J. Pharm. Pharmacol., 1964, **16**, 725–732 Received June 30, 1964

The relation between heat activation and colony formation for the spores of *Bacillus stearothermophilus*

A. M. COOK AND M. R. W. BROWN*

Spores suspended in water were heated at 73·5, 100, 115 and 121° and colony counts made. These showed an initial increase followed by an exponential decline. Spores in phosphate buffer showed a reduced activation. Comparison of total (microscopic) and colony counts showed that only about 5% of unheated spores developed into colonies. This increased to about 50% after optimum heat activation.

LITTLE attention has been drawn explicitly to the relationship between total and viable counts of spore suspensions (Valentine & Bradfield, 1954; Bufton, 1959; Powell, 1956). Sublethal heating of spore suspensions of some bacterial species results in an increased viable count (Evans & Curran, 1943; Curran & Evans, 1945; 1947). It is implied from the results of these and other studies (Murrell, 1961) that without preheating only a small proportion of spores of some species developed into colonies under the conditions tested. It is the purpose of this work to study the kinetics of heat activation and kill and to relate total with viable count for aqueous suspensions of *Bacillus stearothermophilus* spores.

Experimental

PREPARATION OF SPORE SUSPENSIONS

Spores of *B. stearothermophilus* NCIB 8919 were obtained from cells grown at 55° on a medium containing Bactotryptone 3 g, Oxoid peptone 6 g, Yeastrel 3 g, Lab-Lemco 1·5 g, agar 25 g. Mn^{2+} 1 ppm, water to 1,000 ml, pH 7 (Kelsey, 1960). The method of Long & Williams (1958) was used to separate vegetative cells from spores which were washed five times using a refrigerated centrifuge. Details of the spore suspension are given in Table 1.

COUNTING METHOD

A spread plate colony count method (Roberts, 1961) was used to screen several counting media, using five replicate plates for each count. These were Oxoid dextrose tryptone agar with and without 0·1% starch; Oxoid tryptone glucose extract agar with 0·1% starch; Oxoid tryptone soya agar with 0·1% starch and 0·5% dextrose; Antibiotic Assay medium with 0·1% starch, pH 6·6.

The latter medium gave the highest counts and was chosen as the basic medium for subsequent experiments. Its composition is based upon that recommended in the B.P. 1958 and is as follows: Peptone (Oxoid) 6 g, tryptone (Oxoid) 4 g, yeast extract (Yeastrel) 3 g, beef extract (Oxoid

From the School of Pharmacy, University of London, Brunswick Square, London W.C.1.

* Present address: School of Pharmacy, College of Science and Technology, Ashley Down, Bristol, 7.

Lab-Lemco) 1·5 g, Dextrose B.P. 1 g, agar 15 g, soluble starch (Analar) 1 g, water to 1,000 ml.

Fig. 1 illustrates the effect of presence or absence of 0·1% starch in the recovery medium on the count of heat-activated spores.

Maximum colony formation occurred within the range 55°–68°. Routine incubation was at 56° for 3 days.

TABLE 1. DETAILS OF SPORE SUSPENSIONS

Suspension	Diluent	Sporulation time (days)	Total count	Counting chamber size (mm)	% forming colonies
TW	Water	2	10^8	0·1	5
TWH	Water	8	$7{\cdot}8 \times 10^8$	0·1	4·7
TR	Ringers Solution ..	2	$9{\cdot}8 \times 10^7$	0·1	3·3
TP	Phosphate Buffer pH 7..	2	$2{\cdot}1 \times 10^8$	0·1	2
E	Water	5	10^8	0·2	1·4
G	Water (Unwashed) ..	5	4×10^8	0·2	1·2

Variability of counts. Nine replicate counts of a spore suspension were made and the results subjected to an Analysis of Variance (Bailey, 1959) which showed that the between counts variance was not significantly greater than that within counts. The coefficient of variation of the means was 6·1% (Brown, 1962).

METHOD OF TESTING HEAT RESISTANCE

Five drops of spore suspension were introduced into each of several 1 ml sterile glass ampoules using a standard dropping pipette (Cook & Yousef, 1953). The ampoules were then sealed and totally immersed in a Townson & Mercer X27* constant temperature bath. Ampoules were removed after measured time intervals, immediately cooled, and the contents diluted and plated out. Thermocouple readings indicated that 100° was reached in less than 30 sec; 115° and 121° in less than 50 sec.

