Effect of pH on sporicidal and microbicidal activity of buffered mixtures of alcohol and sodium hypochlorite

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SUMMARY The effect of pH on the activity of buffered sodium hypochlorite solution, and a buffered methanol/sodium hypochlorite mixture, against *Bacillus subtilis* spores was investigated. The best results, considering both sporicidal activity and stability, were achieved in the pH range 7·6-8·1. The sporicidal activity and stability of five alcohol/hypochlorite mixtures, each containing a different alcohol and buffered to pH 7·6, and of hypochlorite alone buffered to pH 7·6, were compared. The mixtures were marginally more sporicidal than hypochlorite alone when fresh but were much less stable. An unbuffered methanol/hypochlorite mixture, a methanol/hypochlorite mixture buffered to pH 7·6, and hypochlorite alone buffered to pH 7·6 were all found to be effective against six vegetative organisms and spores of *B. subtilis* and *Clostridium sporogenes*. By buffering alcohol/hypochlorite mixtures or hypochlorite solution alone in the pH range 7·6-8·1, high sporicidal activity can be achieved with low concentrations of alcohol and hypochlorite. Such formulations show promise for the disinfection of heat-sensitive medical equipment.

In a previous paper (Coates and Death, 1978) we described the sporicidal activity of various unbuffered alcohol/sodium hypochlorite mixtures against *Bacillus subtilis* NCTC 10073 spores. The best performance was achieved by a mixture containing methanol (50% v/v) and hypochlorite (2000 parts per million of available chlorine (ppm av. Cl)) in distilled water. This mixture effected a five-log reduction in spore count in 10 minutes, both when freshly prepared and for at least 8 hours after preparation. However, the usefulness of such mixtures is limited by the high concentrations of alcohol and available chlorine present; alcohol is a solvent, and concentrated sodium hypochlorite is corrosive. It has long been known (Sykes, 1965) that, within limits, the sporicidal activity of chlorine disinfectants may be markedly increased by lowering the pH. Hence we decided to investigate the effect of pH on the sporicidal activity and stability of buffered alcohol/hypochlorite mixtures and buffered hypochlorite alone. By buffering to a lower pH, we hoped to obtain highly sporicidal mixtures that contained low concentrations of alcohol and hypochlorite. A

buffered mixture was selected, and its activity against a range of organisms was compared with that of buffered hypochlorite alone and with an unbuffered, relatively concentrated methanol/hypochlorite mixture.

Material and methods

Spores of *B. subtilis* NCTC 10073 and *Clostridium sporogenes* NCTC 276 were used in sporicidal studies. Vegetative cells of *Mycobacterium fortuitum* NCTC 8573, *Pseudomonas aeruginosa* NCTC 6749, *Proteus vulgaris* NCTC 4635, *Escherichia coli* NCTC 8196, *Staphylococcus aureus* NCTC 4163, and *Candida albicans* NCPF 3153 were used in microbicidal studies.

An aqueous suspension of *B. subtilis* spores was prepared by the method of Beeby and Whitehouse (1965) and stored at 4°C. An aqueous suspension of *Cl. sporogenes* spores was prepared as follows. The organism was grown on cooked meat agar (Dye and Mead, 1972) and incubated anaerobically (90% hydrogen/10% carbon dioxide) at 32°C for four days. The harvested aqueous suspension was filtered through glass wool and washed three times in sterile

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distilled water by means of centrifugation and resuspension. It was heated to 70-75°C for 10 minutes and, when cool, was refrigerated at 4°C for a week. After a final wash it was refrigerated at 4°C. *Ps. aeruginosa*, *Pr. vulgaris*, *E. coli*, and *Staph. aureus* were grown and subcultured daily in the synthetic medium of Wright and Mundy (1960), and *C. albicans* in Sabouraud's dextrose broth, which contains 10 g/l mycological peptone and 40 g/l dextrose and has a pH of approximately 5.6. For tests, an overnight culture was centrifuged, and the packed cells were resuspended in sterile WHO standard hard water (Kelsey and Maurer, 1974) by shaking with glass beads. *M. fortuitum* was grown as above (Wright and Mundy medium) except that it was subcultured every three days, and a 3-day-old culture was used for preparing a test-suspension. The vegetative cell suspensions were used in tests in the same way as spore suspensions.

