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Dynamic light-scattering studies on the effect of heat and disinfectants on spores of *Bacillus cereus*

Antonio D. MOLINA-GARCIA,* Stephen E. HARDING,*‡ Lucrezia DE PIERI,† Navid JAN*§ and William M. WAITES*

*Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington, Loughborough, Leics. LE12 5RD, and †Division of Electrical Engineering, Hatfield Polytechnic, College Lane, Hatfield, Herts. AL10 9AB, U.K.

The relative stability of spores of *Bacillus cereus* grown at three different temperatures was examined by using quasi-elastic light scattering (q.l.s.) in conjunction with turbidity and scanning electron microscopy (s.e.m.). Cultures grown at 20, 30 and 40 °C (BC20, BC30 and BC40 respectively) were compared in terms of (i) their effective hydrodynamic radius, r_H, as determined from q.l.s. and (ii) their gross morphology, as determined from s.e.m. The effects of autoclaving at 121.1 °C on both these properties was also examined. We observed (1) that cultures BC20 and BC30 appeared to have similar values for r_H, whereas that of BC40 appeared some 50% higher, and (2) BC40 had a correspondingly much lower heat resistance (its structural integrity was lost after about 20 min autoclaving, whereas that of BC20 and BC30 was retained even after 80 min autoclaving). These data were in good agreement with independent measurements of heat-resistance coefficients. Changes in the hydrodynamic radius, polydispersity (both using q.l.s.) and turbidity were monitored with time on addition of the disinfectants sodium hypochlorite and peracetic acid; again BC40 appeared to have a lower resistance.

INTRODUCTION

The utility of quasi-elastic light scattering (q.l.s.) as a non-destructive probe to examine the morphology of bacterial spores has been established by previous studies (Harding & Johnson, 1984*a*,*b*, 1986; Harding, 1986). Q.l.s. (also known as 'photon correlation spectroscopy' or 'intensity fluctuation spectroscopy') has had a considerable impact for the analysis of a wide range of biological systems, with particular regard to their dynamic properties [see, e.g., Bloomfield (1981) for a review of these applications]. For the analysis of bacterial spores, q.l.s. provides a valuable non-destructive probe into spore structure (see, e.g., Harding & Johnson, 1984*b*; Harding, 1986). Previous studies have, however, been made with spores (*Bacillus subtilis* and *B. megaterium* KM), which lack exosporia, because of the potential anomalous hydrodynamic properties the latter may induce. The objective of the present study was to examine the feasibility of applying q.l.s. procedures to spores of *B. cereus*, which are contained within exosporia, and specifically to compare the relative stability of three cultures grown at different temperatures 20.0(±2.0) °C, 30.0(±2.0) °C and 40.0(±2.0) °C, henceforth referred to as 'BC20', 'BC30' and 'BC40' respectively.

Q.l.s. was used to estimate the apparent (*z*-average) translational diffusion coefficient, D_z and hence the equivalent hydrodynamic or 'Stokes' radius, r_H, for these different cultures before and after periods of autoclaving at 121.1 °C and to compare this with their viability and gross morphology as revealed (albeit destructively) using scanning electron microscopy (s.e.m.).

We also used q.l.s., in conjunction with turbidity, to monitor the effects with time on the addition of disinfectants (namely sodium hypochlorite and peracetic acid) in the manner used previously for the time-course germination behaviour of *B. subtilis* (Harding & Johnson, 1984*b*) and *B. megaterium* KM (Harding & Johnson, 1986).

