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Aspects of the action of chlorhexidine on bacterial spores

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Summary

At low concentrations, chlorhexidine diacetate (CHA) was sporostatic towards *Bacillus subtilis* spores. Concentrations of CHA up to 25 μg/ml had little sporicidal activity at 20, 30 or 37°C, but the effect increased when the temperature was increased to 60 or 70°C. CHA at low concentrations inhibited spore outgrowth but not germination; this inhibitory effect could, to some extent, be reversed by Tween 80. The outgrowth-inhibiting effect was also shown by two other cationic bactericides, a quaternary ammonium compound (cetylpyridinium chloride) and a diamidine (dibromopropamidine isethionate). In contrast, phenol was an effective inhibitor of germination.

Introduction

Chlorhexidine diacetate (CHA) is a potent antimicrobial agent, showing activity at low concentrations against non-sporulating bacteria (Hugo and Russell, 1982). It is also effective against some types of yeasts and fungi (D'Arcy, 1971) and its mechanism of action against bacteria resides at the cytoplasmic membrane level (Longworth, 1971). However, interaction with cytoplasmic constituents appears to be associated with its lethal effects, which occur only at CHA concentrations considerably higher than those needed to achieve a bacteriostatic effect (Longworth, 1971).

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Chlorhexidine is not considered as being a sporicidal agent (Hugo and Russell, 1982). There is, however, a paucity of information about its effects on bacterial spores, so much so that a comprehensive discussion of chemical and physical methods used to destroy spores did not include a consideration of CHA (Russell, 1982).

In a recent paper (Russell et al., 1985) we examined the effects of some antimicrobial agents on the germination and outgrowth of *Bacillus subtilis* spores. One of the inhibitors tested was a quaternary ammonium compound (QAC), benzalkonium chloride. In the present report, we have studied the effects of CHA on the viability of *B. subtilis* spores at different temperatures; in addition, we have compared the effects of CHA with two other cationic bactericides, viz. a QAC (cetylpyridinium chloride, CPC) and a diamidine (dibromopropamidine isethionate, DBPI) on the germination and outgrowth of the spores.

Materials and Methods

Chemicals

These consisted of chlorhexidine diacetate, B.P., purchased from ICI Pharmaceuticals, Macclesfield, Cheshire, cetylpyridinium chloride, and Tween 80 from BDH Chemicals, Poole, Dorset. Dibromopropamidine isethionate was a gift from May and Baker, Dagenham, Essex.

Spores

Bacillus subtilis NCTC 8236 was employed as the test organism. It was grown on the surface of Nutrient agar (Oxoid, London) in Roux flasks for 7 days at 37°C. The growth was washed off the surface with sterile Water-for-Injections, B.P. and washed 6 times with this vehicle, before being resuspended in sterile water to a density of ca. 2.5×10^8 viable spores/ml. The suspension was stored at 4°C until required for use.

Inhibitory drug concentrations

These were determined in two ways, using undiluted and diluted spore suspensions: (a) broth dilution method (El-Falaha et al., 1985); (b) overdried agar method, using a Denley multipoint inocular (Denley Instruments, Billingshurst, Essex), as described by Russell et al. (1985). Broth containers and plates were incubated at 37°C for 24 h, and the presence or absence of growth noted. The sporostatic concentration was taken as the lowest concentration of a drug completely preventing growth.

Sporicidal studies

1-ml vols. of spore suspension were added to 9-ml vols. of appropriate final concentrations of CHA, previously equilibrated to the desired temperature. At appropriate intervals, 1-ml vols were removed, serially diluted in 9 ml Letheen broth (Oxoid, Basingstoke) and 1-ml vols. plated into 20 ml Letheen agar (Difco, Detroit). Colonies were counted after incubation at 37°C for 24 and 48 h. Preliminary experiments demonstrated that Letheen broth and Letheen agar had no inhibitory effect on colony counts, and that they quenched the action of CHA (see Table 1).