Reproducibility of exposure time/survivor curves. Time/survivor curves typically showed activation followed by an exponential death rate. Duplicate heat resistance experiments were made at 115° and 121° (Figs 2 and 3) and the analysis of Yousef (1954) used to test if the replicate regression lines could be represented by a common regression. The residual variance between replicate regressions was not significantly greater than that within regressions in each case.

Effect of heating spores at different temperatures. Spore suspensions were heated at 73·5°, 100°, 115° and 121° (Figs 4–7).

The highest colony count caused by heat stimulation was estimated from the results shown in Figs 4–7 for suspension TW and the corresponding time noted for each exposure temperature. Time for maximum heat activation was plotted against exposure temperature (Fig. 8). The exposure times given in the figures were calculated from the time the ampoules were immersed in the heating-bath. This was because the lag period was found to be important in stimulating an increase in the viable count at 115° and 121°.

* Townson & Mercer, Ltd., Croydon.

METHOD OF MAKING TOTAL COUNTS

Helber slides were used of stated depth 0·1 mm ± 0·001 mm with improved Neubauer* ruling. The spores in 64 preselected squares were counted using a phase contrast microscope. The experimental details have been described previously (Cook & Lund, 1962; Lund, 1962).

Replicate counts of several spore suspensions were made using five slides in each case and the mean coefficient of variation was calculated to be 5%. Earlier counts using slides of 0·02 mm depth gave a mean coefficient of variation of 16%.

PERCENTAGE OF SPORES FORMING COLONIES

Total and colony counts were made of several suspensions of spores cultured on different occasions and the results used to calculate the percentage of spores forming colonies (Table 1).

Results and discussion

The presence of starch in the recovery medium increased the counts of both heated and unheated spores (Fig. 1). This has been noted by other workers (Olsen & Scott, 1950; Murrell, Olsen & Scott, 1950).

The incubation period necessary for maximum colony formation for all the suspensions tested was inversely proportional to the heating period and to the reaction temperature (Brown, 1962). Curran & Evans (1954; 1947) found that heat activation resulted in the earlier development of colonies. Conversely, heating at temperatures close to lethal temperature has substantially increased the time necessary for incubation of thermophilic anaerobes such as *Clostridium botulinum* and also for *Bacillus* species under certain conditions (Schmidt, 1954). It would seem that the recovery medium used in this work does not contain inhibitors which make an increased incubation time necessary for heated spores.

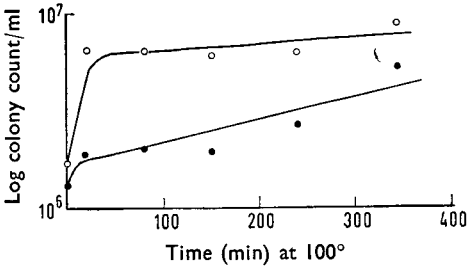

FIG. 1. Effect of presence or absence of starch in the recovery medium for heated *B. stearothermophilus* spores (Suspension E). ○ With starch. ● Without starch.

The heating of suspensions of *B. stearothermophilus* spores in water, but not phosphate buffer, gave characteristic time/survivor curves. The colony count rose to a maximum as exposure time increased after which there was an apparent exponential death rate. No "tail off" was observed.

* Messrs Hawksley and Sons Ltd., 17 New Cavendish Street, London, W.1.

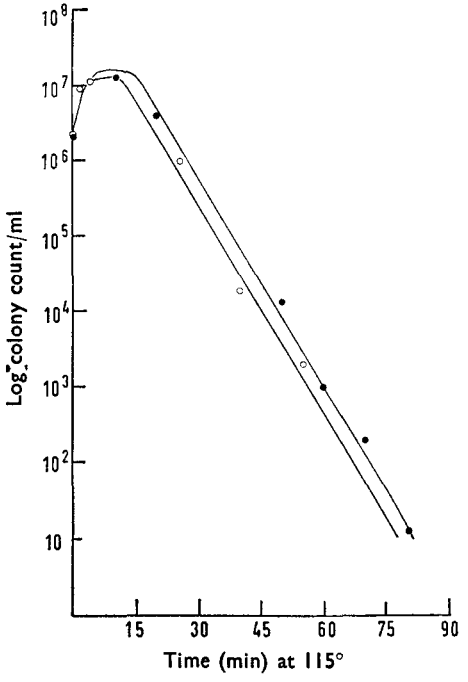

FIG. 2. Replicate heat resistance experiments at 115° with *B. stearothermophilus* spores (Suspension TW). ● Experiment 1. ○ Experiment 2.

