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Reversal of the inhibition of bacterial spore germination and outgrowth by antibacterial agents

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Summary

The effects of some antibiotics and other antibacterial agents on the germination and outgrowth of *Bacillus subtilis* are described. The antibiotics tested (benzylpenicillin, cephalothin, chloramphenicol, minocycline, actinomycin D and mitomycin C) had no effect on germination but inhibited outgrowth to varying degrees. Of the preservative-type compounds, phenol and cresol acted predominantly at the germination stage, whereas the quaternary ammonium compound, benzalkonium chloride (BZK), and the organomercurial, phenylmercuric nitrate, inhibited outgrowth but not germination. The inhibitory effect of phenol was reversed by membrane filtration, but not by 2% polysorbate 80, whereas BZK appeared to be tightly bound to the cells and could not be removed by filtration.

Introduction

Some antibacterial agents, e.g. glutaraldehyde (pentanedial) and ethylene oxide, are sporicidal to a variety of bacterial spores. Others, such as phenols, quaternary ammonium compounds and mercurials, are not sporicidal at ambient temperatures, but may inhibit specific stages of sporulation or prevent germination and/or outgrowth (Russell, 1982, 1983).

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Several important pharmaceutical, cosmetic and food preservatives are known to be sporostatic in nature (Russell, 1982; Sofos and Busta, 1982). In this communication, the effects of some of these agents added at different periods in the germination and outgrowth processes (as measured spectrophotometrically: Gould, 1971, 1984) have been studied, together with different methods of assessing the reversible nature of any inhibition. Some important antibiotics have also been investigated briefly.

Materials and Methods

Test organism

This was *Bacillus subtilis* NCTC 8236. It was grown for 7 days at 37°C on the surface of nutrient agar (Oxoid, London). Growth was washed off the surface with sterile glass-distilled water. The suspension was washed three times with sterile water, and resuspended in sterile water to a density of ca. 5×10^8 spores/ml.

Inhibitors

These consisted of various antibiotics (benzylpenicillin, cephalothin, actinomycin D, mitomycin C, minocycline, chloramphenicol) and other antibacterial agents (phenylmercuric nitrate (PMN), benzalkonium chloride (BZK), phenol, cresol, chlorocresol and glutaraldehyde). Concentrated solutions were prepared in sterile water, and sterilized by appropriate means.

Minimum inhibitory concentrations (MICs) were determined with a final spore density of ca. 5×10^6/ml by a broth dilution method with the presence or absence of growth noted after 48 h at 37°C. The MIC was the lowest concentration preventing growth.

Inhibition of germination and outgrowth

0.2 ml of the spore suspension was incorporated into 20 ml (final volume) of nutrient broth (Oxoid) containing 0.01 M L-alanine and 0.01 M D-glucose prewarmed at 37°C. Optical density (O.D.) measurements during subsequent incubation in a shaking water-bath at 37°C were made in a Unicam SP600 spectrophotometer. When necessary, an antibacterial agent was incorporated into the medium at zero time, or during the germination or outgrowth processes and the O.D. monitored.

Reversal of inhibition by filtration

Aliquots of drug-treated spores in broth containing 0.01 M L-alanine and 0.01 M D-glucose were filtered through a Swinnex membrane filter (0.22 μm pore size), and the spores retained on the filter were washed in situ with broth. The membrane was immediately placed in 20 ml broth containing L-alanine and D-glucose, but with no inhibitor, prewarmed at 37°C. The spores were shaken from the membrane, which was then removed, and O.D. monitored during subsequent incubation of the spore suspension at 37°C.

Use of neutralizers

0.15% w/v phenol and 2% Tween 80 were incorporated into broth containing

L-alanine and D-glucose, and the medium incubated at 37°C. 0.2 ml of spore suspension was then added to give a final volume of 20 ml, and O.D. changes during incubation at 37°C measured. In other experiments, phenol (0.15%) was added at zero time and 2% Tween 80 was introduced 20 min later.

When benzylpenicillin was studied, β-lactamase was added during the outgrowth phase or at the end of the germination phase, and further changes in O.D. measured.

