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The effect of sodium hypochlorite–methanol combinations on spores and spore-forms of *Bacillus subtilis*

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Summary

The resistance of spores of *Bacillus subtilis* NCTC 10073 to sodium hypochlorite and sodium hypochlorite–methanol combinations was compared with reference to concentration and pH. Activity against ion-exchange spores (H- and Ca-form) and coat-defective spores (SLS-, UME-, UMS-, UDT- and UDS-treated) was examined in relation to degree of coat protein solubilization. Methanol greatly enhanced hypochlorite activity at all stages of coat extraction. The degree of spore penetration and site of action is discussed.

Introduction

Hypochlorites have a wide antimicrobial spectrum and are among the most potent sporicidal agents. Their antimicrobial activity can, however, be markedly affected by organic matter and pH. Increasing hypochlorite concentration can overcome to some extent the former problem whilst buffering freshly prepared solutions to approximately pH 7.6 provides rapid sporicidal activity with optimum stability (Babb et al., 1980). Potentiation of sporicidal activity by addition of various agents to hypochlorite solutions is one other method of overcoming these problems. Weber and Levine (1944) found this to be the case on addition of low concentrations of ammonia and Farkas-Hinsley (1964) achieved similar potentiation in the presence of bromine. Cousins and Allan (1967) using mixtures of sodium hydroxide and hypochlorite observed a potentiation of sporicidal effect which may be linked to the

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effect of the alkali on the spore coats from which protein is removed (Wyatt and Waites, 1974; Kulikovsky et al., 1975).

Several authors have recently studied the potentiation observed on addition of alcohols to hypochlorite solutions (Kelsey et al., 1974; Coates and Death, 1978; Death and Coates, 1979). The mechanism of this potentiation is unexplained though Coates and Death (1978) suggested a softening effect of the alcohol on the spore coats allowing greater penetration by hypochlorite. We have previously reported on the sporicidal activity of a hypochlorite–methanol mixture (McErlean et al., 1980); details of the nature of this activity are currently presented.

Materials and Methods

Preparation of spores

Bacillus subtilis NCTC 10073 was maintained on nutrient agar slopes at 4°C. Spores were produced either on the medium of Beeby and Whitehouse (1965) for 5 days at 30°C (Method A), or on the chemically defined medium of Ellar and Posgate (1974) for 24 h at 37°C (Method B). Spores were harvested, washed 4 times with sterile distilled water and incubated in phosphate buffer (0.066 M, pH 7.0) containing 200 μg/ml lysozyme for 60 min at 37°C. Spores were washed a further twice with sterile distilled water and stored in dense suspensions at 4°C.

Preparation of ion-exchange spore forms

Cleaned spores were converted to the calcium (Ca-form) and hydrogen (H-form) forms by the method of Kovacs-Proszt and Farkas (1976). Ion-exchange spores were washed 3 times with phosphate buffer (pH 7.0) followed by 3 washes with sterile water to remove any adhering acid or calcium acetate, presence of which could influence the sporicidal activity of the combinations under study.

Preparation of coat-defective forms

Spore coats were extracted to varying extents by use of the following methods: 10.0% (v/v) 2-mercaptoethanol (Sigma Chemicals) +8 M urea (UME) pH 7.0, 60°C, 60 min; 50 mM dithiothreitol (Aldrich Chemicals) +8 M urea (UDT) pH 10.3, 37°C, 180 min; 1.0% (w/v) sodium lauryl sulphate (SLS) pH 7.0, 60°C, 60 min; 8 M urea 10% (v/v) mercaptoethanol + 1.0% (w/v) sodium lauryl sulphate (UMS) pH 7, 60°C, 60 min; 8 M urea + 50 mM dithiothreitol + 1.0% sodium lauryl sulphate (UDS) pH 10.3, 37°C, 180 min. SLS, UME and UMS spores were washed twice with sterile distilled water whereas UDT and UDS spores washed twice with sterile 0.14 M saline, pH 9.0.

