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Science Papers

Method of making spore papers of reproducible resistance

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A method of making papers impregnated with *Bacillus stearothermophilus* spores is described. Replicate experiments testing the resistance of these papers to steam at different temperatures have given reproducible results.

A PREVIOUS report (Cook & Brown, 1960) gave details of tests on a commercial brand of spore papers. The present work is mainly concerned with papers made in this laboratory.

The apparatus and method for testing heat resistance were those of Cook & Brown (1960). The method consists essentially of heating spore impregnated paper discs in a modified autoclave. Replicate discs recovered after suitable time intervals were then incubated in separate tubes of broth.

PREPARATION OF SPORE PAPERS

Spore suspensions of *Bacillus stearothermophilus* N.C.I.B. 8919 in water were prepared and viable counts made as described previously (Brown, 1962). Paper discs† were impregnated with a spore suspension in one of two ways.

Method A. A dropping pipette and needle (Cook & Yousef, 1953) was used to deliver a known volume of a standardised spore suspension separately to each disc. The discs were then dried at ambient room temperature before being stored in dark coloured screw-cap glass jars.

Method B. Sufficient discs were added to a standardised spore suspension present in a glass dish until only a small volume of suspension was left unabsorbed by the discs. Care was taken that each disc was thoroughly wet. The unabsorbed suspension was discarded and the discs then dried and put into jars as described above.

All spore papers were stored at ambient room temperature on the bench. Details of non-commercial spore papers are listed in Table 1.

Recovery conditions. Previous findings with commercial spore papers (Brown, 1962; Cook & Brown, 1960) have shown that the presence of bromocresol purple as an indicator in the broth recommended by the makers of these papers resulted in significantly reduced recovery after

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† Whatman Antibiotic Assay Discs, W. and R. Balston Ltd. Obtained from H. Reeve Angel and Co. Ltd., 9, Bridewell Place, London, E.C.4.

heat treatment. The medium used in this work contained dextrose 0·5%, tryptone (Oxoid) 1% in water and was used for both commercial and non-commercial papers. The spore papers were incubated for 3 days at 56° after heat treatment in the autoclave described by Brown (1962).

TABLE 1. DETAILS OF SPORE PAPERS

Paper code	Sporulation time of spores (days)	Method of preparation	Number of spores per paper
A	2	A	3×10^4
B	2	A	3×10^4
C	8	B	10^7
D	8	B	10^6

Presentation of results. When the percentage of tubes showing growth (positive) was plotted against time of exposure to steam at constant temperature the graph was typically of the form in Fig. 1. The "middle" straight line portion of the graph is important in the present work. In attempting to calculate the equation of this middle portion, it is necessary to exclude from the calculation points which occur on the distal portions of asymptotes of the curve. Consequently, where possible, the percentage of positive results was converted to probits and plotted against exposure time. This transformation enabled a better assessment of the linearity of the graph to be made.

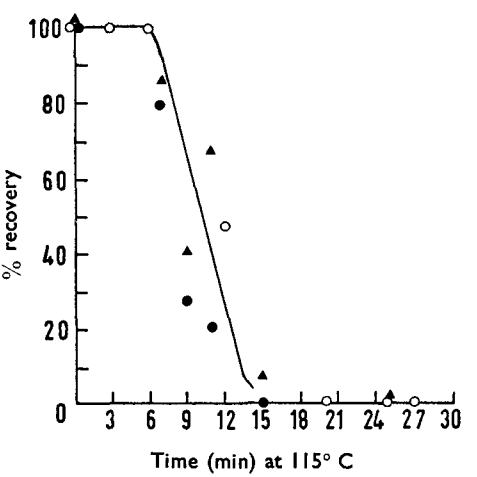

FIG. 1. Reproducibility of results of heat resistance tests on spore papers coded B. ○, Expt. 1. ▲, Expt. 2. ●, Expt. 3.

The equations calculated to fit these lines are not put forward as precise measurements of the rates of kill, but are useful for comparative purposes. This is because in most cases the number of degrees of freedom associated with these lines is small and the smallest interval possible on the % survivor or probit axis is large.