Results and Discussion

MIC values of the various preservative-type agents are presented Table 1. Of the three phenolic-type compounds, chlorocresol was the most, and phenol the least, effective. Inhibitory values (sporostatic concentrations) of these agents and of PMN, BZK and glutaraldehyde corresponded to concentrations preventing growth of non-sporulating bacteria (Russell, 1982).

Studies on germination and outgrowth were carried out by a spectrophotometric method. None of the antibiotics tested inhibited germination (Table 2) whereas inhibitors of cell wall synthesis (β-lactams), protein synthesis (minocycline, chloramphenicol), RNA synthesis (actinomycin D) and DNA synthesis (mitomycin C) were all active during the outgrowth stage (Table 3; reviewed by Russell, 1982). Reversal of the effects of penicillin (by β-lactamase from *Bacillus cereus*), and, by membrane filtration, of chloramphenicol and actinomycin D was also studied. Preliminary experiments suggested that the action of all three antibiotics during outgrowth could be reversed to some extent, but that additional work was needed to quantify these findings.

In contrast, of the four preservative-type agents investigated in detail, only two (phenol and cresol) acted predominantly at the germination stage with a greatly reduced effect on outgrowth (Fig. 1, Table 4), whereas PMN (Fig. 2) and BZK (Fig. 3) both allowed germination to proceed whilst having a very marked effect, at low concentrations, on outgrowth (Table 4). In view of the known mechanisms of action of these compounds, viz. an interaction of PMN with –SH groups (Fildes, 1940) and of quaternary ammonium compounds with the cytoplasmic membrane (Hugo, 1982),

TABLE 1

INHIBITORY CONCENTRATIONS OF PRESERVATIVES ON *B. SUBTILIS*

Preservative	MIC (% w/v)
Phenol	0.15
Cresol	0.1
Chlorocresol	0.02
PMN	0.00004
BZK	0.0002
Glutaraldehyde	0.025

TABLE 2

EFFECT OF ANTIBIOTICS ON GERMINATION AND OUTGROWTH OF *B. SUBTILIS*

Time (min)	% original O.D. ** when spores exposed to antibiotics * (μg/ml)								
	None (control)	Pen		Cet		CMP		Act D	Mit C
		2	5	0.15	0.4	4	10	3	0.8
0	100	100	100	100	100	100	100	100	100
40	41	39	38	40	38	38	37	37	38
50	42	41	39	41	40	38	37	38	38
60	45	44	42	42	41	40	38	40	39
70	53	48	44	49	44	43	39	43	40
80	63	55	50	55	48	45	40	45	41
90	76	63	54	61	51	46	41	46	42
120	>100								

* Pen, benzylpenicillin; Cet, cephalothin; CMP, chloramphenicol; Act D, Actinomycin D; Mit C, mitomycin C.

** Original O.D., i.e. at zero time and when antibiotics added, taken as 100% in each case.

it is unlikely that the apparent similiarity of their inhibition of *B. subtilis* spores does, in fact, result from a similar type of effect. An interesting difference also arises between the inhibition of outgrowth noted here and elsewhere (Parker, 1969) for the organomercurial, PMN, and the inhibition of germination described previously with the inorganic mercury compound, mercuric chloride, on *Clostridium botulinum* (Ando, 1973) and *Bacillus* spp. (Gould and Sale, 1970; Vinter, 1970). Hsieh and Vary (1975) have shown that this inorganic mercury compound can inhibit some reactions in germination before the loss of heat resistance but not the subsequent release of peptidoglycan. Vinter (1970) has also pointed out that Hg^{2+} has a high degree of affinity to the spore surface and may inhibit post-germination development even after thorough washing.

TABLE 3

INHIBITORY EFFECTS OF ANTIBIOTICS ADDED AT DIFFERENT TIMES DURING OUTGROWTH OF *B. SUBTILIS*

Antibiotic	Concentration (μg/ml)	Effect * on outgrowth when added at (min) **		
		30	80	90
Penicillin	5	(+)	(+)	Nd ***
Cephalothin	0.4	(+)	(+)	Nd
Chloramphenicol	10	+	(+)	Nd
Minocycline	4	+	(+)	Nd
Actinomycin D	3	+	Nd	(+)
Mitomycin C	0.8	+	Nd	(+)

* (+), partial inhibition; +, total inhibition.

