The Effect of Hydrogen Peroxide on Spores of
Clostridium bifermentans

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SUMMARY

The effect of hydrogen peroxide on the germination, colony formation and structure of spores of *Clostridium bifermentans* was examined. Treatment with 0·35 M-hydrogen peroxide increased the germination rate at 25 °C but increasing the temperature or concentration of hydrogen peroxide decreased both the germination rate and colony formation. The presence of Cu^{2+} increased the lethal effect of hydrogen peroxide on colony formation as much as 3000-fold. Pre-incubation of spores with Cu^{2+} before treatment with hydrogen peroxide produced a similar increase, but this could be eliminated by washing the spores with dilute acid or ethylenediamine tetraacetate. Hydrogen peroxide removed protein from spores—apparently from the coat—and treatment with dithiothreitol, which also removes spore-coat protein, increased the lethal effect of hydrogen peroxide 500-fold, suggesting that spore-coat protein has a protective effect against hydrogen peroxide.

INTRODUCTION

Hydrogen peroxide rapidly kills vegetative bacteria (Dittmar, Baldwin & Miller, 1930) but bacterial spores are much more resistant (for a review, see Roberts & Hitchins, 1969) although the reasons for this are not understood. Most previous studies on spores have been confined to the effect of hydrogen peroxide on colony-forming ability. Curran, Evans & Leviton (1940) first showed that the rate of loss of viability of spores in the presence of hydrogen peroxide depends on temperature and pH. Copper and cobalt ions increase the lysis of spores by hydrogen peroxide (Gould & Hitchins, 1963) but their effect on spore viability and structure has not been examined. We have therefore studied the effect of pH, temperature and the concentration of hydrogen peroxide and metal ions on colony formation, germination and the structure of spores of *Clostridium bifermentans*.

METHODS

Organism, spore preparation and maintenance of culture. The strain of *Clostridium bifermentans* used and the preparation and storage of spores were as described previously (Waites & Wyatt, 1971) except that spores were produced on a trypticase agar containing (g l⁻¹): Trypticase (BBL), 30; yeast extract (Difco), 1·0; ammonium sulphate, 10; agar, 12; adjusted to pH 7·3 with 1 M-NaOH. The organism was maintained in the reinforced clostridial medium of Hirsch & Grinsted (1954).

Treatment of spores with hydrogen peroxide. Hydrogen peroxide (BDH) at the concentrations described was added to spores (about 0·7 mg dry wt ml⁻¹) in sodium phosphate buffer (100 mM in phosphate at the stated pH) which had been pre-incubated at the required

temperature in polypropylene centrifuge tubes. After incubation, the tubes were cooled rapidly in ice–water, the contents were diluted tenfold with glass-distilled water and centrifuged for 10 min at 15000 g and 4° C, and the spores were washed twice with glass-distilled water by centrifugation at 4 °C.

Treatment with dithiothreitol. Spores (0·7 mg dry wt ml^{-1}) were incubated with 10 mM-dithiothreitol for 15 min at pH 10·5 and 37 °C (Aronson & Fitz-James, 1971) and then washed twice with glass-distilled water.

Colony formation and germination. Colony formation was tested on reinforced clostridial medium solidified with 1·2 % (w/v) agar as described previously (Waites & Wyatt, 1974 *a*). Germination was measured spectrophotometrically as described by Waites & Wyatt (1971). Germination media contained (mM): for the 'Ala +' system, L-alanine (50), L-arginine (5), L-phenylalanine (5), L-lactate (25), sodium chloride (100) and sodium phosphate buffer pH 7·5 (83 mM in phosphate); and for the 'Ala' system, L-alanine (50), sodium chloride (100) and sodium phosphate buffer pH 8·0 (83 mM in phosphate). Previously we have shown that the 'Ala +' system is optimal for spores produced on the reinforced clostridial medium agar of Hirsch & Grinsted (1954) (Waites & Wyatt, 1974 *b*). With the spores produced on the trypticase medium, the concentration of L-phenylalanine in the 'Ala +' system was slightly inhibitory; germination rates for treated and untreated spores were 7 % and 15 % less than the maximum, obtained with 3 mM L-phenylalanine. As mild treatment with hydrogen peroxide caused spores to germinate optimally with the 'Ala' system, they sometimes germinated slightly faster with the 'Ala' system than with the 'Ala +' system.