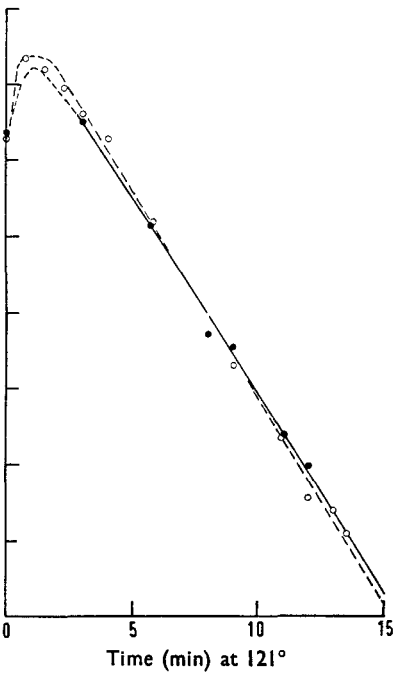

FIG. 3. Replicate heat resistance experiments at 121° with *B. stearothermophilus* spores (Suspension TW). ● Experiment 1. ○ Experiment 2.

Vas & Proszt (1957) obtained similar curves with dilute suspensions of *Bacillus cereus* spores. They found an exponential decline followed by a reduced rate of kill with concentrations exceeding about 10^8/ml. They found evidence suggesting that this "tail off" was due to the presence of a minute and constant fraction (about 1 in 10^7 or 10^8) of very resistant spores.

Amaha & Ordal (1957) found that the logarithm of survivors/time curve for heated spores of *Bacillus coagulans* showed an initial shoulder. They

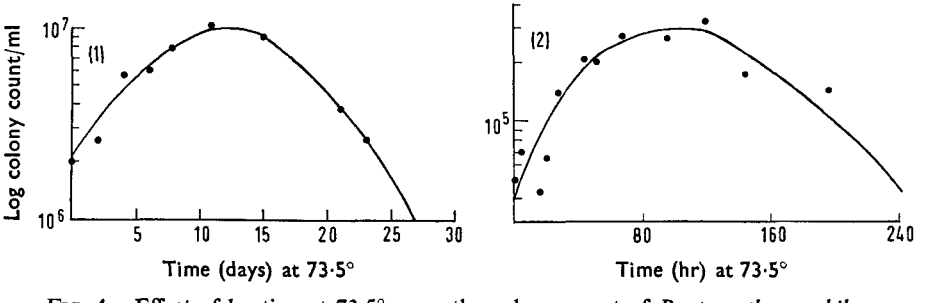

FIG. 4. Effect of heating at 73·5° upon the colony count of *B. stearothermophilus*. Experiment 1, suspension TW. Experiment 2, suspension TP.

made both plate and direct microscopic counts throughout their work and found no significant difference between them. They suggested that the shoulder was due to changes in the resistance of the spores.

The time required to reduce the colony count to unity (single survivor time), for suspension TW at each exposure temperature was calculated from the equation for the exponential part of the curve at 100°, 115° and 121° and there was an exponential relationship between temperature and the calculated single survivor time. This time will depend also upon the initial concentration which was about 2×10^6/ml (Brown, 1962).

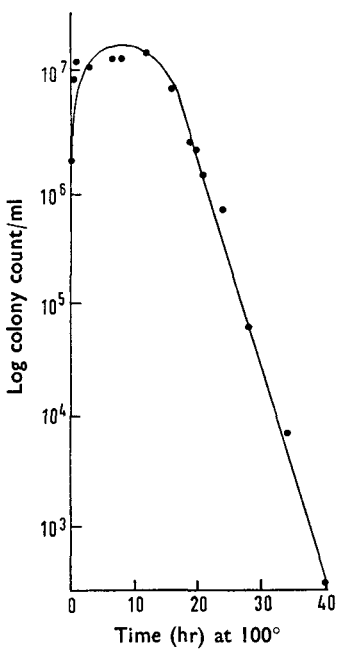

FIG. 5. Effect of heating at 100° upon the colony count of *B. stearothermophilus* (suspension TW).

