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Sporicidal action of peracetic acid and protective effects of transition metal ions

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Although peracetic acid (PAA) is used widely for cold sterilization and disinfection, its mechanisms of sporicidal action are poorly understood. PAA at high concentrations (5–10%) can cause major loss of optical absorbance and microscopically-visible damage to bacterial spores. Spores killed by lower levels of PAA (0.02–0.05%) showed no visible damage and remained refractile. Treatment of spores of *Bacillus megaterium* ATCC 19213 with PAA at concentrations close to the lethal level sensitized the cells to subsequent heat killing. In addition, PAA was found to act in concert with hypochlorite and iodine to kill spores. Antioxidant sulfhydryl compounds or ascorbate protected spores against PAA killing. Trolox, a water-soluble form of α-tocopherol, was somewhat protective, while other antioxidants, including α-tocopherol, urate, bilirubin, ampicillin and ethanol were not protective. Chelators, including dipicolinate, were not protective, but transition metal ions, especially the reduced forms (Co^{2+}, Cu^{+} and Fe^{2+}) were highly protective. The net conclusions are that organic radicals formed from PAA are sporicidal and that they may act as reducing agents for spores that are normally in a highly oxidized state, in addition to their well known actions as oxidizing agents in causing damage to vegetative cells.

Keywords: peracetic acid; sporicides; transition metal ions; radicals

Introduction

The use of peracetic acid (PAA) for sterilization and disinfection has grown over the past years as concerns about environmental spread of infectious agents have intensified and needs for so-called cold sterilization have increased. PAA offers the advantages of being sporicidal at room temperature or even lower temperatures [13] and of leaving only non-toxic residues. Moreover, the use of buffered PAA solutions with anticorrosives has reduced concerns about its corrosive properties. Much of the basic information on the bactericidal actions of PAA was reviewed by Block [6]. Spores are more resistant to PAA than are vegetative cells, although sporicidal concentrations are generally only ten times those required for killing of vegetative cells. Thus, differences in resistance to PAA between vegetative cells and spores are small compared with differences in resistance to heat. The sporicidal action of PAA is greater at acid pH than at alkaline pH values, and this effect is considered to be due to PAA being a weak acid, although this same type of increased sensitivity has been found for the sporicidal actions of hydrogen peroxide [11] and tertiary butyl hydroperoxide [23]. PAA acts synergistically with alcohols as a sporicide [15]. There is a hierarchy of resistance to PAA among spores, and for example, Botzenhart and Jaax [8] found that spores of *Bacillus licheniformis* were more resistant than spores of *B. cereus*, *B. subtilis* or *B. megaterium*.

The sporicidal activity of peracetic acid does not appear to involve formation of H_2O_2 because catalase is not protective [3]. Moreover, at room temperature H_2O_2 is sporicidal only at high concentrations. Results of recent studies of killing of vegetative cells by PAA [10] indicate that radicals are involved, and hydroxyl radicals (OH·) appeared to be of prime importance. Vegetative cells are metabolically active, and radicals could be formed from PAA through metabolic electron transfer or by transfer from reduced transition metal ions at the cell surface, as has been found for *Escherichia coli* [21]. In contrast, spores are in a highly oxidized state [22], and so electron transfer to hydroperoxides is much less likely.

There are still major questions regarding the mechanisms of the sporicidal action of PAA. We explore the interactions of PAA with other sporicides and with modulators of oxidative damage in an attempt to gain a better understanding of the mechanisms by which PAA kills spores.

Materials and methods

Spores

Spores of *B. megaterium* ATCC 19213, *B. subtilis* subsp *niger* and *B. stearothermophilus* ATCC 7953 were prepared as described previously [5]. Spores of *Clostridium sporogenes* ATCC 7955 were prepared following the procedures described by Löwik and Anema [16]. All spores were washed free of vegetative debris by means of differential centrifugation, and the cleaned spores were generally stored under 95% ethanol until needed. Spores of *B. megaterium* ATCC 19213 were demineralized by acid titration and remineralized by means of base titration, as described previously [5]. The spores were decoated by extraction with dithiothreitol and sodium dodecyl sulfate following the procedures of Aronson and Horn [2].