Polyacrylamide gel electrophoresis of spore extracts. Extracts were made from 14 mg dry wt spores by treatment with 3 M-H_2O_2 for 60 min at 37 °C either with or without preincubation with 100 μM-$CuSO_4$. The suspension was centrifuged for 10 min at 15000 g and the supernatant was dialysed against glass-distilled water before concentration and addition of NaOH: 0·2 ml containing 100 μg protein in 0·1 M-NaOH and 10 % (w/v) sucrose was added to polyacrylamide gels. Electrophoresis was carried out as described by Davis & Ornstein (1961), but without the overlayer of large-pore gel, in vertical columns for about 60 min at 5 mA/gel and 4 °C. Bromophenol blue (0·001 %, w/v) was used as a marker and amido black (1·0 %, w/v) in acetic acid (7 %, v/v) as a stain. Gels were destained with several changes of acetic acid (7 %, v/v) during 36 h at room temperature.

Spore dry weight. Dry weights were estimated spectrophotometrically. Extinction was measured at 600 nm and converted to spore dry weight using a standard calibration curve relating E_{600} to spore weight.

Protein estimations. These were made by the method of Lowry *et al.* (1951) with bovine plasma albumin fraction V (Armour Pharmaceuticals, Eastbourne, Sussex) as a standard.

RESULTS

Effect of pH, temperature and hydrogen peroxide concentration on germination and colony formation

When spores were treated with 1·42 M-H_2O_2 at 25 °C for 30 min at different hydrogen-ion concentrations, those treated in sodium phosphate buffer pH 7·0 (which gave a final pH of about 6·7) germinated most slowly with either the 'Ala +' or 'Ala' germination system (Fig. 1). This suggested that the greatest lethal effect occurred at this pH and it was used for all subsequent treatments with H_2O_2. Treatment with different concentrations of H_2O_2 at 25 °C for 30 min showed that the colony-forming ability was not reduced by up to 0·43 M-H_2O_2 but was markedly reduced by higher concentrations (Fig. 2). With the 'Ala' germin-
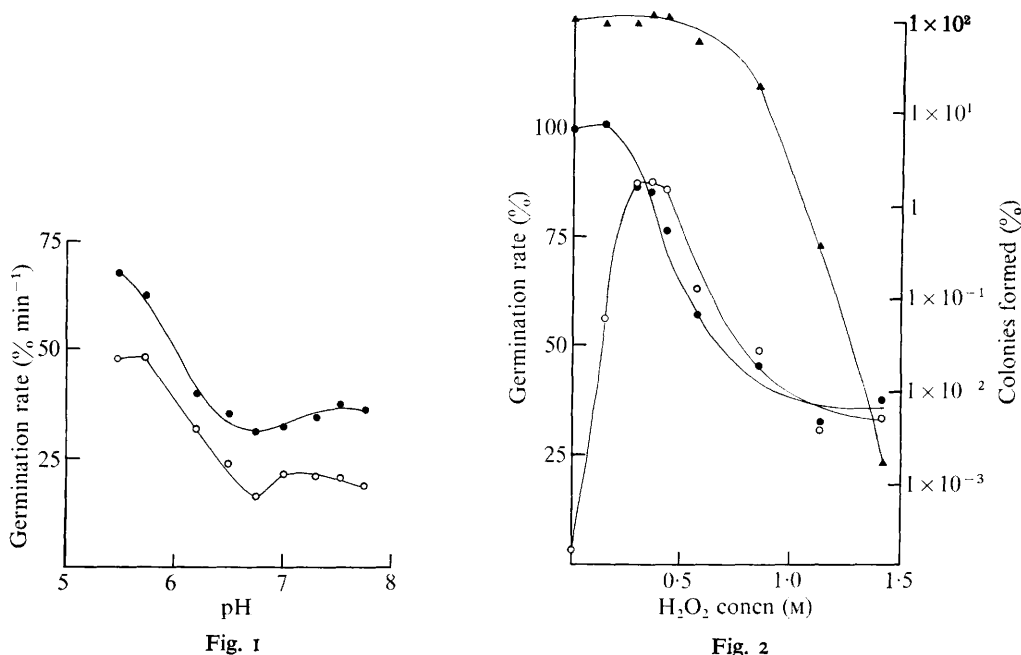
Fig. 1