Germination and outgrowth

Four inhibitors were examined, using the method of Russell et al. (1985). These were chlorhexidine diacetate, cetylpyridinium chloride and

TABLE 1

SPOROSTATIC AND INHIBITORY CONCENTRATIONS OF DRUGS

Organism	Inoculum size [a]	Broth or Agar	Sporostatic [a] conc. or MIC [b] value (μg/ml)		
			CHA	CPC	DBPI
B. subtilis spores	U	Agar	4.75	8	40
	10^{-2}	Agar	4.0	6	30
	10^{-4}	Agar	2.5	4	15
	10^{-5}	Agar	1	2.5	5
	U	Broth	0.8	ND	ND
	10^{-2}	Broth	0.5	ND	ND
	10^{-4}	Broth	0.4	0.8	14
	10^{-4}	Letheen broth	15	ND	ND
S. aureus			1		1
Strep. pyogenes			0.5		1
Strep. viridans					2

[a] Sporostatic conc. effective against *B. subtilis* spores, as determined in this study. Undiluted incoculum (U) contained ca. 2.5×10^5 viable spores per 1 μl drop (agar method) or ca. 2.5×10^6 viable spores/ml (broth method). ND = not done.

[b] MIC = minimum inhibitory conc. against Gram-positive bacteria (values taken from the following references: for CHA, Hugo and Russell (1982); for CPC, unpublished data; for DBPI, Hugo (1971)).

CHA = chlorhexidine diacetate; CPC = cetylpyridinium chloride; DBPI = dibromopropamidine isethionate.

dibromopropamidine isethionate and, for comparison, phenol. In some experiments, the effect of Tween 80 was also examined.

Results and Discussion

Inhibitory drug concentrations

Sporostatic concentrations of CHA, CPC and DBPI are presented in Table 1, with minimum inhibitory concentrations (MICs) of the three inhibitors against Gram-positive bacteria listed for comparative purposes.

Low concentrations of CHA were effective sporostatic agents, especially in nutrient broth as opposed to nutrient agar (Table 1). Letheen broth, containing lecithin (0.07% w/v) and Tween 80 (2% w/v) was an effective inactivator of the inhibitory effect of chlorhexidine.

The inhibitory effect of CPC depended on the number of spores present and also, to a considerable effect, on the method, a fact also observed in studies with non-sporulating bacteria (El-Falaha et al., 1985).

DBPI did not have a particularly marked effect on spores, since high concentrations were needed to effect inhibition.

Sporicidal effects

The effects of CHA on *B. subtilis* spores at various temperatures are provided in Table 2 and Figs. 1a–e and 2. At 20, 30 and 37°C, CHA had little effect on the viability of the spores over a period of 120 min, even when the biguanide was used at a concentration of 25 μg/ml which corresponded to a value > 30 times the sporostatic concentration (Table 1). The effectiveness of CHA increased as the temperature rose (Fig. 1). However, even at the highest temperature tested, 70°C, the drug could hardly be considered as being highly sporicidal, since there was only a 1.8 log fall in viability after 120 min (Fig. 2) when 15 μg/ml was employed, although at 25 μg/ml and 70°C no survivors could be detected at 120 min (Fig. 2).