** Times are minutes after commencement of germination.

*** Nd, not done.

Fig. 1.

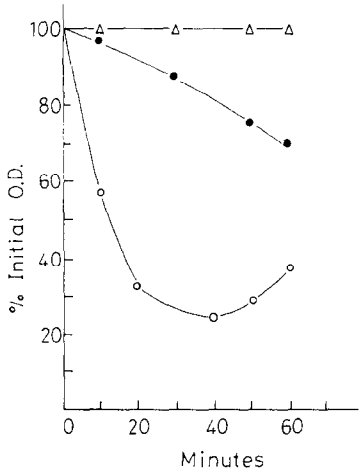

Fig. 2.

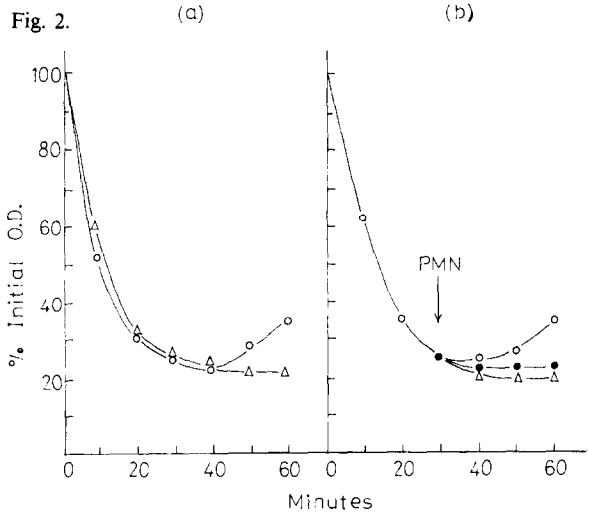

Fig. 1. Effect of cresol, added at zero time, on germination of *B. subtilis* spores. Cresol concentration (% w/v): O——O, control (cresol absent): ●——●, 0.05; △——△, 0.1. (Similar results were obtained with phenol over the range 0–0.15% w/v.)

Fig. 2. Effect of phenylmercuric nitrate (PMN) on germination and outgrowth of *B. subtilis* spores. (a) PMN added at zero time; (b) PMN added at 30 min. PMN concentration (% w/v): O——O, control (PMN absent): ●——●, 0.0002; △——△, 0.0004.

The reversibility of preservative action was studied with an inhibitor of germination (phenol) and of outgrowth (BZK). As before, phenol (0.15% w/v) totally inhibited germination; however, its removal by filtration after a 20-min contact period allowed germination to proceed normally (Fig. 4a). This finding is similar to the results obtained with chlorocresol and *B. subtilis* spores (Parker, 1969). Phenol at this concentration introduced towards the end of germination reduced the rate of outgrowth; if, however, it was removed by filtration after contact with the spores for 10 min, outgrowth proceeded at a more rapid rate (Fig. 4b). Attempts to reverse phenol inhibition by the addition to the system of 2% Tween 80 (added with 0.15% phenol at zero time or 20 min after phenol) were unsuccessful. Higher concentrations of Tween have not been investigated.

BZK had no effect on germination (Table 4). Thus, it was introduced into the system towards the end of germination or during outgrowth, with filtration at an appropriate interval. When added at the end of germination (Fig. 5a), BZK inhibited subsequent outgrowth; this was not overcome to any great extent following "removal" of the inhibitor by filtration. Introduction of BZK during outgrowth caused an immediate cessation of the process; filtration 20 min later did not reverse the effect (Fig. 5b). These findings are reminiscent of those of Chiori et al. (1965) which showed that another quaternary ammonium compound, cetrimide, was retained by the spores and that its removal from the spores could only be achieved by washing the cells with an appropriate neutraliser (Lubrol W plus lecithin). This suggests that

TABLE 4

EFFECT OF INHIBITORS ON GERMINATION OR OUTGROWTH OF *B. SUBTILIS*

Preservative	Concentration (% w/v)	Time of addition (min)	Effect * on:	
			Germination	Outgrowth
Cresol	0.05	0	+	
	0.1	0	+ +	
	0.05	20 or 30		(+)
	0.1	20 or 30		(+)
Phenol	0.05	0	+	
	0.15	0	+ +	
	0.05	20 or 30		(+)
	0.15	20 or 30		+
PMN	0.0002	0	–	+
	0.0004	0	–	+ +
	0.0002	20		+
	0.0004	30		+ +
	0.0002	30		+ +
	0.0004	30		+ +
BZK	0.0001	0	–	+
	0.0002	0	–	+ +
	0.0001	20 or 50		+
	0.0002	20 or 50		+ +
None	Zero		–	–

*–, no inhibition; + + total inhibition; + partial inhibition; (+) slight inhibition.