Fig. 2

Fig. 1. Effect of H_2O_2 at different hydrogen-ion concentrations on germination rate. Spores were treated with 1·42 M-H_2O_2 at 25 °C for 30 min, as described in Methods. After washing, the spores were germinated with the 'Ala +' (●) or 'Ala' (○) systems. Germination rates for untreated spores were 109 % min^{-1} ('Ala +' system) and 5·4 % min^{-1} ('Ala' system).

Fig. 2. Effect of H_2O_2 concentration on colony-forming ability and germination. Spores in sodium phosphate buffer pH 7·0 were treated with H_2O_2 at 25 °C for 30 min, as described in Methods. The percentage of spores able to form colonies (▲) and the germination rates with 'Ala +' (●) and 'Ala' (○) germination systems are expressed as a percentage of the number of colonies formed and the germination rate (with 'Ala +'), respectively, of spores incubated in the absence of H_2O_2. Such spores formed 1·04 × 10^9 colonies ml^{-1} and germinated at a rate of 84 % min^{-1}.

ation system the germination rate was increased 26-fold by 0·35 M-H_2O_2, but with the 'Ala +' system no increase in rate was detected. With 1·42 M-H_2O_2 the germination rate with both systems was markedly reduced, but the decrease in turbidity during germination showed that about 80 % of the spores germinated even though less than 1 in 10^4 spores were able to form colonies.

Treatment with 1·42 M-H_2O_2 at 25 °C required about 20 min for a 99 % reduction in colony-forming ability and no kill occurred within the first 2·5 min (Fig. 3a). However, with 0·142 M-H_2O_2 at 70 °C less than 5 min was required for a 99 % kill (Fig. 3b). After 6 min at 70 °C there was little further decrease in colony formation, but the decrease resumed after 10 min. This 'plateau' effect did not occur at 25 °C. The increased rate of kill after 10 min at 70 °C may therefore have been due to the effect of heat on spores damaged with hydrogen peroxide. Treatment at 25 °C resulted, as before, in an initial increase in germination rate with the 'Ala' but not with the 'Ala +' system (Fig. 3a). Treatment at 70 °C resulted in similar changes, but the rate decreased more rapidly than at 25 °C and reached a lower level (Fig. 3b).
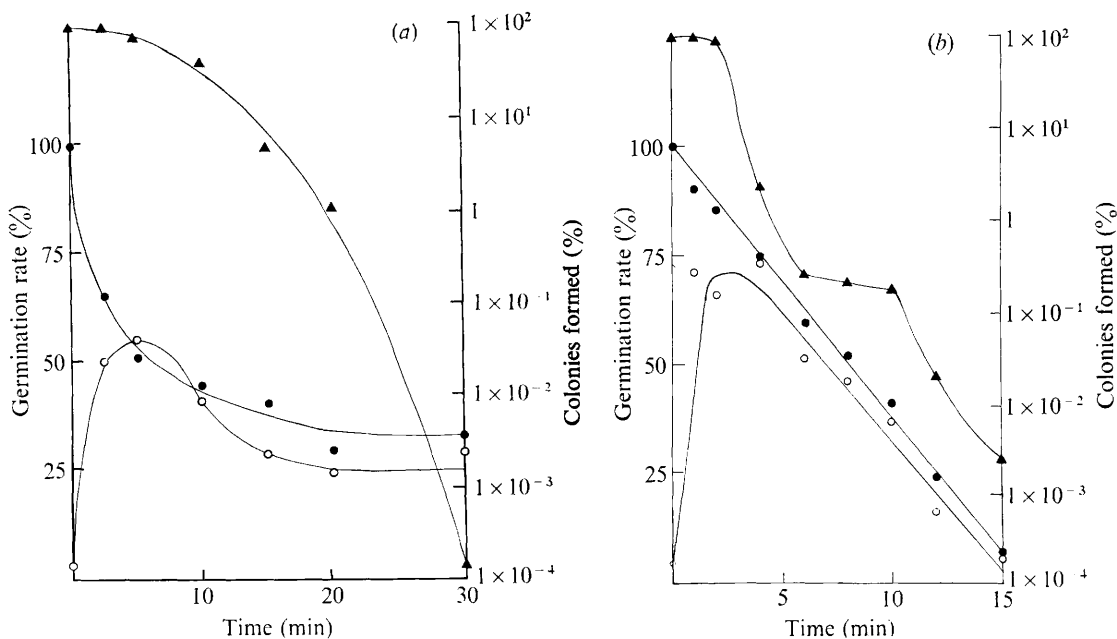
Fig. 3. Effect of H_2O_2 at 25 °C and 70 °C on germination rate and colony-forming ability. Spores were incubated, as for Fig. 2, either (a) with 1·42 M-H_2O_2 at 25 °C or (b) with 0·142 M-H_2O_2 at 70 °C. Samples were removed at intervals and the colony-forming ability (▲) and germination rates with the 'Ala +' (●) and 'Ala' (○) germination systems were determined as described in Methods. Results are expressed as in Fig. 2. Unincubated spores formed (a) 1·2 × 10^9 and (b) 9·7 × 10^8 colonies ml^{-1} and germinated at a rate of (a) 86 % min^{-1} and (b) 118 % min^{-1}; spores incubated in the absence of H_2O_2 formed (a) 9·5 × 10^8 and (b) 9·4 × 10^8 colonies ml^{-1} and germinated at (a) 80 % min^{-1} and (b) 95 % min^{-1}.

