J. Pharm. Pharmac., 1966, **18**, *Suppl.*, 39S–41S Received May 2, 1966

SHORT COMMUNICATION

Sizing *Bacillus megaterium* spore populations as a basis for studying their viability

H. R. HIBBERT AND A. TALLENTIRE

SEVERAL reports have described microbiological applications of the Coulter Counter, particularly for determining the total count of viable and non-viable vegetative bacteria (Garrett & Miller, 1965) or bacterial spores (Kubitschek, 1958) in suspension. The value of the instrument would be greatly increased if it could be used to differentiate between viable and non-viable bacteria.

Germination of bacterial spores, commonly taken as a criterion of their viability, is accompanied by an increase in cell volume, increases in packed cell volume being as much as 100% during pre-emergence swelling of *Bacillus cereus* and *B. subtilis* spores (Hitchins, Gould & Hurst, 1963). If, during germination, changes in the volume of individual cells are sufficiently large, the Coulter Counter might be employed to enumerate either germinated spores or unchanged spores, and hence yield an estimate of the viability of the original population. The object of the reported work was to recognise the limits of a volume distribution of resting spores of *B. megaterium* and to test whether or not this distribution could be measured in the presence of germinated spores of the same organism.

MATERIALS AND METHODS

The Model B Coulter counter used was fitted with a 30 μ aperture tube and was calibrated with 0·796 μ diameter polystyrene latex spheres (Dow Chemical Company). Size distributions were obtained from a Model J plotter which automatically records particle counts in selected size ranges.

Electrolyte. Aqueous solutions of sodium chloride with concentrations ranging from 0·5 to 10% were tested for instrument sensitivity and count stability; a 2% solution was satisfactory and was used in the reported experiments. It was freshly prepared each day.

Medium. MR–VP granules (Difco) 3·4 g, distilled water to 100 ml; 20 ml volumes were sterilised by autoclaving for 5 min at 121°.

Electrolyte used for counting, distilled water and medium were all filtered through a stack of cellulose ester membranes (Millipore Filter Corporation) consisting of two of mean pore size 0·8 μ on one of 0·22 μ. This treatment gave a stable background count for the electrolyte of less than 1% of the spore count. All glassware was routinely cleaned with "chromic acid mixture" and washed with tap water followed by filtered distilled water; it was then stored in closed containers. Blank determinations showed that sampling and diluting procedures did not increase the background count beyond the arbitrary 1% level.

From the Department of Pharmacy, University of Manchester, Manchester 13.

Organism. Spores of *Bacillus megaterium* (ATCC 8245) were produced on potato extract agar (Powers, Ehret & Bannon, 1957). Surface growth from six plates was collected in filtered distilled water, washed three times and then suspended in water.

Incubation and sizing. Sufficient stock spore suspension was added to medium maintained at 37° to give a total spore count of about 2×10^7/ml. At intervals, samples (1 ml) were withdrawn and immediately diluted with 19 ml of electrolyte. Volume distributions of particles in these suspensions were plotted between the limits of 0·14 and 0·88 μ^3 within 5 min of their preparation. An aperture current setting of $\frac{1}{2}$ and an amplification setting of $\frac{1}{8}$ were used.

RESULTS AND DISCUSSION

A typical plot of the volume distribution of unincubated spores is shown in Fig. 1a. Counts recorded between 0·14 to 0·26 μ^3 represent