Hypochlorite–alcohol combinations

Sodium hypochlorite solution (10–14% w/v av. Cl_2) was obtained from BDH, methanol was of analar grade from Hopkin and Williams. Hypochlorite solutions (pH 10.8–11.0) were prepared freshly at the start of each experiment; available chlorine assays were made by an arsenite titration method (Coates, 1977).

Sporicidal activity

A 9 ml volume of hypochlorite solution was equilibrated at 20°C before addition of 1 ml of standardized spore suspension. At timed intervals, 1 ml samples of the mixture were withdrawn, serially diluted in 0.5 % (w/v) sodium thiosulphate solution (Trueman, 1971) and the number of colony-forming units (c.f.u.) per ml determined by the drop-plate method of Miles and Misra (1938). Tryptone soya agar plates employed were incubated for 48 h at 32°C.

When hypochlorite–methanol combinations were employed it was necessary to equilibrate both reagents separately in the water bath prior to mixing and addition of spore suspension.

Protein estimation

Protein was determined by the method of Lowry et al. (1951). Spores or spore forms were broken in a Braun MSK cell homogenizer with 0.1 mm glass beads (4000 rev./min, 10 min) using 1 min pulses with 1 min cooling between each pulse. Percentage breakage was checked microscopically. The supernatant was pipetted off, centrifuged (15,000 *g*, 45 min) and then analyzed for protein content. The insoluble pellet was resuspended in 2 M NaOH and heated for 30 min in a boiling water bath before analysis. The supernatant represented core protein whereas the insoluble pellet contained the coat protein (Spudich and Kornberg, 1968).

Results

Sodium hypochlorite (1000 parts/10^6 av. Cl) combined with methanol (25%) at pH 11 proved to be considerably more effective than hypochlorite only, in reducing the viability of the spore population by 90% (Table 1). Spores produced on defined medium (Method B) exhibited much less resistance to hypochlorite–methanol than spores produced on solid medium (Method A) emphasizing the need for standardized spore suspensions in sporicidal studies. Two patterns emerged in the resistance

TABLE 1

SPORICIDAL ACTIVITY OF HYPOCHLORITE FORMULATIONS ON NORMAL AND ION-EXCHANGE SPORES OF *B. subtilis* PRODUCED UNDER DIFFERENT CONDITIONS

Formulation	Method of spore production [b]	Time (min) to kill 90% of spores [a] or spore forms		
		Untreated	Ca-form	H-form
Hypochlorite (1000 parts/10^6)	A	19	21	11
+ methanol (25%) pH 11	B	7	8	5
Hypochlorite (1000 parts/10^6) pH 11	A	110	95	72

[a] Initial count: 1×10^8 c.f.u./ml.

[b] Spores were prepared as in Materials and Methods by Method A, 5 days at 30°C, or by Method B, 24 h at 37°C.

TABLE 2

EFFECT OF ALTERING pH AND CONCENTRATION ON THE SPORICIDAL ACTIVITY OF HYPOCHLORITE–METHANOL COMBINATIONS WITH RESPECT TO ION-EXCHANGE SPORES OF *B. subtilis*

Formulation	Spore type [b]	Minimum log reduction in viable count [a] after time of contact (min)					
		2.5	5	7.5	10	15	20
1000 parts/10^6 av. Cl	Untreated	–	0	–	<1	<1	1
+25% methanol	Ca-form	–	0	–	<1	<1	1
pH = 11	H-form	–	<1	–	<1	1	2
100 parts/10^6 av. Cl	Untreated	–	4	>6	>6	–	–
+1% methanol	Ca-form	–	4	>6	>6	–	–
pH = 6	H-form	>6	>6	>6	>6	–	–

[a] Initial count: 1×10^8 c.f.u./ml.