The buffers used were prepared as described by Gomori (1955) and were as follows: pH 5.0-7.0, citrate/phosphate buffer; pH 7.3-8.0, phosphate buffer; pH 8.0-9.0, boric acid/borax buffer; and pH 9.9-10.0, borax/sodium hydroxide buffer. Stock solutions were made up in sterile distilled water. Most work was carried out at pH 7.6. Stock buffer solution of pH 7.6 was prepared by mixing 13 ml of an 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml) with 87.0 ml of an 0.2 M solution of dibasic sodium phosphate (53.65 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ or 71.7 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1000 ml) and diluting to a total volume of 200 ml. The buffered hypochlorite solutions and buffered alcohol/hypochlorite mixtures tested were always prepared freshly at the start of each experiment. pH measurements were made by means of a Philips PW9418 pH meter. Available chlorine assays were made by an arsenite titration method (Coates, 1977).

Five alcohols were used in the tests: methanol (AnalaR), propan-1-ol (AnalaR) and propan-2-ol (AnalaR) (Hopkin and Williams Ltd), ethanol (absolute) (James Burrough Ltd), and ethanediol (SLR) (Fisons Ltd). The hypochlorite used was sodium hypochlorite solution (10-14% w/v av. Cl) (BDH Ltd). For tests on mixtures containing 1% v/v alcohol, the mixtures were prepared by adding 1 ml of neat alcohol and 1 ml of appropriately diluted hypochlorite solution to 98 ml of buffer solution. Other solutions and mixtures were similarly prepared.

Microbicidal tests were carried out at $25^\circ \pm 0.1^\circ\text{C}$ in a thermostatically controlled water bath, as previously described (Coates and Death, 1978). At timed intervals after the addition of inoculum to test mixtures, 1-ml samples were taken and added to

9 ml of an 0.5% aqueous solution of sodium thio-sulphate. Tenfold dilutions of sample/inactivator were made in one-quarter strength Ringer's solution, as necessary. A sample of each dilution was taken with a 50-dropper pipette, and 10 drops were seeded onto the surface of each of three nutrient agar plates. After incubation at 32°C for two to seven days the colonies were counted, and the mean was determined. Log reductions in viable count were calculated from the initial viable count of the inoculum and the final viable count of the survivors. The activity of each mixture was tested on at least three separate occasions.

Results

EFFECT OF pH ON SPORICIDAL ACTIVITY AND STABILITY

In preliminary control experiments, it was established that aqueous solutions of the buffers had no detectable sporicidal effect over the experimental times involved. Furthermore, mixtures buffered to pH 8 with phosphate buffer had the same sporicidal activity as mixtures buffered to pH 8 with boric acid/borax buffer.

The activity against *B. subtilis* spores of buffered hypochlorite solution (100 ppm av. Cl) was compared with that of a buffered mixture of methanol (1% v/v) and hypochlorite (100 ppm av. Cl) over the pH range 5-10. Experiments were done in triplicate. The minimum log reductions in spore count effected at each pH investigated are given in Tables 1 and 2. The scatter of the results obtained fell in the range n to $n + 1$, where n is the minimum log reduction achieved. With both the hypochlorite solution and the mixture containing alcohol and hypochlorite, as the pH was lowered so the sporicidal activity increased, reaching a maximum at around pH 6. In the hypochlorite solution, the increase in activity with decrease in pH is due to the increase in concentration of hypochlorous acid (HOCl) relative to hypochlorite ion (OCl^-). Hypochlorous acid is approximately 100 times more sporicidal than hypochlorite ion (Brazis *et al.*, 1958). The same may apply in mixtures of alcohol and hypochlorite. Hypochlorous acid concentration reaches a peak around pH 6, below which av. Cl increasingly takes the form of chlorine gas. A comparison of Tables 1 and 2 shows that a mixture of methanol and hypochlorite is slightly more sporicidal than hypochlorite alone at the same pH.