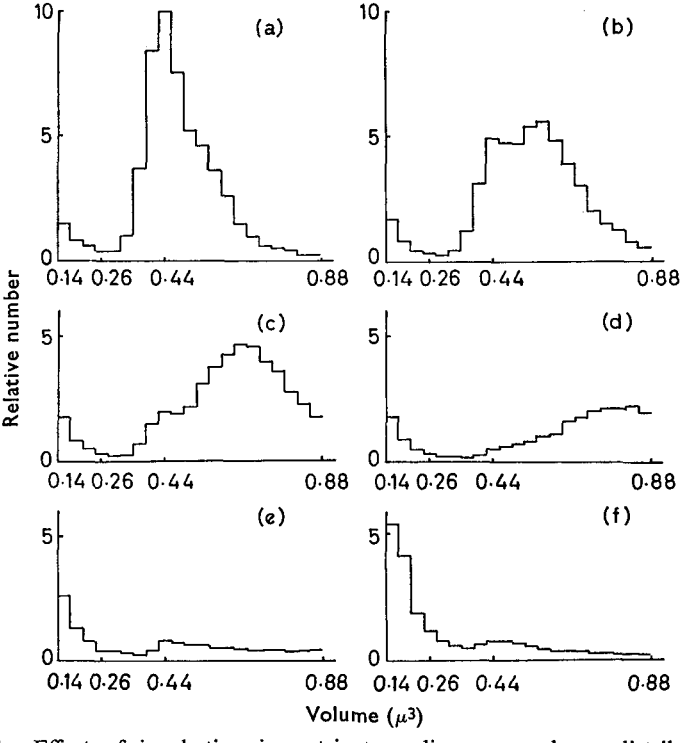

FIG. 1. Effect of incubation in nutrient medium on volume distributions of *B. megaterium* spores. (a) 0 min. (b) 30 min. (c) 45 min. (d) 60 min. (e) 90 min. (f) 120 min.

background count and instrument noise. Above a volume of 0·26 μ^3 the counts show an asymmetric distribution of cell volumes with a single peak lying at about 0·44 μ^3, the count at 0·88 μ^3 being essentially zero. Distributions of cell volumes for spores incubated for increasing periods

in nutrient medium are given in Fig. 1b–f. During incubation cells undergo an asynchronous increase in volume, and after 30 and 45 min incubation bimodal volume distributions are observed (Fig. 1b, c). After 90 min incubation increases are such that more than 85% of the cells have volumes greater than 0·88 μ^3; Fig. 1e shows that the volumes of the remaining 15% are asymmetrically distributed around a peak of 0·44 μ^3 like the unincubated spores. The same peak is seen after 120 min incubation (Fig. 1f), although a greater background count than after 90 min is recorded. With further incubation the background count continues to rise and this is probably caused by cell debris shed during emergence and cell multiplication. The appearance of a constant number of cells with unchanged volumes after incubation for between 90 and 120 min suggests that these cells constitute the inactive fraction of the original spore population. Heating the spore suspension at 65° for 6 hr before 90 min incubation, results in disappearance of the 0·44 μ^3 peak.

These results indicate that estimates of the number of inactive spores in suspension may be made by counting cells within the volume range 0·26 to 0·88 μ^3 after a period of incubation in a suitable medium. To test this, samples of the spore suspension were heated at 90° for graded times (between 4 and 20 min) known from plate counts to yield survival levels ranging from 75 to 30%. These samples were incubated for 90 min and plots of the volume distributions of inactive spores were then made. The distributions were all typical of that for unincubated spores and the areas under the plots increased with heating time as expected from the plate counts. These experiments show that the Model B Coulter counter can be used to count unchanged *B. megaterium* spores in the presence of germinated spores, and that the absence of a change in cell volume on incubation can probably be used as a criterion of spore inactivation. As yet the fate or cells undergoing volume changes is not known.

Acknowledgements. This work was supported by a grant from the S.R.C. and one of us (H.R.H.) is a recipient of a S.R.C. Research Studentship.

References

Garrett, E. R. & Miller, G. H. (1965). *J. pharm. Sci.*, **54**, 427–431.
Hitchins, A. D., Gould, G. W. & Hurst, A. (1963). *J. gen. Microbiol.*, **30**, 445–453.
Kubitschek, H. E. (1958). *Nature, Lond.*, **182**, 234–235.
Powers, E. L., Ehret, C. F. & Bannon, A. (1957). *Appl. Microbiol.*, **5**, 61–64.