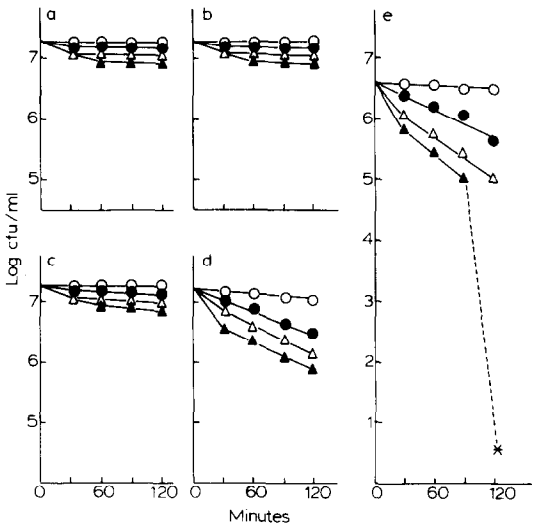

Fig. 1. Sporicidal activity of chlorhexidine diacetate (CHA) at different temperatures: (a) 20°C; (b) 30°C; (c) 37°C; (d) 60°C; (e) 70°C *. CHA concentrations (μg/ml): ○, 0; ●, 5; △, 15; ▲, 25. * Less than 10 cfu/ml after 120 min with CHA (25 μg/ml) at 70°C.

Thus, CHA is only weakly sporicidal and corresponds to other cationic disinfectants, the QACs, which are also deemed to be without a lethal effect on bacterial spores (Russell, 1982, 1983).

TABLE 2

LETHAL EFFECTS OF CHLORHEXIDINE DIACETATE AT DIFFERENT TEMPERATURES

Chlorhexidine conc. (μg/ml)	Surviving fraction after exposure for 2 h at (°C):				
	20	30	37	60	70
0	1.0	1.0	1.0	1.0	1.0
5	0.79	0.72	0.58	0.21	0.11
15	0.41	0.35	0.29	0.13	0.02
25	0.27	0.23	0.18	0.055	< 0.00001

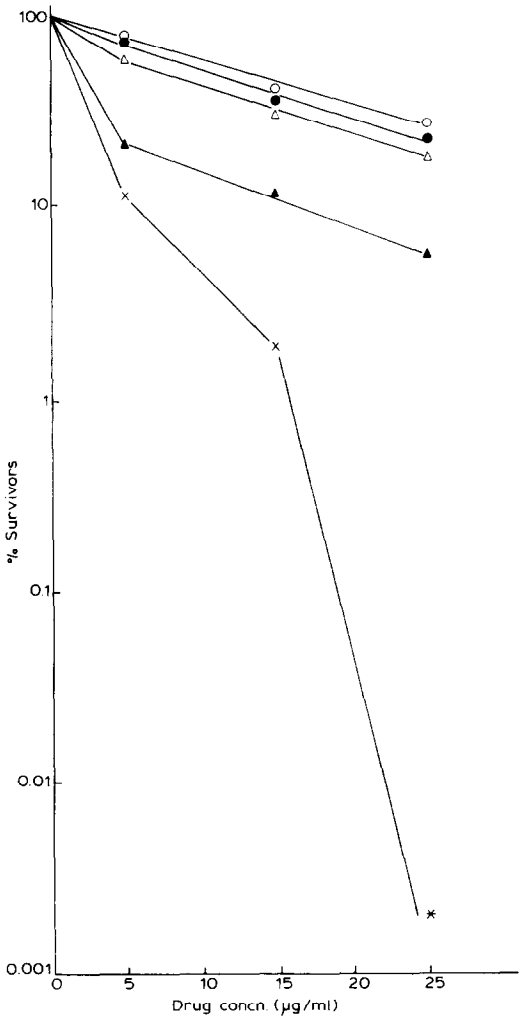

Fig. 2. Comparison of rates of kill of *B. subtilis* spores exposed to chlorhexidine diacetate (CHA) at different temperatures. Temperature: ○, 20°C; ●, 30°C; △, 37°C; ▲, 60°C; ×, 70°C *. * Less than 10 cfu/ml after 120 min with CHA (25 μg/ml) at 70°C.

Germination and outgrowth

Low concentrations of CHA were found to be sporostatic (Table 1). Accordingly, these concentrations were tested against germinating and outgrowing spores by adding CHA at different stages during the overall process.

