus*.

In vivo *activity*

In order to judge the *in vivo* action of DA 136 areas of skin were artificially inoculated with *P. aeruginosa* (1×10^4 organisms), *S. aureus* (2×10^4) and *A.*

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Table 7. *The influence of divalent cations in the diluent on the antimicrobial activity of DA* 136 *against* P. aeruginosa, S. aureus *and* A. anitratus

Dilution of product	Time to kill (min.) against *P. aeruginosa*	*S. aureus*	*A. anitratus*
DA 136 in tap water	< 1	1	< 1
DA 136 in tap water + $MgSO_4$ 0·01%	< 1	1	< 1
DA 136 in tap water + $MgSO_4$ 0·1%	25	1	1
DA 136 in tap water + $CaCl_2$ 0·01%	< 1	1	< 1
DA 136 in tap water + $CaCl_2$ 0·1%	30	1	1

Table 8. *Antibacterial activity of RBA* 777 *and DA* 136 *under influence of pH, serum, organic matter and hard water at various temperatures*

		Time to kill (min.) at various temperatures							
		RBA 777 (2·5% solution)				DA 136 (10 ml. per litre)			
		P. aeruginosa		*S. aureus*		*P. aeruginosa*		*S. aureus*	
	pH	20° C.	30° C.	20° C.	30° C.	20° C.	30° C.	20° C.	30°C.
Demineralized water	7	15	2	2·5	0·5	< 0·5	< 0·5	2·5	0·5
	9	3	< 0·5	2·5	1	< 0·5	< 0·5	2·5	1
	10	< 0·5	< 0·5	2·5	1	< 0·5	< 0·5	2·5	1
Demineralized water and serum	7	> 30	> 30	> 5	1	< 0·5	< 0·5	> 5	1
	9	> 30	2·5	2·5	1	< 0·5	< 0·5	2·5	1
	10	10	1·0	2·5	1	< 0·5	< 0·5	2·5	1
Tap water	7	> 30	> 30	< 0·5	< 0·5	< 0·5	< 0·5	2·5	1
	9	> 30	3	< 0·5	< 0·5	< 0·5	< 0·5	1	< 0·5
	10	4	< 0·5	< 0·5	< 0·5	< 0·5	< 0·5	1	< 0·5
Tap water and serum	7	> 30	> 30	5	2·5	< 0·5	< 0·5	> 5	2·5
	9	> 30	> 30	> 5	2·5	< 0·5	< 0·5	1	2·5
	10	> 30	> 30	> 50	2·5	< 0·5	< 0·5	> 5	2·5
Artificial hard water	7	> 30	> 30	< 0·5	3	> 5	< 1	2·5	2·5
	9	> 30	> 30	< 0·5	2	< 0·5	< 0·5	2·5	1
	10	> 30	6	< 0·5	< 0·5	< 0·5	< 0·5	1	0·5
Artificial hard water and serum	7	> 30	> 30	2·5	1·0	> 5	2·5	2·5	5
	9	> 30	> 30	5·0	1·0	< 2·5	< 0·5	2·5	2·5
	10	> 30	> 30	2·5	1·0	< 0·5	< 0·5	> 5	1

anitratus (1×10^4). The immediate and persistent activity expressed as the killing percentage was measured by comparing the resultant agar slice count from areas tested with DA 136 with the untreated control (Table 9). DA 136 gave a high percentage reduction on immediate application to contaminated skin, and even when DA 136 was applied to the skin two hours before bacterial contamination, the antibacterial activity was maintained at a very high level.

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Table 9. *The antibacterial activity of DA* 136 *on skin artificially infected with micro-organisms*

	Percentage reduction in bacteria after various contact times			
	Immediate activity			Persistent activity
Test organism	5 min.	10 min.	15 min.	2 hr.
P. aeruginosa	94	91	94·5	97
S. aureus	97·7	98·9	98·5	90
A. anitratum	97	98·7	98	95

DISCUSSION

The results have shown that the antibacterial activity of the chloroxylenol product RBA 777 varies with both cultural and environmental test conditions. This variation is particularly observable against *P. aeruginosa* and to a lesser extent against *S. aureus* while virtually no variation is apparent with *A. anitratus*.