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Sporicidal assays

Suspensions of spores containing approximately 10^9 colony-forming units (CFU) per ml in water were used. The assays were carried out routinely with 1% Difco peptone (Detroit, MI, USA) in the final mix. Samples of the treated spore suspensions were removed at intervals and diluted initially at least 1 : 10 in 1% (w/v) Difco peptone broth prior to further dilution in 1% Difco peptone broth. Samples (0.1 ml) of the diluted suspensions were spread plated on tryptic-soy agar plates. For some experiments the diluent also contained 20 mM sodium thiosulfate, which did not result in higher plate counts (data not shown). However, when 20 mM thiosulfate was added to suspensions of spores of *B. megaterium* ATCC 19213 prior to addition of 0.05% PAA, it was highly protective against killing (data not shown). The pour-plate, rather than the streak-plate, procedure was used for spores of *Clostridium sporogenes*. Similar procedures were used for assessing killing of vegetative cells.

All experiments were repeated at least once with consistent results. Generally, the results of representative single experiments are presented.

Chemicals

Peracetic acid was obtained from Sigma Chemical Co (St Louis, MO, USA) as a 32% solution, which is approximately 4.75 M. It contained less than 6% H_2O_2, and the hydrogen peroxide at the levels in the diluted solutions used would not be sporicidal at the temperatures (below 45° C) of our experiments. Other hydroperoxides were obtained from Aldrich Chemical Co (St Louis, MO, USA).

Results

Characterization of the sporicidal action of PAA and relation of heat resistance to resistance to PAA

As shown in Figure 1a, PAA killed spores of *B. megaterium* ATCC 19713 at concentrations higher than about 0.025% (*ca* 3.7 mM) at 25° C. The biphasic nature of the curve relating PAA concentration and killing rate might be related to protective effects of materials such as spore coats at lower concentrations of PAA, or alternatively to multi-hit kinetics for killing.

Figure 1b shows effective dose (mM concentration of PAA multiplied by time in minutes for 90% killing or 1 D value) versus temperature. The plot shows that the sporicidal action of PAA is much less affected by changes in temperature than is that of H_2O_2 [24,25] or tertiary butyl hydroperoxide [23].

Spores were more resistant to PAA than vegetative cells (Table 1), even those of *Deinococcus radiodurans* ATCC 13939, which has a remarkably high resistance to H_2O_2. The resistance of *D. radiodurans* could be reduced with cyanide or azide. However, the organism did not have a high level of resistance to PAA, presumably because PAA is not a substrate for catalase.

The range of resistance to PAA was narrow and not well correlated with heat resistance. For example, spores of *B. stearothermophilus* ATCC 7953 are over 1000 times more heat resistant than *B. megaterium* ATCC 19213 spores, based on D values at 120° C [17]. However, as shown by the comparative data in Figure 2 on sensitivity to 0.025% PAA, *B. stearothermophilus* spores were if anything slightly less resistant than were *B. megaterium* spores.

Table 1 Comparative sensitivities to hydroperoxides at 25° C

Cells	Approximate concentration required for rapid[a] killing		
	H_2O_2 (%)[b]	t-butyl hydroperoxide (mM)	PAA (%)[c]
Spores of *B. megaterium*	0.5	720	0.020
Vegetative cells of *B. megaterium*	0.1	7.2	0.004
Deinococcus radiodurans	10	200	0.005
Streptococcus mutans	0.1	7.2	0.005

[a] D value between approximately 15 and 30 min
[b] 1% H_2O_2 (*ca* 326 mM)
[c] 1% PAA (*ca* 149 mM)