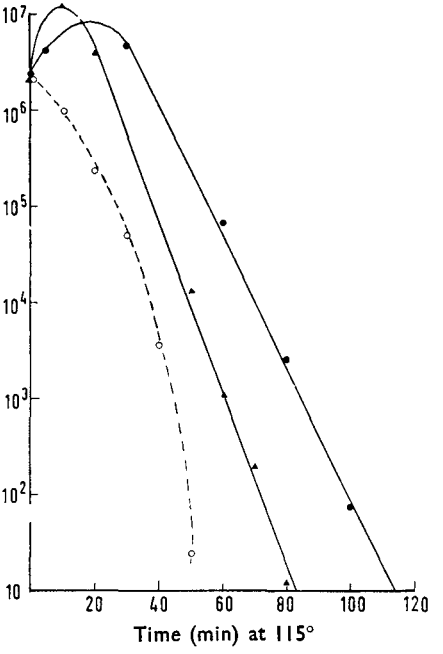

FIG. 6. Effect of heating at 115° upon the colony count of *B. stearothermophilus*. ● Suspension E. ▲ Suspension TW. ○ Suspension TP.

There is a smooth relationship between temperature and logarithm of the exposure time necessary to produce maximum activation (Fig. 8). This graph could be used to predict the approximate time for maximum activation within the range plotted for spores suspended in water. A similar relationship may be calculated from the results given by Murrell (1961, Fig. 1) of log viable count/heating time for spores of *B. coagulans* heated at different temperatures.

Spores in 0·1M phosphate, pH 7 at 115° showed no activation and gave a time/survivor curve concave downwards (TP in Fig. 6).

This latter effect was possibly because at the final exposure times, when counts were low, there was little and eventually no dilution of the spores suspended in phosphate buffer and consequently initiation of germination

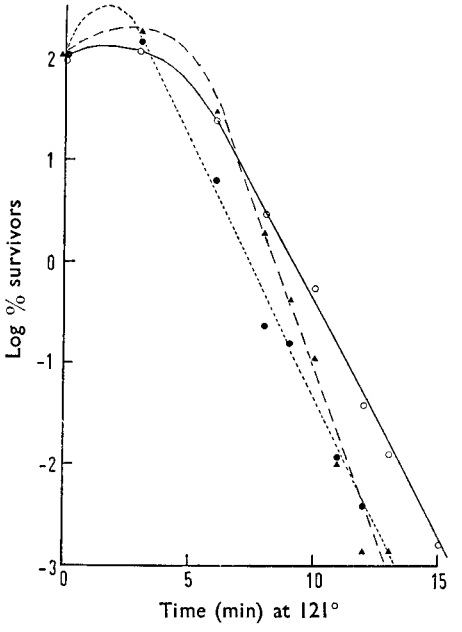

FIG. 7. Effect of heating at 121° upon the colony count of *B. stearothermophilus*. ○ Suspension E. ▲ Suspension TH. ● Suspension TW.

in the recovery medium may have been inhibited by the phosphate (Williams & Hennessee, 1956; Murty & Halvorson, 1957). Spores heated for only a short period were much diluted in distilled water before plating out and the concentration of phosphate in the recovery medium was likely to have been insignificant.

Activation in the presence of 0·1M phosphate did take place at 73·5° although the time for maximum activation of suspension TP was about 4 days. The time for maximum activation for suspensions in water

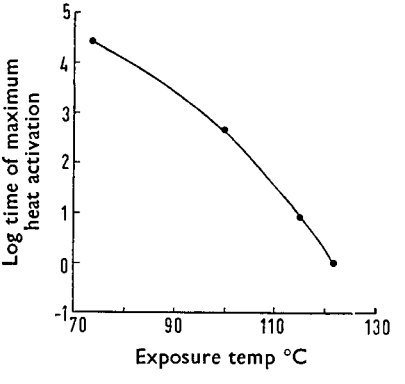

FIG. 8. Relationship between exposure temperature and time for maximum heat activation for *B. stearothermophilus* spores (TW).

(TW) of similar spore concentrations was about 12 days (Fig. 4). The increase in count which occurred in each case was of the same order (6-fold).