The age of the culture in particular affects the resistance of *P. aeruginosa* to RBA 777; rather surprisingly the older culture was found to be less resistant than younger cultures. Normally increased resistance of organisms to phenols is associated with increasing culture age (Cook & Wills, 1958). Cowen (1974) has shown that resistance of *Pseudomonas* spp. to chloroxylenol is influenced by the magnesium content of the growth medium and this may be an important factor in our current findings since an 8 hr. culture which is not fully developed will have available more magnesium ions per cell than a fully grown culture. The fact that *P. aeruginosa* is unique in accumulating Mg^{2+} over and above the minimum required for growth (Webb, 1949) accounts for the finding that neither *S. aureus* nor *A. anitratus* showed any relation between resistance and age of culture. Further evidence for the involvement of divalent cations comes from the effect of EDTA on the performance of RBA 777 against *P. aeruginosa* where the potentiation of bactericidal activity is greater for increasing concentrations of cations in the growth medium.

The addition of divalent cations to the diluent used with RBA 777 likewise has a profound effect on the bactericidal activity against *P. aeruginosa*, but not on the other two test organisms. The specificity of this effect is surprising since cations in the diluent are widely regarded (Myers, 1968) as having a generally adverse effect on activity against most bacterial species.

An attempt to increase the resistance of the three test organisms to RBA 777 and DA 136 by growing the organism in nutrient broth supplemented with glucose resulted in an increase in resistance as far as *S. aureus* was concerned but not *P. aeruginosa* or *A. anitratus*.

Previous authors (Hugo & Stretton, 1966; Hugo & Franklin, 1968) have shown that glycerol or glucose incorporated into the growth medium of *Staphylococcus* spp. results in a more resistant culture to certain antibiotics and, moreover, in a higher lipid content of the cell wall. The small increase in resistance to RBA 777 apparent with *S. aureus* when grown in medium supplemented with glucose may be due to a high lipid content of the cell wall which could provide an additional

barrier to penetration of chloroxylenol to the site of action. *P. aeruginosa* does not usually incorporate supplemental glucose as extra lipid in the cell wall (Brown, 1971) and this may explain our observation that no increase in resistance was observed with this organism. *A. anitratus* did not show any increase in resistance either but it is not known whether this organism is capable of utilizing excess glucose in a manner similar to staphylococci.

An increase in pH is generally associated with a slight increase in the bactericidal activity of RBA 777 against *P. aeruginosa*; however, pH has been shown not to affect the germicidal activity against *S. aureus*. This finding may be due to the influence of pH on the viability of the organism rather than any potentiating effect on RBA 777 since dissociation of the chloroxylenol molecule at high pH would lead to a loss of bactericidal activity.

The effect of DA 136 on *P. aeruginosa* shows fairly uniform killing times under different test conditions.

The reduced effect of DA 136 on *P. aeruginosa* in artificial hard water at pH 7 at 20 ° C. may be due to a neutralization effect of EDTA. At a higher pH the preparation was fully active. This effect may be due to the influence of pH on the viability of the organisms. Another explanation may be found in the fact that EDTA as a chelating agent has a greater effect at pH 9 and 10 than at pH 7 (Gray & Wilkinson, 1965*b*; Haque & Russell, 1974).

In common with all antibacterial agents (Russell, 1974) the presence of organic material impairs antibacterial performance, presumably owing to competition between the organic matter and the bacterial cell for the antimicrobial agent. As expected an increase in temperature produces an increase in bactericidal activity.

Some of these results, particularly those associated with the effect of cations on activity against *P. aeruginosa*, may explain the varying results reported by earlier workers for the activity of chloroxylenol preparations against this bacterium. In addition, the effect of additional glucose in the growth medium of *S. aureus* also indicates that the resistance of this organism can be varied if growth conditions are modified. Again, this type of situation may explain the anomalous results reported in the literature.

Ethylenediaminetetra-acetic acid is known to alter the permeability of *P. aeruginosa* cells as illustrated by the potentiating effect it exhibits on lysozyme against this organism (Repaske, 1958). The action of EDTA is most probably on the cell wall (Gray & Wilkinson, 1965*a*) and is probably connected with the removal of divalent cations (Gray and Wilkinson, 1965*b*; Eagon & Carson, 1965; Brown & Melling, 1969*b*), and possible release of lipopolysaccharide from the cell wall.

Our work has shown that EDTA potentiates the performance of RBA 777 (chloroxylenol) against *P. aeruginosa* but not against the two other organisms evaluated. This work supports the findings of earlier workers (Gray & Wilkinson, 1965*a*; Brown, 1971). Potentiation, however, does not occur to the same extent when the organism is grown in a divalent cation-depleted medium as opposed to nutrient broth (Gilleland, Stinnett & Eagon, 1974). A similar observation has been shown for the potentiating effect of EDTA on lysozyme (Brown & Richards, 1965).