Next, a comparison was made of the pH and sporicidal activity of a freshly prepared, unbuffered solution in distilled water of hypochlorite containing 100 ppm av. Cl and freshly prepared Milton fluid

Table 1 *Effect of pH on sporicidal activity of a fresh solution of hypochlorite (100 ppm av. Cl)*

pH	Minimum log reductions in <i>B. subtilis</i> spore count in: (minutes)					
	2	5	10	15	20	30
5.2	4	>5	>5	>5	>5	>5
6.1	>5	>5	>5	>5	>5	>5
7.0	4	>5	>5	>5	>5	>5
7.3	4	>5	>5	>5	>5	>5
7.5	1	>5	>5	>5	>5	>5
7.6	1	5	>5	>5	>5	>5
7.7	<1	4	>5	>5	>5	>5
7.8	<1	3	>5	>5	>5	>5
8.1	<1	3	>5	>5	>5	>5
8.2	<1	<1	4	>5	>5	>5
8.4	<1	<1	3	4	>5	>5
9.0	<1	<1	<1	<1	1	3
9.9	<1	<1	<1	<1	<1	>1

Table 2 *Effect of pH on sporicidal activity of a fresh mixture containing methanol (1% v/v) and hypochlorite (100 ppm av. Cl)*

pH	Minimum log reductions in <i>B. subtilis</i> spore count in: (minutes)					
	2	5	10	15	20	30
5.3	3	>5	>5	>5	>5	>5
6.2	>5	>5	>5	>5	>5	>5
7.0	5	>5	>5	>5	>5	>5
7.6	3	>5	>5	>5	>5	>5
8.1	<1	5	>5	>5	>5	>5
8.9	<1	<1	<1	1	3	5
9.9	<1	<1	<1	<1	<1	>1

(Richardson-Merrell Ltd) diluted in tap water as directed, and containing 128 ppm av. Cl. The unbuffered hypochlorite solution had a pH of 9.6 and possessed little sporicidal activity. In contrast, the Milton fluid had a pH of 8.5 and achieved a 5-log reduction in spore count within 15 minutes.

The stability of hypochlorite solution (100 ppm av. Cl), both alone and when mixed with methanol (1% v/v), was also affected by pH. Lowering the pH increased the rate at which available chlorine was lost (Table 3). As with sporicidal activity, the effect was greater with the mixture than with hypochlorite alone. Thus, with both the hypochlorite solution and the alcohol/hypochlorite mixture, as the pH fell so the sporicidal activity increased and the stability decreased. Our aim was to find a disinfectant that would effect a 5-log reduction in spore count in less than 15 minutes, and which would be active for at least 24 hours after preparation. We found optimum sporicidal activity coupled with stability in the pH range 7.6-8.1. Both with hypochlorite alone and with the mixture, at least a 5-log reduction in spore count was obtained in 10 minutes, and the av. Cl was not all lost for at least 30 days with hypochlorite alone and for seven days with the mixture.

Table 3 *Effect of pH on stability of hypochlorite solution, and a methanol/hypochlorite mixture, at 25°C*

pH	Time taken for av. Cl level to fall below 10 ppm from:	
	Hypochlorite (100 ppm av. Cl)	Methanol (1% v/v) + hypochlorite (100 ppm av. Cl)
5.0	20 minutes	20 minutes
6.0	1 hour	1 hour
7.0	3 weeks	4 days
7.6	>1 month	1 week
8.0	>1 month	1-2 weeks
9.0	>1 month	1 month
10.0	>1 month	>1 month