CHA was not inhibitory at the commencement of, or during, the germination phase (Fig. 3a), whereas when added at the end of germination or during outgrowth it had an immediate effect (Fig. 3b, c). The same type of effect was observed when preheated spores (60°C, 10 min) were studied. This inhibitory effect on outgrowth was compared with that of CPC, a QAC with a known inhibition of this stage (Russell, 1971, 1982, 1983) and the diamidine, DBPI, which despite its antibacterial activity (Hugo, 1971) does not appear to have been studied previously in this manner. Both CPC and DBPI inhibited outgrowth but not germination (Fig. 4a, b), whereas phenol (not shown here, but see Russell et al., 1985) inhibited germination immediately.

Thus, the three cationic bactericides all inhibited outgrowth and not germination. It is, however, noticeable that there is quite a wide range in effective concentrations with 0.3 μg/ml required for CHA, 0.8 μg/ml (2.67 times) for CPC, and 14 μg/ml (47 times) for DBPI to achieve this inhibition, all on a weight basis. The corresponding molar concentrations are 4.7×10^{-7}M for CHA, 2.23×10^{-6}M for CPC (4.7 times) and 1.937×10^{-5} for DBPI (41.2 times).

The effect of CHA could be reversed by the addition of 2% Tween 80 added at zero time or some 20 min after the addition of CHA (Fig. 5). This suggests that at least some of the CHA can be removed from its binding with the spore surface. Chiori et al. (1965) found that the QAC, cetrimide, could only be removed from treated spores by washing the cells with an appropriate neutraliser (Lubrol W plus lecithin: see also Parker and Bradley, 1968).

It has been pointed out previously (Parker, 1969; Russell 1982, 1983; Russell et al., 1985) that there are two types of inhibitory agents acting against spores. In the first type, exemplified by phenols, cresols, parabens, glutaraldehyde, alcohols and mercuric chloride, germination is inhibited, whereas in the second group, which comprises ethylene oxide, chlorine, QACs, organomercurials, diamidines and chlorhexidine, germination is not inhibited, although outgrowth is. Germination is a complex biochemical process which involves the alteration of peripheral spore layers, hydration of spore structures, activation of lytic enzymes, degradation of cortex and the release of peptidoglycan and calcium dipicolinate (Russell, 1982). It is not immediately apparent why one

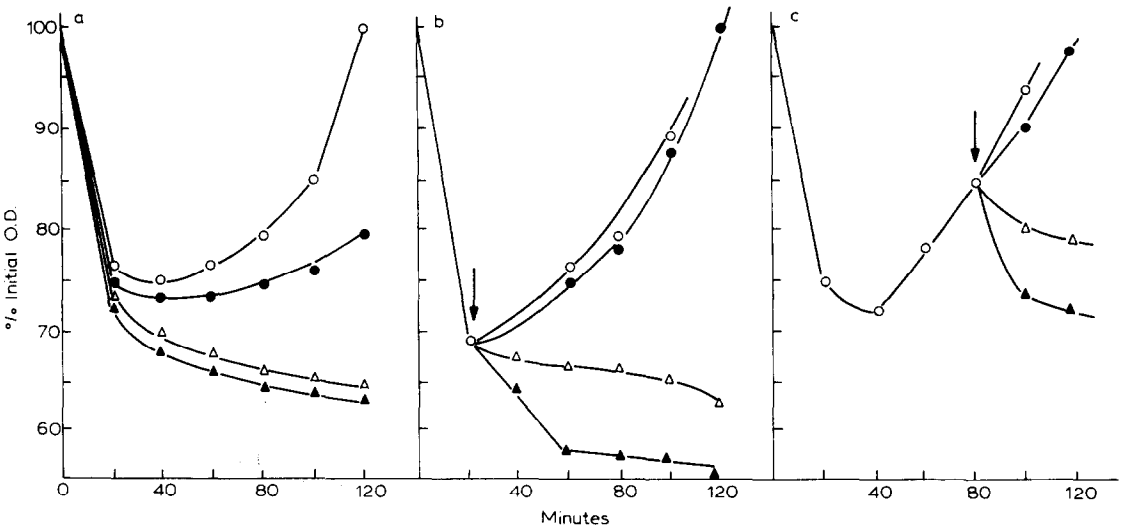

Fig. 3. Effect of chlorhexidine diacetate (CHA) on the germination and outgrowth of *B. subtilis* spores at 37°C. CHA was added at: (a) zero time; (b) at 20 min; (c) at 80 min. CHA concentrations (μg/ml): ○, 0; ●, 0.1; △, 0.3; ▲, 0.5.