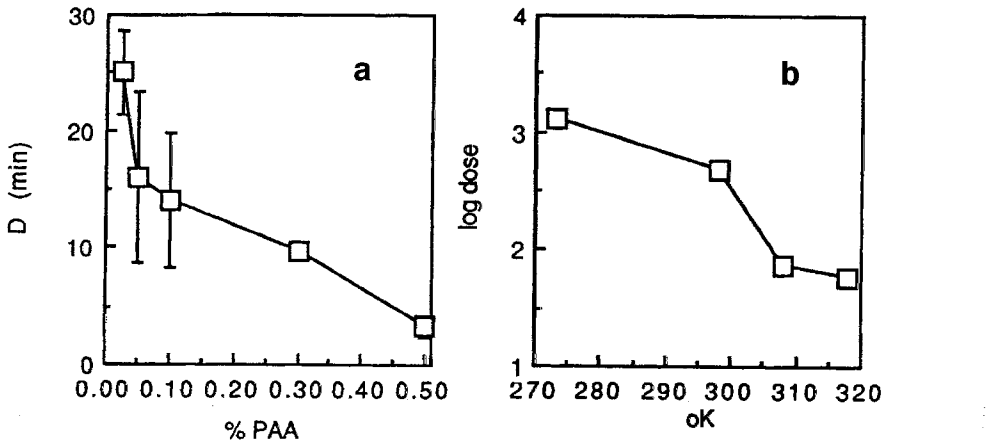

Figure 1 Killing of spores of *B. megaterium* ATCC 19213 by PAA. (a) Plot of D value in minutes versus % PAA for killing at 25° C. The error bars indicate 95% confidence limits. (b) Plot of the logarithm of the dose of PAA (mM concentration multiplied by exposure time in minutes) for 90% killing (or 1 D value) versus Kelvin temperature over the range from 0° C (237° K) to 45° C (318° K)

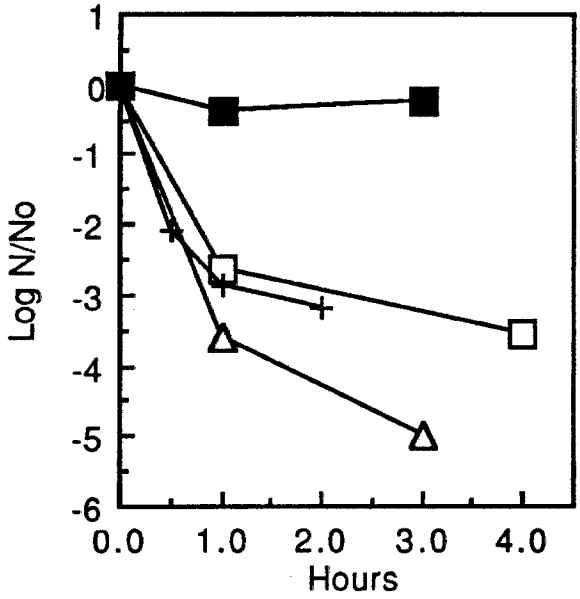

Figure 2 Killing by 0.025% PAA at 25° C of spores of *B. subtilus* subsp *niger* (■), *B. megaterium* ATCC 19213 (□), *B. stearothermophilus* ATCC 7953 (+) and *Cl. sporogenes* ATCC 7955 (△)

This small difference in sensitivity contrasts with the larger differences we found earlier for H_2O_2 and tertiary butyl hydroperoxide. Repeat assessments of H_2O_2 sensitivity with the specific *B. stearothermophilus* spores used in this study confirmed their high level of resistance to H_2O_2.

Spores of *B. subtilis* subsp *niger* have lower heat resistance than spores of *B. stearothermophilus* and yet they were more resistant to 0.025% PAA. However, they were killed rapidly by 0.05% PAA at 25° C. Spores of *Cl. sporogenes* had relatively low resistance to 0.025% PAA (Figure 2).

Native spores of *B. megaterium* ATCC 19213 were insensitive to 0.005% PAA, while decoated spores were killed with a D value of about 12 min. Sensitization occurred also as a result of demineralization. For example, demineralized spores of *B. megaterium* ATCC 19213 were killed by 0.0025% PAA with a D value of about 20 min, whereas native spores were not affected by this low level of the agent. When the spores were remineralized with calcium [5] they showed enhanced resistance to PAA, approaching the resistance of native spores (data not shown).