SPORICIDAL ACTIVITY AND STABILITY AT A FIXED pH

From the above it was decided to carry out further experiments at pH 7.6 ± 0.1 , when maximum sporicidal activity was coupled with minimum stability requirement. The sporicidal activity of hypochlorite solution (100 ppm av. Cl) alone buffered to pH 7.6 was both good and long-lasting; a 5-log reduction in spore count occurred within 10 minutes with solutions up to at least a week old. The addition of alcohol (1% v/v) to the hypochlorite solution slightly enhanced the sporicidal activity initially;

Table 4 Activity against *B. subtilis* spores of alcohol/hypochlorite mixtures at pH 7.6, and 25°C

Mixture	Minutes required for a 5-log reduction		
	Fresh mixture	24-hour-old mixture	48-hour-old mixture
Methanol (1% v/v) + hypochlorite (100 ppm av. Cl)	3	8	15
Ethanol (1% v/v) + hypochlorite (100 ppm av. Cl)	5	10	>30
Propan-2-ol (1% v/v) + hypochlorite (100 ppm av. Cl)	4	10	>30
Propan-1-ol (1% v/v) + hypochlorite (100 ppm av. Cl)	4	5	10
Ethanediol (1% v/v) + hypochlorite (100 ppm av. Cl)	4	8	30
Hypochlorite (100 ppm av. Cl)	5	5	5

however, the activity of the mixtures decreased steadily, and within 24 hours it was less than that of hypochlorite solution alone of the same age (Table 4). In mixtures up to 24 hours old, similar results were obtained with each of the five different alcohols tested. Hypochlorite alone buffered to pH 7.6 was comparatively stable (Table 5); even after a month, 65 ppm of an initial 100 ppm av. Cl remained. Addition of alcohol decreased the stability significantly, and all av. Cl was lost in a few days, the

actual rate of loss varying with the alcohol involved and alcohol concentration.

ACTIVITY AGAINST A RANGE OF ORGANISMS

From our previous results (Coates and Death, 1978) and the current results, we decided to select an unbuffered alcohol/hypochlorite mixture, a buffered alcohol/hypochlorite mixture, and a buffered hypochlorite solution for further tests against a range of test-organisms. The unbuffered mixture contained methanol (25% v/v) and hypochlorite (2000 ppm av. Cl), the buffered mixture methanol (1% v/v) and hypochlorite (100 ppm av. Cl) at pH 7.6, and the buffered hypochlorite solution 100 ppm av. Cl at pH 7.6. These were freshly prepared at the start of experiments. Spores of *B. subtilis* and *Cl. sporogenes* were used in sporidical studies, and vegetative cells of *M. fortuitum*, *Ps. aeruginosa*, *Pr. vulgaris*, *E. coli*, *Staph. aureus*, and *C. albicans* were used in microbicidal studies.

The results obtained are given in Table 6. With regard to homogenous suspensions of spores or vegetative cells of the test-organisms, *B. subtilis* spores proved most difficult to kill. However, *M. fortuitum* readily forms clumps (Bergan and Lystad, 1971) in which interior cells are protected by peripheral cells. The repeatability of results obtained with this organism was low unless the washed suspensions used in tests had been previously shaken vigorously

Table 5 Effect of alcohol, and alcohol concentration, on stability of alcohol/hypochlorite mixtures at pH 7.6, and 25°C

Mixture	Days taken for av. Cl level to fall below 10 ppm
Methanol (10% v/v) + hypochlorite (100 ppm av. Cl)	2
Methanol (5% v/v) + hypochlorite (100 ppm av. Cl)	3
Methanol (1% v/v) + hypochlorite (100 ppm av. Cl)	7
Ethanol (1% v/v) + hypochlorite (100 ppm av. Cl)	2
Propan-2-ol (1% v/v) + hypochlorite (100 ppm av. Cl)	3
Propan-1-ol (1% v/v) + hypochlorite (100 ppm av. Cl)	7
Ethanediol (1% v/v) + hypochlorite (100 ppm av. Cl)	3
Hypochlorite (100 ppm av. Cl)	>30

Table 6 Microbicidal activity of fresh alcohol/hypochlorite mixtures, and hypochlorite solution, at 25°C