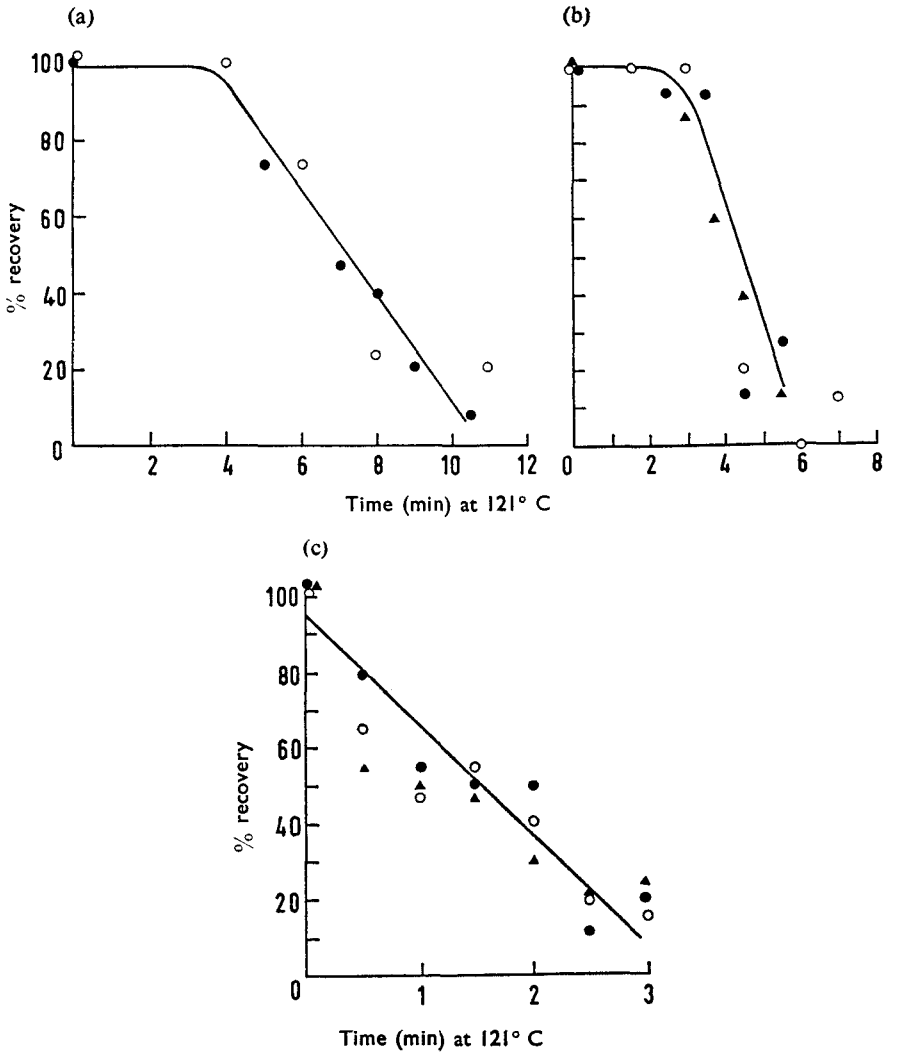

FIG. 2. Reproducibility of results of heat resistance tests on spore papers. (a) Coded C. (b) Coded D. (c) Commercial. ○, Expt. 1. ●, Expt. 2. ▲, Expt. 3.

Variability of results. Replicate experiments were made of the resistance to wet heat at 121° of papers coded C and D and Oxoid* spore papers (Ox. S) and at 115° of papers coded B. The results of these experiments are illustrated in Figs 1–3 and an analysis of variance of these results is recorded in Table 2. The results recorded in Fig. 2(b) show evidence of the reproducibility of the testing method but they are not suitable for probit transformation and are not recorded in Table 2.

*Oxide spore strips, Oxoid Division, Oxo Ltd., London.

TABLE 2. ANALYSIS OF VARIANCE OF RESULTS IN FIGURES 1 AND 2

Figure	Source of variance	Sum of squares	Degrees of freedom	Mean square	F	P	Equation of lines (Probit % +ive/Time)
1	Pooled residual (*a*)	1·8368	5				(1) y=8·47–0·41x
	Residual due to individual regressions (*b*)	1·1256	3	0·3752	1·0551	>0·2	
	(*c*)	0·7112	2	0·3556			(2) y=7·89–0·28x
2 (a)	Pooled residual (*a*)	2·1701	6				(1) y=6·93–0·27x
	Residual due to individual regressions (*b*)	0·4401	4	0·1100	7·8636	0·05–0·01	
	(*c*)	1·7300	2	0·8650			(2) y=7·58–0·19x
2 (c)	Pooled residual (*a*)	1·1420	16				(1) y=5·7–0·28x
	Residual due to individual regressions (*b*)	0·8807	12	0·0734	1·1240	>0·2	(2) y=6·04–0·35x
	(*c*)	0·2613	4	0·0653			(3) y=5·37–0·20x

Resistance at different temperatures. The resistance of A and B papers was tested at 110°, 115° and 121°. One batch of papers was tested at 110° and 121° and a second batch, made from the same spore suspension as the first batch, was tested at 115°. All the papers used in these experiments were stored for less than one week before being tested. The results are illustrated in Fig. 3.

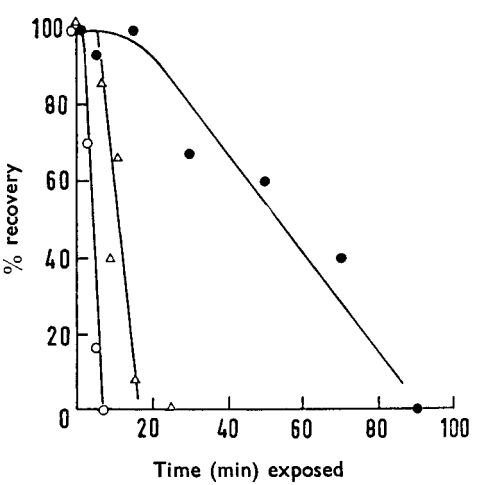

FIG. 3. Effect of steam at different temperatures on the recovery of spore papers. ○, 121° (A). △, 115° (B). ●, 110° (A).