[b] Spores were prepared by Method A and converted to the ion-exchange form as described in Materials and Methods.

of ion-exchange forms to the two formulations in Table 1. With both formulations and under each condition of spore production, the H-form presented least resistance. This clear pattern was not observed with the Ca-form which became less resistant than the untreated spore to the hypochlorite only formulation.

When the pH of hypochlorite–methanol formulations is examined in conjunction with concentration (Table 2), the superior cidal effects of the lower pH formulation, albeit at a much reduced concentration, against untreated spores is observed. Similar patterns to those presented in Table 1 were observed for the ion-exchange forms with the H-form again showing increased susceptibility, especially to the lower pH formulation.

The percentage of spore coat protein solubilized by various agents shown in Table 3 ranged from 10 to 70% in line with the severity of treatment. The relative susceptibilities of these coat-defective forms to the two formulations (Fig. 1a and b) are in quite good agreement with loss of spore coat protein, the UDS-form being most susceptible in each case.

TABLE 3

REMOVAL OF PROTEIN FROM SPORE COATS OF *B. subtilis*

Treatment [a]	Treatment conditions	% coat protein solubilized
SLS	pH 7, 60°C, 60 min	10
UME	pH 7, 60°C, 60 min	38
UMS	pH 7, 60°C, 60 min	54
UDT	pH 10.3, 37°C, 180 min	32
UDS	pH 10.3, 37°C, 180 min	70

[a] Abbreviations of chemicals and concentrations described in Materials and Methods.