The depressant effect of phosphate in the heating medium upon the activation of spores is of particular interest. Gerhardt & Black (1961) have shown that *B. cereus* spores are permeable to phosphate to a considerable extent (40%). It seems possible that if heat activation involves the stimulation of some mechanism connected with initiation of metabolism, then the presence of phosphate within the cell at the time of heating may prevent this stimulation.

It is apparent that the heating up time necessary for the suspension to achieve either 115° or 121° was important for activation. During this period of about 50 sec there was a substantial increase in colony count when compared to that of the unheated control (Figs 2 and 3).

0·02 mm depth counting chambers gave significantly more variable total counts than those obtained with 0·1 mm slides. Norris & Powell (1961) found that total counts with 0·02 mm slides were systematically in excess of the true values by 10–50%.

5% or less of the unheated spores germinated and gave colonies (Table 1). These results indicate that after maximum heat activation (10-fold) about 50% of the spores developed into colonies after subculture.

Microscopic examination of the heat activated spores showed that they retained their refractility under phase contrast illumination. Consequently it seems possible that under other conditions substantially all of the spores may be capable of colony formation. It is interesting to note that a similar low percentage colony formation has been observed with *Rhizopus nigrificans* spores (Brown & Bullock, 1960).

The practice in the literature of quoting only the time necessary for maximum heat activation may be misleading. It would be more informative also to state the percentage of spores which produce colonies.

References

Amaha, M., & Ordal, Z. J. (1957). *J. Bacteriol.*, **74**, 596–604.
Bailey, N. T. J. (1959). *Statistical Methods in Biology*, p. 102. London: English Universities Press.
Brown, M. R. W. (1962). Ph.D. thesis, University of London.
Brown, M. R. W. & Bullock, K. (1960). *J. Pharm. Pharmacol.*, **12**, *Suppl.* 119*T*–126*T*.
Bufton, A. W. J. (1959). *J. appl. Bact.*, **22**, 278–280.
Cook, A. M. & Lund, B. M. (1962). *J. gen. Microbiol.*, **29**, 97–104.
Cook, A. M. & Yousef, R. T. (1953). *J. Pharm. Pharmacol.*, **5**, 141–144.
Curran, H. R. & Evans, F. R. (1945). *J. Bacteriol.*, **49**, 335–346.
Curran, H. R. & Evans, F. R. (1947). *Ibid.*, **53**, 103–113.
Evans, F. R. & Curran, H. R. (1943). *Ibid.*, **46**, 513.
Gerhardt, P., & Black, S. H. (1961). *Ibid.*, **82**, 750–760.
Kelsey, J. C. (1960). *Thermophilic spore papers for testing steam sterilisers.* Roneo sheet, Bact. Dept., Guy's Hospital Medical School, London.
Long, S. K. & Williams, O. B. (1958). *J. Bacteriol.*, **76**, 332.
Lund, B. M. (1962). Ph.D. thesis, University of London.
Murrell, W. G. (1961). 11th Symposium of the Society for General Microbiology, Cambridge: The University Press.
Murrell, W. G., Olsen, A. M. & Scott, W. J. (1950). *Aust. J. Sci. Res. B.*, **3**, 234–244.

Murty, G. G. K. & Halvorson, H. O. (1957). *J. Bact.*, **73**, 230–234.
Norris, K. P. & Powell, E. D. (1961). *J. Roy. Microscopical Soc.*, **80**, 107–119.
Olsen, A. M. & Scott, W. J. (1950). *Aust. J. Sci. Res., B.*, **3**, 219.
Powell, E. O. (1956). *J. gen. Microbiol.*, **14**, 153.
Roberts, T. A. (1961). Ph.D. thesis, University of London.
Schmidt, C. F. (1954). *Antiseptics, disinfectants, fungicides and chemical and physical sterilisation.* Editor, Reddish, London: H. Kimpton.
Valentine, R. C. & Bradfield, J. R. G. (1954). *J. gen. Microbiol.*, **11**, 349.
Vas, K. & Proszt, G. (1957). *J. appl. Bact.*, **20**, 431–441.
Williams, O. B., & Hennessee, A. D. (1956). *Food Research*, **21**, 112–116.
Yousef, R. T. (1954). Ph.D. thesis, University of London.