Finally, spores killed by PAA did not appear to be superdormant. Decoating after exposure to PAA and plating on agar with 10 μg lysozyme (hen egg white lysozyme, Sigma Chemical Co, St Louis, MO, USA) per ml, as described by Peck *et al* [18], did not increase the numbers of survivors (data not shown).

Other organic hydroperoxides

PAA was more potent than cumene hydroperoxide, which is often used for assessing vegetative cell sensitivity to organic hydroperoxides. At 25° C, 525 mM cumene hydroperoxide was not sporicidal for *B. megaterium* ATCC 19213 (data not shown). However, if the temperature was increased to 50° C, the agent was lethal with a lag of about 1 h before killing began, and the subsequent killing was characterized by a D value of 30 min. Benzoyl hydroperoxide, even at a concentration of 210 mM at 50° C, was not sporicidal for *B. megaterium* ATCC 19213. However, magnesium monoperoxyphthalate was effective, and at 25° C, at a level of 10 mM, it induced spore killing characterized by a D value of some 20 min.

Spore lytic effects of PAA

PAA acted as a lytic agent for spores in a manner similar to that described previously for H_2O_2 [14, 24]. Thus, PAA at levels of 5 or 10% at pH 4 caused nearly complete loss of optical absorbance at 700 nm of suspensions of spores of *B. megaterium* ATCC 19213 over a 1- to 2-h incubation at 25° C (Figure 3). H_2O_2 at equivalent concentrations caused lysis only at higher temperatures, and therefore H_2O_2 in commercial preparations of PAA was not likely to be responsible for the lytic activity. Killing of spores by PAA, like that by H_2O_2, appeared not to require lysis, and for example, 0.1% PAA caused rapid killing of spores but no lysis as indicated by no change in microscopic appearance or light scattering. Changes in light absorbance were somewhat greater at pH 4 than at pH 7, but titration of untreated spores at acid pH did not cause loss of light absorbance. Spores subjected to 5% PAA were observed in the phase microscope to have lost refractility, initially with darkening around the periphery of the spore, and then complete darkening, prior to lysis.

Interactions of PAA with other sporicides

The results of previous studies indicate that PAA acts synergistically with alcohols as a biocide [15]. PAA also potentiated the actions of hypochlorite (Figure 4). Mixing of 0.05% PAA with 0.2% hypochlorite resulted in greatly enhanced killing of spores of *B. megaterium* ATCC 19213 at 25° C over that caused by either agent alone. When PAA and hypochlorite at concentrations that leave high levels of residual, viable spores were combined, the resulting sol-

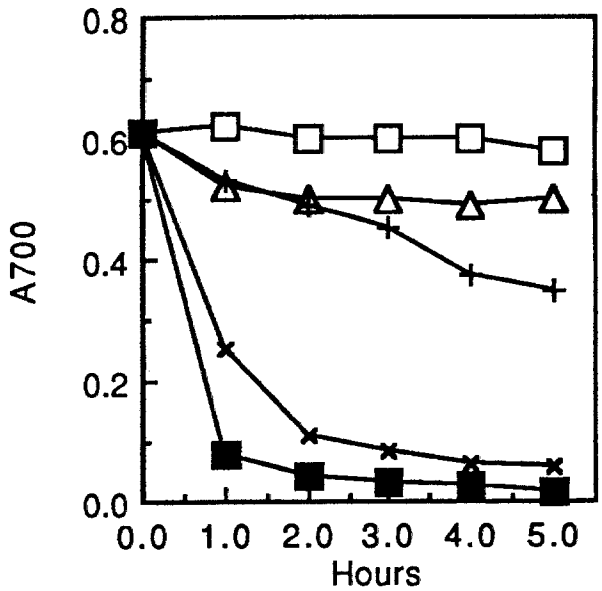

Figure 3 Light absorbance (700 nm) at 25° C and pH 4 by spores of *B. megaterium* ATCC 19213 treated with 0 (□), 0.1 (△), 1.0 (+), 5.0 (×) and 10.0 (■) % PAA at 25° C