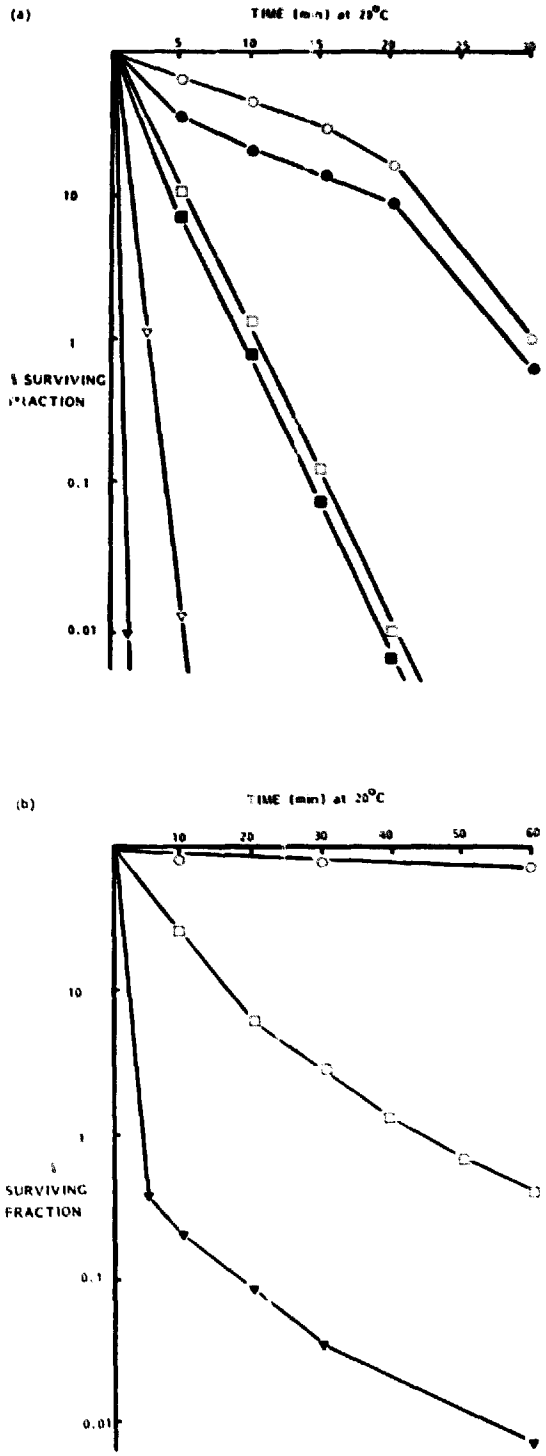

Fig. 1. Resistance of untreated and coat-extracted spores of *Bacillus subtilis* to: (a) sodium hypochlorite (1000 parts/10^6 av. Cl) combined with methanol (25% v/v); and (b) sodium hypochlorite (1000 parts/10^6 av. Cl). Method A spores as described in Materials and Methods. ○, untreated spores; ●, SLS-treated; □, UME-treated; ■, UDT-treated; ▽, UMS-treated; ▼, UDS-treated. Initial spore count: 1×10^8 c.f.u./ml.

Discussion

The possibility that the perceived susceptibility of the H-form to both formulations might be the result of a pH influence in the form of a microenvironmental residue from formation of the ion-exchange form can be discounted by reference to Table 2. The relative susceptibilities of the 3 types, untreated, Ca-form and H-form, remain unchanged at both pH 11 and pH 6. The occurrence of a pronounced lag or shoulder before a rapid, logarithmic loss in viability was observed for *B. metiens* spores by Rudoff and Levine (1941) who assumed this represented time for penetration of chlorine. The same lag was apparent in the untreated and Ca-form spores of *B. subtilis* exposed to both formulations though a much reduced lag was observed on chemical treatment of the H-form. This lag phase was also noticeable to some extent in SLS-treated spores in contact with hypochlorite–methanol indicating some difficulty still in penetration. Increasing severity of treatment resulting in greater removal from spore coats progressively increased the, now logarithmic, rate of kill with this formulation. In comparison, with hypochlorite only although the lag phase has also disappeared on treatment of the UME- and UDS-treated spores the initial logarithmic kill is lost and tailing results. This may indicate a degree of heterogeneity in the population with the possibility of incomplete coat removal.

UME treatment of *B. subtilis* spores was shown to remove approximately 38% coat protein though the degree of kill experienced with hypochlorite only was still less at 30 min than that obtained on exposing untreated spores to the hypochlorite-methanol formulation. Similarly, a more rapid kill was obtained with the latter formulation against UDS-treated spores than with hypochlorite, even when consideration is given only to the logarithmic portions of the kill.

Consideration of these facts points towards a role for methanol in aiding penetration of hypochlorite, whether as far only as the cortex or further to the core. However, further information (results not shown) shows that pre-treatment of the spore for 30 min with methanol, with removal of this by filtration and washing, followed by hypochlorite treatment does not significantly increase sporicidal activity. Furthermore, reversing this sequence with a pre-treatment of 30 min with hypochlorite followed by 30 min treatment with methanol, after filtration and washing, resulted in no further reduction in c.f.u. from that experienced at termination of the hypochlorite pre-treatment. Similar results have been shown by Cousins and Allan (1967) who found an insignificant reduction in viable *B. subtilis* spores after treatment with NaOH compared to the effect of a combined hypochlorite NaOH treatment which was much superior to hypochlorite only. Coulthard and Sykes (1936) also found a solution of 1% NaOH in 70% ethyl alcohol killed spores whereas these chemicals alone were ineffective.

If hypochlorite penetration, and therefore activity, is facilitated by a potentiating or synergistic effect of methanol akin to the 'softening' of the spore coat suggested by Coates and Death (1978), it is interesting to speculate on the degree of penetration and site of action compared with hypochlorite. Wyatt and Waites (1975) suggested that high concentrations of chlorine disrupt the spore coat and inactivate the germination mechanism. Gorman et al. (1983) have shown that revival, i.e.

increased germination, of hypochlorite-treated spores is possible up until the end of the lag phase. The much reduced lag phase and faster rate of kill observed on exposing spores to hypochlorite–methanol coupled with the absence of tailing with coat-defective forms suggests a faster and further penetration, possibly to the core.

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