The exposure time necessary to produce zero % positive results was estimated from the graph by eye for the three temperatures and the logarithm of this time was plotted against temperature (Fig. 4).

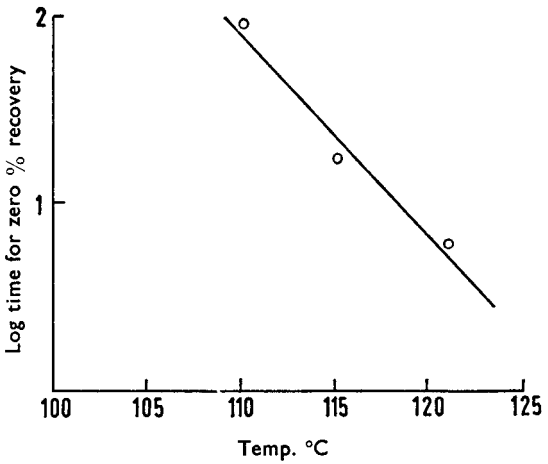

FIG. 4. Relationship between temperature and the log of exposure time for zero % positive results.

Discussion

The method of preparation of spore papers used in this work differs from that suggested by Kelsey (1961), who recommended that spore suspensions intended for impregnation of papers be heat activated immediately after harvesting, to kill vegetative bacteria. If the suspension had been stored, he recommended a second heat activation to kill extraneous vegetative organisms. He stated that "the pre-impregnation viable count of spores will be maximal" and also that this activation is important.

The suspensions used to impregnate papers in this work were not heat activated. Although Kelsey (1961) quoted Evans & Curran (1960) as showing that heat activated spores retain viability after prolonged storage at low temperatures (and at neutral or slightly alkaline pH), nevertheless these authors used buffered solutions. The suspensions used in this work and by Kelsey were made with distilled water.

Retention of heat resistance as well as viability is important with spore papers. In earlier papers, Curran & Evans (1945, 1947) have shown that there was a tendency for heat-activated spores in a nutritionally incomplete medium to lose viability, and that heat activated spores are more sensitive to heat than non-activated spores. If a count of heat-activated spores is required, this may be made with a sample of the suspension, leaving the bulk of the spores with their resistance unaltered.

Kelsey (1961), found that the cultural conditions he used did not affect survival from heated spore papers. However, he compared Oxoid dextrose tryptone broth (containing bromocresol purple) with nutrient broth. He varied the amount of broth by using "$\frac{1}{2}$ inch" and "3 inch" quantities. He did not test the effect of using dextrose tryptone broth without any dye—the medium we found to be most satisfactory in the present work.

Figs. 1–3 and Table 2 show that heat resistance experiments gave results which were satisfactorily reproducible.

It is necessary that the spores on the papers be sufficiently resistant to the lethal effects of heat to be of practical value. In hospital sterilisation practise, the main concern is the killing of pathogenic organisms such as tetanus and the gas-gangrene group. The M.R.C. report (1959) suggested that the minimum heat resistance for spore papers should be 5 min at 121° or 1 min at 130° wet heat. The review by Perkins (1954) of quoted thermal death times from the literature indicates that 5 min at 121° wet heat would be sufficient to kill pathogenic organisms.

Papers coded C which contained about 10^7 spores showed 100% positive results on subculture after exposure to steam at 121° for over 4 min (Fig. 2a). With these papers 90% and 10% positive recoveries occurred after 5 and 10 min respectively at 121°. Thus, if each of two C papers showed growth on subculture after exposure to steam at 121° then there would be a 100 to 1 chance ($P = 0{\cdot}99$) that they had been exposed for less than 10 min. Conversely if neither of the 2 papers showed growth then the probability that they had been exposed for at least 5 min would be $P = 0{\cdot}99$.

The maximum exposure times at 121° resulting in 100% recovery for other papers tested were as follows: Ox.S—3½ min, D (10^6 spores per paper)—3 min, A (3×10^4 spores per paper)—2 min.

Kelsey (1961) showed that bulk impregnation of spore papers did not significantly increase the variation compared to that obtained by individual impregnation. The results obtained here confirm this finding. With A and B (Figs 1 and 3), which were impregnated individually, results were similar to those obtained with spore papers prepared in bulk. The absence of an excessive number of "wild" negative or positive results with bulked papers is an indication that they are not more variable than the individually impregnated papers.

This work shows that papers impregnated with aqueous suspensions of *B. stearothermophilus* N.C.I.B. 8919, such that each paper contained about 10^7 spores, were of satisfactorily high and reproducible resistance.

The relatively low resistance shown by Ox.S papers (Fig. 2c) is probably correlated with the fact that this batch was stored in the laboratory for 4 months before this experiment.

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