MATERIALS AND METHODS

Production of *B. cereus* spores

A strain of *B. cereus* was isolated at Hatfield Polytechnic and identified by comparison with a reference strain using morphological observations and biochemical tests. These included the API 50 CHB kit for the identification of bacilli. The spores were grown in modified 'SP' medium (Setlow *et al.*, 1982) containing: Lab-Lemco (beef extract) 3.64 g; yeast extract, 0.64 g; peptone, 4.14 g; dextrose, 0.43 g; NaCl, 1.50 g; K_2HPO_4, 1.58 g; KH_2PO_4, 0.57 g; 'spore salts' solution, 10.0 ml; water, 1000 ml. The spore salts solution contained: $CaCl_2,7H_2O$, 7.50 g; $MgCl_2,6H_2O$, 5.00 g; $MnSO_4,H_2O$, 1.50 g; $ZnSO_4,7H_2O$, 0.35 g; $FeSO_4,7H_2O$, 0.09 g; $CuSO_4$, 0.05 g; water, 500 ml. This spore salts solution was made up and autoclaved separately before aseptically adding it to the sterile broth. Thiamin (2.5 mg/ml), lysine (100 μg/ml) and biotin (0.5 μg/ml) were filter-sterilized and aseptically added to the broth.

SP medium (10 ml) was inoculated with a single colony of *B. cereus* from a nutrient-agar plate and grown overnight at 30 °C before being used to inoculate nine 250 ml conical flasks each containing 70 ml of SP media. The inoculated flasks were incubated in a Nobel orbital

Abbreviations used: q.l.s., quasi-elastic light scattering; s.e.m., scanning electron microscopy; PF, polydispersity factor.

‡ To whom correspondence and reprint requests should be addressed.

§ Present address: Northern Foods, Nottingham NG7 2NS, U.K.

incubator (LH Engineering Co. Ltd., Stoke Poges, Slough, Berks., U.K.), set at (30.0±2.0)°C or (40.0±2.0)°C and at approx. 180.0 rev./min. The flasks grown at (20.0±2.0)°C were incubated instead in a shaking water bath set at ~ 100 rev./min.

After 14 days incubation for BC30 and BC40 and 17 days for BC20, > 95% fully released spores were observed under a phase-contrast microscope.

Spores were harvested by centrifugation at ~ 6000 *g* for 10 min at 4 °C. The supernatant was removed and the pellet resuspended in 10 ml of sterile distilled water and centrifuged again at 980 *g* for 20 min using an MSE Minor bench centrifuge (MSE Instruments, Crawley, Sussex, U.K.). This washing procedure was repeated six times. The spores were finally resuspended in 10 ml of sterile distilled water.

Preparation of spore suspensions

Spores for light-scattering analysis were suspended in a sodium/potassium phosphate buffer (50 mM, pH 7.5) to a concentration corresponding to an absorbance of 0.35 at 450 nm as measured using an LKB Ultraspec 4050 spectrophotometer. All spore suspensions were ultrasonicated before any study for periods of up to 2 min to reduce the effects of aggregation phenomena (see, e.g., Harding & Johnson, 1984*b*). A Sonicleaner (Ultrasonics Ltd.) bath was employed, filled with deionized water. Ultrasonication did not have a noticeable effect on the hydrodynamic data for suspensions of BC20 or BC40, but it was found necessary for the adequate dispersal of suspensions of BC30 (see Harding & Johnson, 1986).

Q.l.s. measurements

Q.l.s. measurements were performed by using a Malvern 4700 light-scattering apparatus, equipped with a Siemens 40 mW He/Ne laser [wavelength (λ) 632.8 nm]. The beam was focused on to the centre of a 1 cm × 1 cm cuvette which was itself placed at the centre of the goniometer so that the scattering angle could be varied from 5° to 90°. A 90° scattering angle was deemed appropriate [since Chen *et al.* (1977) have demonstrated that, for particles of this size range, rotational motion is so slow in comparison with translational motion as to be negligible, except at low scattering angles].