Effect of hydrogen peroxide and metal ions on germination and colony formation

Gould & Hitchins (1963) showed that certain divalent metal ions increased the lysis of spores by H_2O_2. We therefore incubated spores with 100 μM metal ions with or without 0·28 M-H_2O_2 for 30 min at 25 °C (Table 1). Metal ions alone had little effect on colony formation or germination but addition of Cu^{2+} to H_2O_2 markedly reduced both the number of colonies formed and the germination rate. Other metal ions were much less effective although Fe^{2+} in H_2O_2 slightly reduced the number of colonies formed. The lethal effect of H_2O_2 on subsequent colony formation was increased 4- and 28-fold by pre-incubation for 5 min at 37 °C with 5 and 20 μM-Cu^{2+} respectively, and germination rates were also decreased although to a lesser extent (Fig. 4). Pre-incubation with Cu^{2+} for 2 min at 0 °C or 1 min at 37 °C also markedly reduced the resistance to subsequent treatment with H_2O_2 and this suggested that Cu^{2+} was rapidly absorbed by the spores even at low temperature. However, washing the treated spores with 0·04 M-HCl (Table 2) or 250 μM-EDTA eliminated most of the effect of 5 or 10 μM-Cu^{2+}, presumably by removing the Cu^{2+}.

Effect of hydrogen peroxide and Cu^{2+} on spore structure

Treatment of spores with H_2O_2 in the presence or absence of Cu^{2+} removed protein which, on polyacrylamide gel electrophoresis, had a mobility similar to that of the protein removed from spore coats by chlorine (Wyatt & Waites, 1975). Spores also lost refractility from their peripheries when treated successively with 10 μM-Cu^{2+} at 37 °C for 5 min and 0·03 M-H_2O_2

Table 1. *Effect of divalent metal ions and hydrogen peroxide on spore viability and germination rate*