group of agents should act at one stage and a second group at another stage, bearing in mind the diverse types of molecules involved and their differing mechanisms of action. It is, however, conceivable that some agents can alter susceptibility to germination markedly by changing the surface of the spore. This is an aspect worth exploring and is one upon which experiments are currently in progress.

Gould (1971, 1983) made the pertinent point

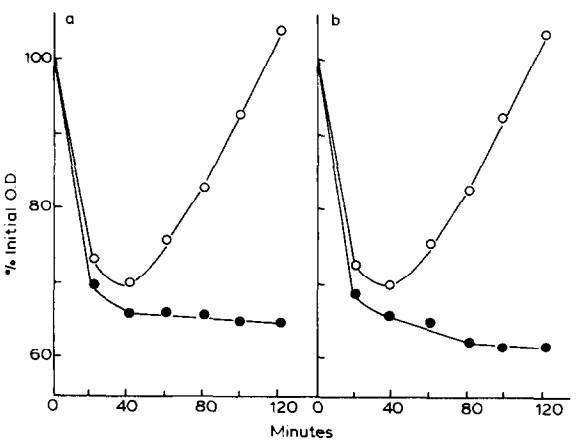

Fig. 4. Effect of (a) cetylpyridinium chloride (CPC), and (b) dibromopropamidine isethionate (DBPI) on the germination and outgrowth of *B. subtilis* spores at 37°C. CPC concentration (μg/ml): ○, 0; ●, 0.8. DBPI concentration (μg/ml): ○, 0; ●, 14. (Drugs were added at zero time.)

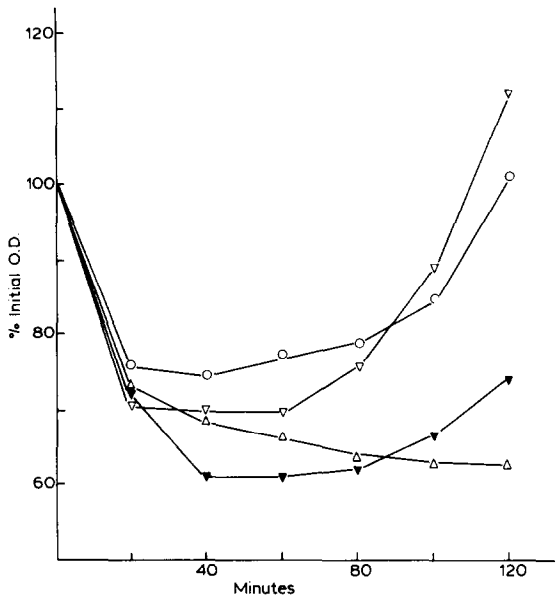

Fig. 5. Reversal of the effect of chlorhexidine diacetate (CHA) on *B. subtilis* spores by Tween 80 (2%). ○———○, control; △———△, CHA (0.3 μg/ml) at zero time; ▽———▽, CHA (0.3 μg/ml) and Tween 80, both at zero time; ▼———▼, CHA (0.3 μg/ml) at zero time and Tween 80 at 20 min.

that although the mechanisms of action of most chemicals on spores are unknown, there is a strong argument in favour of the spore coats playing an important role in bacterial spore resistance. It is likely that the high resistance of intact spores to chlorhexidine results from their impermeability to the biguanide. Future studies will examine the importance of the coats in this resistance.

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