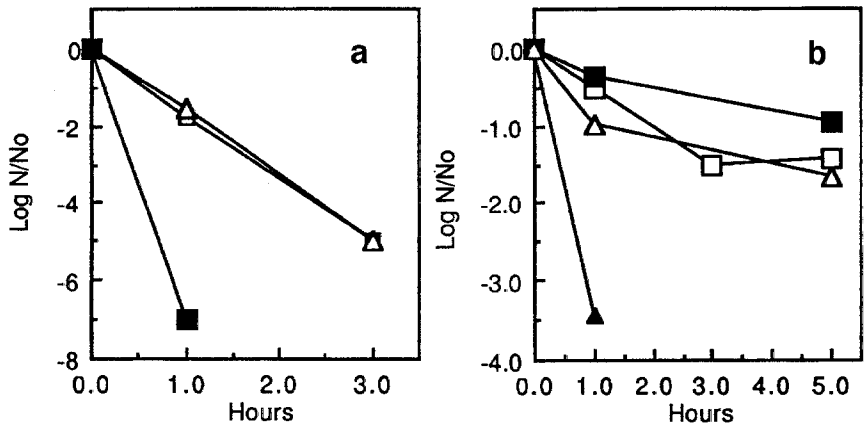

Figure 4 Sporicidal actions of PAA and hypochlorite for spores of *B. megaterium* ATCC 19213 at 50° C. (a) Killing by 0.05% PAA (□), 0.2% hypochlorite (△), or a combination of the two (■). (b) Killing by 0.025% PAA (□), 0.1% hypochlorite (△), a combination of the two (▲) or the combination diluted 1 : 2 with water prior to addition to the spores (■)

ution was highly active with no detectable survivors (Figure 4b). The agents acted in concert but were not highly synergistic. When 0.025% PAA plus 0.1% sodium hypochlorite was diluted 1 : 2 with water, the diluted solution was only weakly sporicidal. Similar data were obtained for killing by iodine in combination with PAA. For example, a solution containing 250 ppm available I_2 (prepared following the procedure of Bloomfield and Megid [7]) had minimal effect on spores of *B. megaterium* ATCC 19213. When combined with 0.025% PAA, the mixture killed the spores at 25° C with a D value of only a few minutes.

In addition, PAA can act in concert with other hydroperoxides such as H_2O_2 or tertiary butyl hydroperoxide to kill spores (Figure 5). When tertiary butyl hydroperoxide at a marginally lethal concentration of 720 mM was combined with 0.01% PAA at 25° C, the mixture was potently sporicidal.

Hydroperoxides can act synergistically with heat for killing spores [24]. However, PAA is not sufficiently stable to be used at killing temperatures for spores of *B. megaterium* ATCC 19213 [6]. Still, PAA treatment of spores sensitized the cells to subsequent heat killing (Figure 6). Spores were exposed to 0.005% PAA prior to being washed and then exposed to 85° C. The initial exposure to PAA did not decrease viable count. However, PAA exposure resulted in increased sensitivity to heat, so much so that cells exposed to 0.005% PAA for 3 h prior to heating at 85° C were nearly all killed in less than 2 h at 85° C. In contrast, spores not exposed to PAA, showed only a little reduction in viable count due to heating for 2 h at 85° C. Similar sensitization could be shown for spores heated to 80° C for periods longer than 3 h. To show sensitization, we had to use levels of PAA close to the lethal level and temperatures close to the minimal lethal temperature.