Test organism	Minutes required to achieve a 5-log reduction in		
	Methanol (25% v/v) + hypochlorite (2000 ppm av. Cl) at pH 11.2	Methanol (1% v/v) + hypochlorite (100 ppm av. Cl) at pH 7.6	Hypochlorite (100 ppm av. Cl) at pH 7.6
Spores of <i>Bacillus subtilis</i>	15	3	5
Spores of <i>Clostridium sporogenes</i>	7	<1	<1
<i>Mycobacterium fortuitum</i>	<1	2* to >10	3* to >10
<i>Pseudomonas aeruginosa</i>	<1	<1	<1
<i>Proteus vulgaris</i>	<1	<1	<1
<i>Escherichia coli</i>	<1	<1	<1
<i>Staphylococcus aureus</i>	<1	<1	<1
<i>Candida albicans</i>	<1	<1	<1

*Obtained with clump-free suspensions.

with glass beads to break down the clumps. Such clump-free suspensions were readily killed. In contrast, suspensions of *M. fortuitum* containing clumps proved more resistant than homogenous suspensions of *B. subtilis* spores to both the buffered alcohol/hypochlorite mixture and the buffered hypochlorite solution, although not to the unbuffered, relatively concentrated alcohol/hypochlorite mixture.

Discussion

In our previous paper (Coates and Death, 1978) we showed that the sporicidal activity of a sodium hypochlorite solution was potentiated by the addition of an alcohol. The sporicidal activity of alcohol/hypochlorite mixtures containing the same initial level of available chlorine (2000 ppm av. Cl) was shown to vary with the particular alcohol used, the concentration of alcohol, and the age of the mixture. A mixture of methanol (50% v/v) and hypochlorite (2000 ppm av. Cl) was found to be the most sporicidal of the various mixtures tested against *B. subtilis* spores. Unfortunately, the applications of such a mixture are limited because of the high concentrations of both alcohol and hypochlorite present. Furthermore, the mixture is unstable, and although the sporicidal activity is maintained for at least eight hours after preparation, it is all gone within 24 hours.

The aim of the research described in this paper has been to find an alcohol/hypochlorite mixture, or a hypochlorite solution, which is as sporicidal as a mixture of methanol (50% v/v) and hypochlorite (2000 ppm av. Cl) but which contains low concentrations of both alcohol and hypochlorite or hypochlorite alone, and which is active for at least 24 hours. We hoped to achieve this by lowering the pH of alcohol/hypochlorite mixtures or hypochlorite solution alone by means of buffering. As anticipated, we have found that as the pH of mixtures and hypochlorite solution falls, so the sporicidal activity greatly increases. Therefore, it is possible to lower the concentrations of alcohol and hypochlorite present. However, as the pH falls, so the mixtures and the hypochlorite solution become increasingly unstable. Hence a compromise must be made between increasing the sporicidal activity and decreasing the stability. In our first experiments, we found an acceptable level of sporicidal activity coupled with an acceptable degree of stability in the pH range 7.6-8.1, the optimum occurring around pH 7.6. Hence pH 7.6 was selected as the standard pH for subsequent studies.

Mixtures containing alcohol (1% v/v of either

methanol, ethanol, propan-1-ol, propan-2-ol, or ethanediol) and hypochlorite (100 ppm av. Cl), buffered to pH 7.6, have been shown to be highly sporicidal. Such mixtures have several advantages over the unbuffered mixtures previously described (Coates and Death, 1978), which contain alcohol (up to 50% v/v) and hypochlorite (2000 ppm av. Cl). The very much lower concentrations of both alcohol and hypochlorite present minimise difficulties arising from the solvent properties of the alcohol and the corrosive, caustic, and bleaching properties of the hypochlorite. Furthermore, the buffered mixtures are cheaper, more sporicidal, and more stable than the unbuffered mixtures. For example, a mixture containing methanol (1% v/v) and hypochlorite (100 ppm av. Cl) buffered to pH 7.6 has been shown to effect a 5-log reduction in viable count of *B. subtilis* spores in 3 minutes when freshly prepared, and in 8 minutes when 24 hours old. In comparison, an unbuffered mixture containing methanol (25% v/v) and hypochlorite (2000 ppm av. Cl), at pH 11.2, effects a 5-log reduction in 15 minutes when freshly prepared but possesses negligible sporicidal activity after 24 hours (Coates and Death, 1978). Buffered mixtures possess the same characteristics as unbuffered mixtures; the addition of alcohol to hypochlorite results in increased sporicidal activity coupled with decreased stability; the greater the alcohol concentration the greater the effect.