Spores (0·7 mg dry wt ml⁻¹) were incubated with or without 0·28 M-H_2O_2 and 100 μM metal sulphates at 25 °C for 30 min in sodium phosphate buffer (100 mM in phosphate) then cooled in ice–water, diluted tenfold with glass-distilled water, and washed twice with distilled water by centrifugation at 4 °C. Colony formation and germination rate were determined as described in Methods. The values obtained for spores incubated with the 'Ala +' system in the absence of H_2O_2 and metal ions are taken as 100 %. Such spores formed $9\cdot4 \times 10^8$ colonies ml⁻¹ and had a germination rate of 130 % min⁻¹.

| | Colony formation (%) | | Germination rate (%) | | | |
| | | | 'Ala +' | | 'Ala' | |
Cation added	Untreated	H_2O_2 treated	Untreated	H_2O_2 treated	Untreated	H_2O_2 treated
None	100	87	100	81	3·2	56
Cu^{2+}	95	0·028	101	7·6	4·4	6·2
Fe^{2+}	72	48	106	66	4·8	57
Ni^{2+}	113	63	91	77	3·2	57
Co^{2+}	98	68	90	34	2·1	24
Zn^{2+}	118	88	97	69	0·9	3·1

Table 2. *Effect of Cu^{2+} and acid washing on the action of hydrogen peroxide*

Spores (0·7 mg dry wt ml⁻¹) were pre-incubated with Cu^{2+} for 5 min at 37 °C, as described in Table 1, and then washed (at 0·15 mg dry wt ml⁻¹) either with 0·04 M-HCl followed by two washes with glass-distilled water at 4 °C, or with glass-distilled water alone, before treatment with 0·142 M-H_2O_2 for 5 min at 70 °C, as described in Methods. Colony-forming ability and germination rate were measured, the values obtained with the 'Ala +' system from spores pre-incubated without Cu^{2+} and washed twice with glass-distilled water before H_2O_2 treatment being taken as 100 %. Such spores formed $5\cdot8 \times 10^8$ colonies ml⁻¹ and germinated at a rate of 130 % min⁻¹. Untreated spores formed $1\cdot09 \times 10^9$ colonies ml⁻¹ and germinated at a rate of 126 % min⁻¹.

| Cu^{2+} concn added (μM) | Colony formation (%) | | Germination rate (%) | | | |
| | | | 'Ala +' | | 'Ala' | |
	Water washed	Acid washed	Water washed	Acid washed	Water washed	Acid washed
0	100	127	100	90	85	82
5	17	94	48	73	43	80
10	9·3	32	33	85	34	84

at 70 °C for 5 min. Similar changes in refractility occurred with 8·5 M-H_2O_2 at 25 °C for 9 h (Waites *et al.*, 1976), suggesting that in the presence of Cu^{2+} less severe treatment is required to cause refractility changes.

Dithiothreitol removes protein from spore coats without affecting colony formation (Aronson & Fitz-James, 1971). We have shown previously that pretreatment of spores with dithiothreitol markedly increases the lethal effect of chlorine on spores (Wyatt & Waites, 1975). Such pretreatment also increased the lethal effect of H_2O_2. Treatment with dithiothreitol, as described in Methods, followed by 1·42 M-H_2O_2 reduced the fraction of spores able to form colonies to 0·082 % (the colonies formed from untreated spores being taken as 100 %) compared with reduction to 93 % for treatment with H_2O_2 alone and to 40 % for dithiothreitol alone.
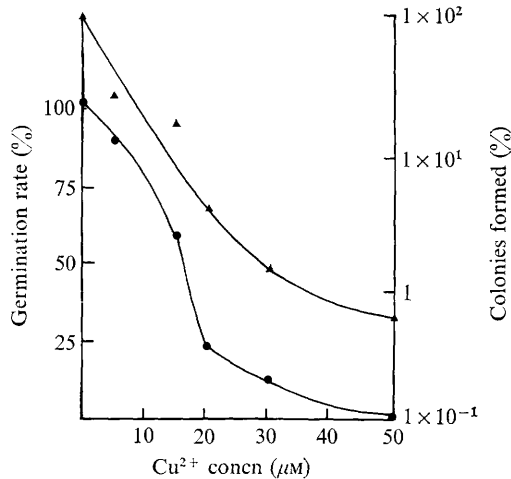
Fig. 4. Effect of Cu^{2+} concentration on activity of H_2O_2. Spores were incubated, as for Fig. 2, with 0·142 M-H_2O_2 for 5 min at 70° C in the presence of different concentrations of Cu^{2+}, as described in Methods. Colony-forming ability (▲) and germination rates with the 'Ala +' system (●) were determined as for Fig. 2. Results are expressed as a percentage of colonies formed and the germination rate produced by spores treated with H_2O_2 in the absence of Cu^{2+}. Such a control formed 1·03 × 10⁷ colonies ml⁻¹, and the germination rate was 74 % min⁻¹; spores incubated in the absence of H_2O_2 formed 7·2 × 10⁸ colonies ml⁻¹ and germinated at a rate of 135 % min⁻¹.