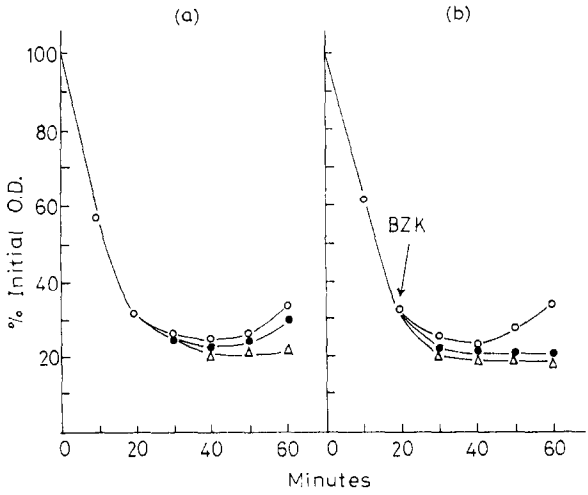

Fig. 3. Effect of benzalkonium chloride (BZK) on germination and outgrowth of *B. subtilis* spores. (a) BZK added at zero time; (b) BZK added at 20 min. BZK concentration (% w/v): ○——○, control (BZK absent): ●——●, 0.0001; △——△, 0.0002.

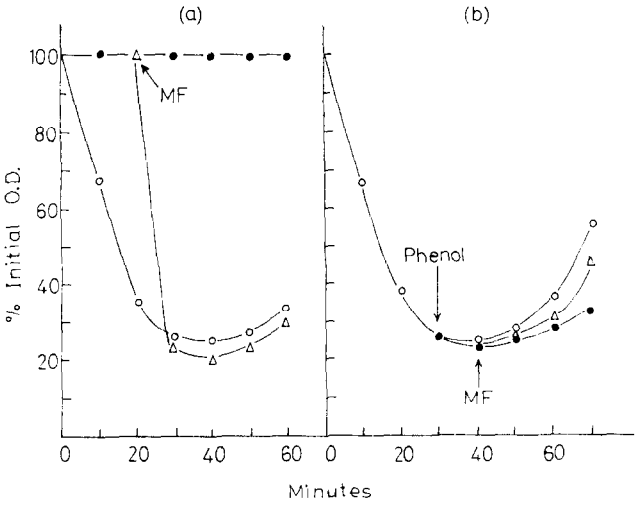

Fig. 4. Reversibility by membrane filtration (MF) of the germination-inhibitory effect of 0.15% w/v phenol on *B. subtilis* spores. (a) Phenol added at zero time; (b) phenol added at 30 min. O——O, control (phenol absent); ●——●, phenol treatment without filtration; △——△, phenol treatment followed by filtration, 10 or 20 min later.

BZK also is bound tightly to spores and that filtration and subsequent washing are insufficient for its removal. In contrast, inhibitors such as phenol and cresol appear to be bound only loosely to the spore surface, because mere washing or filtration is an adequate means of dislodging this type of inhibitor.