This is indicative that divalent cations are important in the function of EDTA – a theory put forward by other workers (Eagon & Carson, 1965; Gray & Wilkinson, 1965*a*; Brown & Richards, 1965).

The potentiating effect of EDTA on RBA 777 and on DA 136 is particularly marked when the product is diluted with tap water containing divalent cations, and only disappears when the concentration of these cations is more than sufficient to neutralize the EDTA present. This concentration is normally in excess of that found in practice as far as DA 136 is concerned.

Of the three organisms used in this study, the action of EDTA in potentiating the chloroxylenol preparation appears specific to *P. aeruginosa*. Thus the major variations associated with the activity of RBA 777 with regard to divalent cations in both the growth media and the diluent have been considerably reduced with DA 136 and in addition this product possesses a potentiated performance against *P. aeruginosa*. The use of EDTA in DA 136 to potentiate the bactericidal activity of chloroxylenol has also considerably reduced the variabilities associated with RBA 777 alone in respect to age of culture, pH and organic matter when applied to *P. aeruginosa*. The variations associated with *S. aureus* however have not been altered to any significant extent but these are relatively minor compared with the gross variabilities associated with *P. aeruginosa*.

It is important to test antiseptics in as near *in vivo* conditions as possible. The surface skin test, based on the sampling method of Ten Cate (1965), was used to meet some of the *in vivo* requirements. Skin environmental factors (e.g. low pH, presence of sebum, traces of soap) are known to modify germicidal performance (Cowen, 1974).

DA 136 has been shown to exert a high degree of bactericidal activity on the skin that is capable of persisting for at least 2 hr. Thus, bacteria present on the skin have been shown to be effectively removed, leaving a potentially low risk of subsequent infection and cross-contamination.

REFERENCES

Ayliffe, G. A. J., Collins, B. J. & Lowbury, E. J. L. (1966). Cleaning and disinfection of hospital floors. *British Medical Journal* ii, 442–5.

Bassett, D. C. J. (1970). Wound infection with *Pseudomonas multivorans*. A water-borne contaminant of disinfectant solution. *Lancet* i, 1188–91.

Beath, T. (1943). The suppression of infection in recent wounds by the use of antiseptics. *Surgery* **13**, 667–76.

Boyce, J. M. H. & Meddick, H. M. (1974). Disinfectant contamination. *British Medical Journal* i, 38.

Brown, M. R. W. (1971). In *Inhibition and Destruction of the Microbial Cell* (ed. W. Hugo), pp. 307–367. London: Academic Press.

Brown, M. R. W. & Melling, J. (1969*a*). Loss of sensitivity to EDTA by *Pseudomonas aeruginosa* grown under conditions of Mg-limitation. *Journal of General Microbiology* **54**, 439–44.

Brown, M. R. W. & Melling, J. (1969*b*). Role of divalent cations in the action of polymyxin B and EDTA on *Pseudomonas aeruginosa*. *Journal of General Microbiology* **59**, 263–74.

Brown, M. R. W. & Richards, R. M. E. (1965). Effect of ethylene diamine tetracetate on the resistance of *Pseudomonas aeruginosa* to antibacterial agents. *Nature, London* **207**, 1391–3.

Burdon, D. W. & Whitby, J. L. (1967). Contamination of hospital disinfectants with *Pseudomonas* species. *British Medical Journal* ii, 153–5.

CALMAN, R. M. & MURRAY, J. (1956). Antiseptics in midwifery. *British Medical Journal* ii, 200–204.

COLEBROOK, L. (1941). Disinfection of the skin. *Bulletin of War Medicine* **2**, 73–9.

COLEBROOK, L. & MAXTED, W. R. (1933). Antiseptics in midwifery. *Journal of Obstetrics and Gynaecology of the British Empire* **40**, 966–90.

COOK, A. M. & WILLS, B. A. (1958). The use of stored suspension of *Escherichia coli* I in the evaluation of bactericidal action. *Journal of Applied Bacteriology* **21**, 180–87.

COWAN, S. T. & STEEL, K. J. (1965). *Manual for the Identification of Medical Bacteria*. Cambridge University Press.

COWEN, R. A. (1974). Relative merits of 'in-use' and laboratory methods for the evaluation of antimicrobial products. *Journal of the Society of Cosmetics and Chemistry* **25**, 307–23.