In practice, it may be considered that hypochlorite solution (100 ppm av. Cl) alone, buffered to around pH 7.6, is overall better than a mixture with alcohol (1% v/v) if factors other than initial sporicidal activity are taken into account. The sporicidal activity of the hypochlorite solution alone is both considerable and prolonged. Solutions up to at least a week old effect a 5-log reduction in spore count within 10 minutes. The available chlorine content of the hypochlorite solution decreases relatively slowly; even after a month 65 ppm of an initial 100 ppm av. Cl remains. The addition of alcohol (1% v/v) to the hypochlorite solution decreases its stability. Although the initial sporicidal activity is marginally greater, this decreases steadily, and within 24 hours it is less than that of buffered hypochlorite alone of the same age. The available chlorine content of mixtures also rapidly diminishes. With a mixture of the buffered hypochlorite and methanol (1% v/v), less than 10 ppm of an initial 100 ppm av. Cl remains after a week.

It is apparent from the above studies that solutions of different sodium hypochlorite products that contain approximately the same level of available chlorine may possess widely different sporicidal activities, depending upon their pH. Thus the

sporicidal activity of BDH sodium hypochlorite solution (100 ppm av. Cl) buffered to pH 7.6 has been shown to be superior to that of Milton Fluid (128 ppm av. Cl) at pH 8.5, which in turn is much superior to that of BDH sodium hypochlorite solution (100 ppm av. Cl) at pH 9.6.

Although most of the work was done with spores of *B. subtilis*, it was also established that an unbuffered mixture of methanol (25% v/v) and hypochlorite (2000 ppm av. Cl) at pH 11.2, a buffered mixture of methanol (1% v/v) and hypochlorite (100 ppm av. Cl) at pH 7.6, and buffered hypochlorite solution (100 ppm av. Cl) alone at pH 7.6 are all highly active against a range of test-organisms. They were found to be effective against spores of an aerobe (*B. subtilis*) and an anaerobe (*Cl. sporogenes*) and vegetative cells of a Gram-positive bacterium (*Staph. aureus*), Gram-negative bacteria (*Ps. aeruginosa*, *Pr. vulgaris*, and *E. coli*), an acid-fast bacillus (*M. fortuitum*), and a yeast (*C. albicans*). Virucidal studies, however, have not been done.

In other experiments, not reported here, it has been found that the hypochlorite solutions and alcohol/hypochlorite mixtures described above are seriously inactivated by organic matter. Hence they should be used only for the treatment of clean objects.

The disinfectant activity of buffered hypochlorite solutions and buffered alcohol/hypochlorite mixtures may have many practical applications. In situations in which hypochlorites are currently recommended for the disinfection of clean surfaces, buffered hypochlorite might be substituted with advantage. In addition, formulations of buffered hypochlorite and possibly alcohol could be developed for the rapid disinfection of clean, heat-sensitive, non-disposable, medical equipment such as endoscopes. At present, glutaraldehyde is commonly used for disinfecting fibrescopes between use on successive patients in a session, but at least 3 hours' contact with glutaraldehyde is recommended for sporicidal activity. The buffered hypochlorite preparations described above are sporicidal in less than 15 minutes, and the

addition of a compatible wetting agent should enhance this activity, but a fresh solution would have to be made each day. However, before they can be recommended for disinfecting fibrescopes it will be necessary to establish that they do not affect adversely any component of these instruments.

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