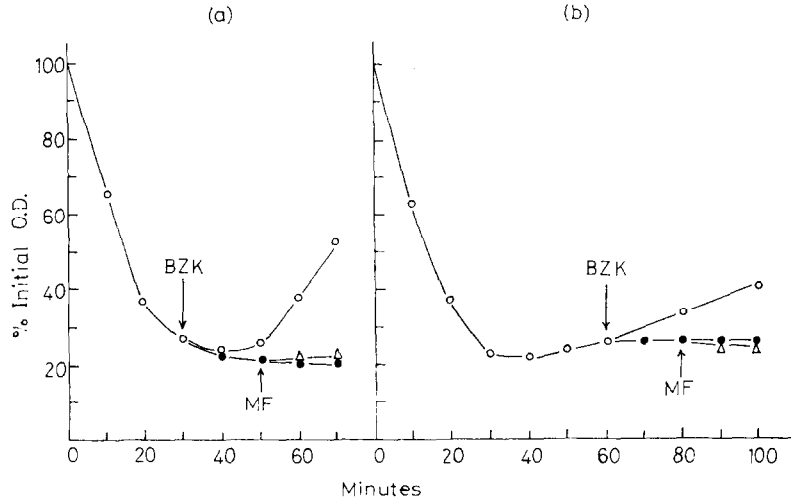

Fig. 5. Effect of membrane filtration (MF) on the action of 0.0002% w/v benzalkonium chloride (BZK) on outgrowing spores of *B. subtilis*. (a) BZK added at 30 min; (b) BZK added at 60 min. O——O, control (BZK absent); ●——●, BZK treatment without filtration; △——△, BZK treatment followed by filtration, 20 min later.

The disinfectant-type agents used in this study are not, with the exception of glutaraldehyde, sporicidal in action. The binding, firmly or loosely, to the cell surface is not, therefore, an important criterion in their lack of sporicidal activity. What is of far greater importance is their ability to penetrate the outer layers of the bacterial spore, and in this context it is likely that all of the disinfectants tested (except glutaraldehyde at alkaline pH) find difficulty in passing through the bacterial spore coat(s) (Russell, 1982; Gorman et al., 1984).

Further experiments will be carried out to investigate the bactericidal effects of these agents at different stages in the germination and outgrowth processes.

References

Ando, Y., Studies on germination of spores of clostridial species capable of causing food poisoning. II. Efect of some chemicals on the germination of spores of *Clostridium botulinum* Type A. (In Japanese.) J. Food-Hyg. Soc. Japan, 14 (1973) 462–466.

Chiori, C.O., Hambleton, R. and Rigby, G.J., The inhibition of spores of *Bacillus subtilis* by cetrimide retained on washed membrane filters and on the washed spores. J. Appl. Bact., 28 (1965) 322–330.

Fildes, P., The mechanism of the antibacterial action of mercury. Br. J. Exp. Path., 21 (1940) 67–73.

Gorman, S.P., Scott, E.M. and Hutchinson, E.P., Emergence and development of resistance to antimicrobial chemicals and heat in spores of *Bacillus subtilis*. J. Appl. Bact., 57 (1984) 153–163.

Gould, G.W., Methods for studying bacterial spores. In Norris, J.R. and Ribbons, D.W. (Eds.), Methods in Microbiology, Vol. 6A, Academic Press, London, 1971, pp. 327–381.

Gould, G.W., Injury and repair mechanisms in bacterial spores. In Andrew, M.H.E. and Russell, A.D. (Eds.), Revival of Injured Microbes, Society for Applied Bacteriology Symposium Series No. 12, Academic Press, London, 1984, pp. 199–220.

Gould, G.W. and Sale, A.J.H., Initiation of germination of bacterial spores by hydrostatic pressure. J. Gen. Microbiol., 60 (1970) 335–346.

Hsieh, L.K. and Vary, J.C., Germination and peptidoglycan solubilization in *Bacillus megaterium* spores. J. Bact., 123 (1975) 463–470.

Hugo, W.B., Disinfection mechanisms. In Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. (Eds.), Principles and Practice of Disinfection, Preservation and Sterilisation, Blackwell Scientific Publications, Oxford, 1982, pp. 158–185.

Parker, M.S., Some effects of preservatives on the development of bacterial spores. J. Appl. Bact., 32 (1969) 322–328.

Russell, A.D., The Destruction of Bacterial Spores, Academic Press, London, 1982, pp. 168–231.

Russell, A.D., Mechanisms of action of chemical sporicidal and sporistatic agents. Int. J. Pharm., 16 (1983) 127–140.

Sofos, J.N. and Busta, F.F., Chemical food preservatives. In Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. (Eds.), Principles and Practice of Disinfection, Preservation and Sterilisation, Blackwell Scientific Publications, Oxford, 1982, pp. 306–342.

Vinter, V., Germination and outgrowth: effect of inhibitors. J. Appl. Bact., 33 (1970) 50–59.