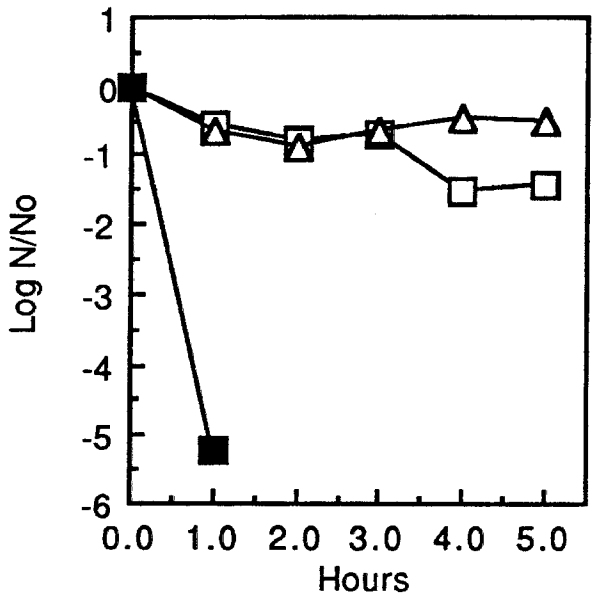

Figure 5 Killing of spores of *B. megaterium* ATCC 19213 at 25° C by 720 mM tertiary butyl hydroperoxide (□), 0.01% PAA (△) or a combination of the two (■)

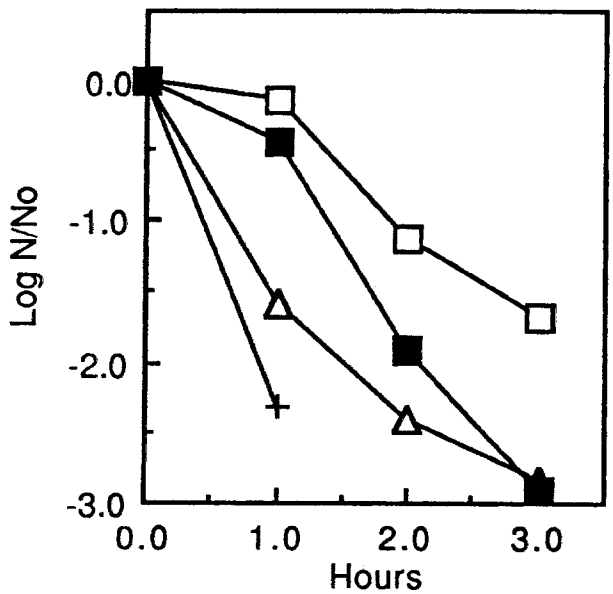

Figure 6 Killing of spores of *B. megaterium* ATCC 19213 at 85° C after prior exposure to 0.01% PAA at 25° C and pH 7 for 0 (□), 1 (■), 2 (△) or 3 (+) h

Effects of antioxidants

Thiosulfate inhibits the sporicidal actions of PAA and is regularly used as a neutralizer [3]. Sulfhydryl compounds, such as cysteine and methionine also were effective neutralizers. For example, 50 mM cysteine or methionine completely protected spores of *B. megaterium* ATCC 19213 from killing by 0.05% PAA at 25° C (data not shown). Ascorbate also was protective, and 50 mM ascorbate completely protected against 0.05% PAA (data not shown).

Many agents that are considered to be radical scavengers proved not to be effective protectors, including 10 mM uric acid, 10 mM bilirubin, 10% ethanol, 20 mM ampicillin or 10 mM α-tocopherol (data not shown). However, a more water-soluble form of α-tocopherol, Trolox, was somewhat protective, and at a concentration of 50 mM reduced killing by 0.025% PAA by nearly tenfold in terms of D value and the residual surviving population level, and also delayed the onset of killing by 1 h (data not shown).

Effects of transition metal ions and chelators

Chelators of metal ions proved not to be effective for protecting spores of *B. megaterium* ATCC 19213 against killing by PAA: 50 mM *o*-phenanthroline, ethylenediaminine tetraacetate or deferroxamine were not significantly protective against 0.05% PAA at 25° C, although these chelators are protective for vegetative cells. In addition, 20 mM dipicolinate was not protective for spores of *B. megaterium* against 0.05% PAA at 25° C.