EAGON, R. G. & CARSON, K. J. (1965). Lysis of cell walls and intact cells of *Pseudomonas aeruginosa* by ethylenediamine tetra acetic acid and by lysozyme. *Canadian Journal of Microbiology* **11**, 193–201.

FEISAL, E. V. & BENNETT, E. O. (1961). The effect of water hardness on the growth of *Pseudomonas aeruginosa* in metal cutting fluids. *Journal of Applied Bacteriology* **24**, 125–30.

GILLELAND, H. E. Jr., STINNETT, J. D. & EAGON, R. G. (1974). Ultrastructural and chemical alteration of the cell envelope of *Pseudomonas aeruginosa*, associated with resistance to ethylenediaminetetracetate resulting from growth in a Mg^{2+}-deficient medium. *Journal of Bacteriology* **117**, 302–11.

GILLIES, R. R. & GOVAN, J. R. W. (1966). Typing of *Pseudomonas pyocyanea* by pyocine production. *Journal of Pathology and Bacteriology* **91**, 339–45.

GRAY, G. W. & WILKINSON, S. G. (1965*a*). The action of ethylenediaminetetra-acetic acid on *Pseudomonas aeruginosa*. *Journal of Applied Bacteriology* **28**, 153–64.

GRAY, G. W. & WILKINSON, S. G. (1965*b*). The effect of ethylenediaminetetra-acetic acid on the cell walls of some Gram-negative bacteria. *Journal of General Microbiology* **39**, 385–99.

HAQUE, H. & RUSSELL, A. D. (1974). Effect of ethylenediaminetetra-acetic acid and related chelating agents on whole cells of gram-negative bacteria. *Antimicrobial Agents and Chemotherapy* **5**, 447.

HARE, R., RAIK, E. & GASH, S. (1963). Efficiency of antiseptics when acting on dried organisms. *British Medical Journal* i, 496–500.

HATCH, E. & COOPER, P. (1948). Sodium hexametaphosphate in emulsions of Dettol for obstetric use. *Pharmaceutical Journal* **161**, 198–9.

HUGO, W. B. & STRETTON, R. J. (1966). The role of cellular lipid in the resistance of Gram-positive bacteria to penicillins. *Journal of General Microbiology* **42**, 133–8.

HUGO, W. B. & FRANKLIN, I. (1968). Cellular lipid and the antistaphylococcal activity of phenols. *Journal of General Microbiology* **52**, 365–73.

KUIPERS, J. S. (1968). Investigation and treatment of floors of patients' rooms: a study with an agar cylinder. *Journal of Hygiene* **66**, 625–31.

KUIPERS, J. S. & DANKERT, J. (1970). Bactericidal properties of Tego 103S and Tego 103 G. *Journal of Hygiene* **68**, 343–8.

LOWBURY, E. J. L. (1951). Contamination of cetrimide and other fluids with *Pseudomonas pyocyanea*. *British Journal of Industrial Medicine* **8**, 22–5.

MYERS, J. A. (1968). Disinfectants and antiseptics. *Pharmaceutical Journal* **200**, 276–7.

NAKAMURA, H., (1967). Changes in sensitivity to acriflavine of *Escherichia coli* grown in media of different glucose contents. *Journal of General Microbiology* **49**, 443–9.

REPASKE, R. (1958). Lysis of Gram-negative organisms and the role of versene. *Biochimica et biophysica acta* **30**, 225–32.

REYBROUCK, G. & VAN DE VOORDE, G. H. (1969). Effect of ethylenediamine tetraacetate on the germicidal action of disinfectants against *Pseudomonas aeruginosa*. *Acta clinica belgica* **24**, 32–41.

RUSSELL, A. D. (1974). Factors influencing the activity of antimicrobial agents, an appraisal. *Microbios* **10**, 151–74.

SMITH, G. (1970). Ethylenediamine tetra-acetic acid and the bactericidal efficiency of some phenolic disinfectants against *Pseudomonas aeruginosa*. *Journal of Medical Laboratory Technology* **27**, 203–6.

TEN CATE, L. (1965). A note on a simple and rapid method of bacteriological sampling by means of agar sausages. *Journal of Applied Bacteriology* **28**, 221–3.

WEBB, M. (1949). The influence of magnesium on cell division. 3. The effect of magnesium on the growth of bacteria in simple chemically defined media. *Journal of General Microbiology* **3**, 418–24.