DISCUSSION

We have shown that H_2O_2 prevents colony formation by spores of *Clostridium bifermentans* but that most of the spores that could not form colonies were still able to germinate. Such spores are therefore unable to form colonies because they cannot outgrow or divide. Similar results were obtained after treatment of spores with chlorine (Wyatt & Waites, 1975). The effect of H_2O_2 on germination was greatest at pH 6·7. With spores of *Bacillus* sp., Curran *et al.* (1940) found that inactivation was greatest at pH 3·0 but Cerf & Hermier (1972) found that inactivation at pH 6·7 was more rapid than at pH 4·6 or 7·7. In this study, as in others (Curran *et al.*, 1940; Roundy, 1958; Swartling & Lindgren, 1968; Cerf & Hermier, 1972; Toledo, Escher & Ayres, 1973), it is apparent that the lethal effect of H_2O_2 increases with increasing concentration and temperature. There was a lag during the first minutes of treatment and a later reduced rate of kill, as observed previously (Cerf & Hermier, 1972). Treatment with H_2O_2 makes spores more sensitive to heat (Toledo *et al.*, 1973) so heat may be the major cause of death during this latter phase.

No previous studies have been made on the effect of H_2O_2 on spore germination. Our work has shown that germination with suboptimal concentrations of germinants was stimulated more than 25-fold by mild treatment with H_2O_2. Other oxidizing agents such as sodium perborate (Cochran & Ordal, 1973) and chlorine (Wyatt & Waites, 1973) also increase the germination rate of spores. Chlorine acts by removing protein, probably from spore coats, and increasing the permeability of the spores to germinants (Wyatt & Waites, 1975). We have shown that H_2O_2 removes a similar protein from spores, which suggests that H_2O_2 and chlorine act in the same way. Dithiothreitol also removes protein from spore coats (Aronson & Fitz-James, 1971), and we have shown that pretreatment of spores with dithiothreitol increased the lethal effect of H_2O_2 500-fold, suggesting that the coat protects spores against H_2O_2. A similar conclusion was reached for the effect of chlorine on spores (Wyatt & Waites, 1975).

The lethal effect of H_2O_2 was increased 3000-fold by the presence of 100 μM-Cu^{2+}. Pre-incubation with Cu^{2+} for as little as 1 min also increased the lethal effect of H_2O_2, which suggests that spores absorbed the ions very rapidly. Washing with dilute acid or EDTA, but not glass-distilled water, eliminated the effect of Cu^{2+}, presumably by removing the ions from the spores. Thus the presence of Cu^{2+} or, to a much lesser extent, Fe^{2+}, during or after spore formation, largely determines the sensitivity of spores of *C. bifermentans* to H_2O_2. Similarly, washing with dilute acid to remove metal precipitates from spore preparations (Slepecky & Foster, 1959) may increase resistance. Cu^{2+} increases the rate of breakdown of H_2O_2 (Walton & Graham, 1928) and the rate of cleavage of peptide bonds by H_2O_2, and, since most bonds seem to be sensitive (Deasy, 1967), the effect on the spore is likely to be a general destruction of protein.

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