Transition metal ions proved to be protective against PAA killing of spores (Figure 7). Cu^{2+} (10 mM) was partially protective against killing by 0.05% PAA, while reduced Cu^{+} completely protected the cells against killing. Mg^{2+} and K^{+} showed no protective effects (data not shown). Other transition metals tested included Fe^{2+} and Fe^{3+} and Co^{2+} and Co^{3+}. For iron and cobalt, the reduced cation was much more effective than the oxidized form (data not shown). For vegetative cells, all the transition metal ions sensitized the cells for killing by PAA. For example, with vegetative cells of *B. megaterium* treated for 4 h with 0.005% PAA, an average D value at 25° C of *ca* 45 min was found with a residual population at 4 h of about 1%. When 10 mM $FeSO_4$ was added, the D value was reduced to *ca* 30 min, and the residual population was only about 0.1% from an initial population at zero time of *ca* 2×10^6 cells ml^{-1}.

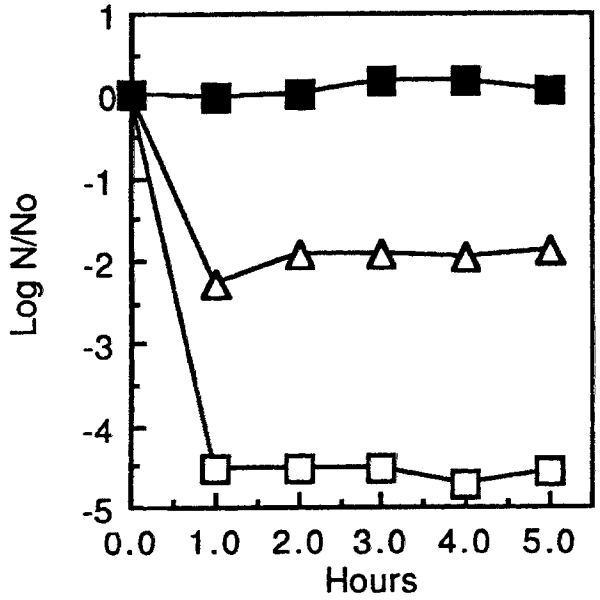

Figure 7 Killing of spores of *B. megaterium* ATCC 19213 at 25° C by 0.05% PAA (□), 0.05% PAA + 10 mM $CuSO_4$ (△), or 0.05% PAA + 10 mM CuCl (▲)

Hemin was also effective in protecting spores against killing by PAA, presumably because of its iron content. For example, the D value for initial killing of spores of *B. megaterium* by 0.05% PAA was increased from some 15 min to nearly 30 min by 10 mM hemin. The residual spore population in the presence of hemin was 10^5 spores ml^{-1} (from an initial population of *ca* 10^8 spores ml^{-1}) compared with fewer than 10^3 spores ml^{-1} without hemin. The protection by hemin of spores in aqueous suspension was limited by the solubility of the compound.

Discussion

A comparison of the sporicidal properties of PAA with those of H_2O_2 [24] or another organic hydroperoxide, tertiary butyl hydroperoxide [23] reveals similarities and differences. The formation of radicals seems to be involved in the damage caused by each agent since ascorbate and sulfhydryl compounds are uniformly protective. Catalase is protective against H_2O_2 but not against PAA. This indicates that H_2O_2 is not an intermediate in the actions of the organic hydroperoxide. The patterns of protection by other agents also reveal differences. For example, *o*-phenanthroline is partially protective against damage caused by tertiary butyl hydroperoxide but not H_2O_2 or PAA. Presumably, the differences have to do with the specific radicals involved in damage and the oxidation-reduction potentials of chelate complexes [9]. For H_2O_2, the damaging radical is considered to be the hydroxyl radical OH·. For tertiary butyl hydroperoxide, the radical most clearly implicated in killing of vegetative cells is the alkylperoxyl radical RCOO· [1]. However, since spore killing appears to differ fundamentally from killing of vegetative cells, other radicals may be involved in sporicidal action.

The high level of resistance to H_2O_2 of *D. radiodurans* appears to be due mainly to the organism's high level of catalase [26] in that it could be reduced dramatically with cyanide or azide. Resistance of *D. radiodurans* to tertiary butyl hydroperoxide appeared also to be related to catalase, for which it is a substrate [19]. The low level of resistance to PAA suggests that it is not a substrate for catalase.

In a study involving E.P.R. spin-trapping, Clapp *et al* [10] concluded that the cidal actions of hydroperoxides, including PAA, involve formation of hydroxyl radicals. Ascorbate and Trolox both were protective for vegetative cells, while transition metal ions strongly potentiated damage. Also, for vegetative cells, chelators such as *o*-phenanthroline and deferroxamine, which sequester transition metal ions, were highly protective. Clapp *et al* [10] also detected carbon-centered radicals, and it seems that radicals such as $CH_3C(=O)O\cdot$ or $CH_3C(=O)\cdot$ may be involved in sporicidal actions of PAA.

Differences in the actions of PAA and those of H_2O_2 or tertiary butyl hydroperoxide were reflected in the enhanced sensitivities of decoated and demineralized spores to PAA compared with smaller changes in sensitivities to H_2O_2 or tertiary butyl hydroperoxide. The increased sensitivity of decoated spores to PAA suggests that the coats may act as a barrier to penetration by the agent or its radicals as they protect spores against other chemical agents. Enhanced sensitivities of demineralized spores may also be related to access to targets since demineralization [4] results in increased water content of the spore protoplast. PAA is a larger molecule than H_2O_2 and more hydrophilic than tertiary butyl hydroperoxide so that it may not move as readily through the integuments into intact spores.

Radicals must be formed outside the spore protoplast because agents such as ascorbate and transition metal ions which do not normally cross cell membranes were protective. In fact, exchange of spore minerals with environmental minerals requires relatively severe acidification, for example, to pH values of about 1.5 to 2.0 for spores of *B. megaterium* ATCC 19213. Small, uncharged molecules can penetrate some 40% of the spore volume [12], presumably through the coats and into the cortex. However, the targets for killing are likely to be in the spore protoplast. Organic peroxyl radicals have much longer half lives than radicals such as OH· [20] and should then be able to move longer distances from point of origin to point of reactivity with cell structures. Thus, organic radicals may be more effective at reaching targets than OH· or $O_2\cdot^-$.

The fundamental differences between hydroperoxide killing of spores and of vegetative cells may have to do with the oxidized nature of spores [22]. It seems that spore components would not readily donate electrons to hydroperoxyl radicals. However, the radicals could donate electrons to spore components. In fact, this sort of reduction may be reflected in the lytic phenomenon, which includes decoating. This decoating may include reductive cleavage of disulfide bonds. Decoating is not lethal for the spores, but the decoating reaction may allow peroxyl radicals to act as reducing agents rather than oxidizing agents. Presumably, added sulhydryl compounds are protective because they act oppositely to reduce peroxyl radicals. Again, the radicals with an unpaired electron can act either as oxidizing agents to accept a single electron or as reducing agents to give up an electron. Further work is needed to define the nature of hydroperoxide killing of spores in terms of specific targets and mechanisms. Setlow and Setlow [22] showed that small, acid-soluble proteins of spores are involved in resistance to H_2O_2, specifically through binding to DNA to protect it against radical attack. However, resistance to H_2O_2 is acquired sequentially in at least two stages during sporulation, the first stage associated with synthesis of small, acid-soluble proteins. Higher-level resistance develops at the time of synthesis and uptake of dipicolinate. Protection by small, acid-soluble proteins is specific for DNA, and some means of protection of other spore components would be needed for full resistance. Presumably, resistance of dormant spores to organic hydroperoxides is closely related to resistance to H_2O_2, although the finding of a different hierarchy of resistance to PAA compared with that for H_2O_2 raises questions about this view. Part of the difference may have to do with protection functions of the coats against PAA. However, there is still much to be learned about the molecular mechanisms of spore killing by hydroperoxides.

Acknowledgements

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