Scattered light was collected by an EMI photomultiplier via a well-collimated pinhole (aperture 100 μm) and via an amplifier–discriminator to a 64-channel Malvern Autocorrelator (K7032-OS). The digital correlator output was stored on floppy disks and then sent, via an Olivetti M24 microcomputer and the inter-University JANET link, to the University of Cambridge IBM 3081/B computer, for full data analysis. The routine used produced an accurate plot of $\ln[g^{(2)}(t)-1]$ versus time, t, where $g^{(2)}(t)$ is the normalized intensity correlation function (Pusey, 1974), the best least-squares fit of this plot to a linear, quadratic or cubic equation and also the 'polydispersity factor', PF (Pusey, 1974) (namely the normalized z-averaged variance of the distribution of diffusion coefficients). A guide to the best fit was provided by the ϵ function [see, e.g., Teller (1973), p. 375].

A sample time of 200 μs was employed. No dependence of diffusion coefficient with sample time was observed, so a correction to zero sample time (Godfrey *et al.*, 1982) was not found necessary. Experimental duration time was fixed at 5 min to ensure a high number of counts (and signal averaging) to be stored in the autocorrelator channels.

A temperature-control unit maintained the cell temperature through a water bath during the experiment to a precision of ±0.1 °C. All measurements were performed at 25.0 °C.

The water in the index-matching bath was filtered, firstly through a coarse filter and then through a 0.45 μm-pore-size Millipore filter HA type for 15 min. Spores scatter light very strongly, so that ultrafiltration of suspensions was unnecessary.

Hydrodynamic radii were determined from the D_z values via the well-known Stokes relationship:

$$r_H = \frac{kT}{6\pi\eta D_z}$$

where k is the Boltzmann constant, η the solvent viscosity and T the absolute temperature.

S.e.m.

S.e.m. experiments were carried out by using an S100 (Cambridge Instruments) scanning electron microscope. The spore suspensions were fixed with glutaraldehyde, dehydrated through increasing concentrations of ethanol and amyl acetate rinses, dried to the critical point on a 0.5 μm-pore-size Millipore filter, using a SAMDIR 780 apparatus, and finally coated with gold/palladium using a Polaron gold sputter coater.

Thermal-stability measurements

Stability was studied by using q.l.s. and s.e.m. measurements on samples autoclaved at 121.1 °C for periods up to 80 min.

Heat-resistance measurements

Spores suspended in sterile distilled water (0.1 ml) were introduced in an ampoule for freeze-drying (Gallenkamp apparatus) and sealed by using a propane burner. Each ampoule was introduced into a small cage and submersed in an oil bath (Grant Instruments, Cambridge, U.K.) equilibrated at 90 °C, for the appropriate exposure time, after which the ampoule was removed and introduced immediately in an ice bath for at least 10 min. The exposure time varied from 0 (control) to 60 min, with 5 min intervals. The cooled ampoule was then opened and 0.9 ml of sterile sodium/potassium phosphate buffer (pH 7.3, I 0.10) was added with a Gilson pipette. Serial dilution followed, and 0.1 ml of three consecutive dilutions were plated in duplicate on fresh and dry nutrient-agar plates and spread with a sealed, bent and sterile Pasteur pipette. The plates were then incubated at 30 °C, and colonies were counted daily until no further increase in their number was recorded. This was usually after 3 days. The heat-resistance test was repeated five times for each culture (BC20, BC30 and BC40). Heat-resistance coefficients at 90 °C (D_{90}) were calculated in the usual way (details are available from L. de P. on request).

Turbidity measurements

Turbidity or absorbance measurements at 580 nm against time were performed in a Beckman DU 50 spectrophotometer interfaced to an Olivetti M24 microcomputer. This spectrophotometer was equipped with a cell jacket through which water at 25.0 °C from a thermostatically controlled bath was pumped. Turbidity measurements were taken automatically using Beckman software supplied for operation with the microcomputer.

Effect of disinfectants by q.l.s. and turbidimetry

Two types of disinfectant were used (sodium hypochlorite and peracetic acid) to investigate the effect of disinfectant on the spores as a function of time. Hydrodynamic radius and turbidity measurements were performed on separate 2 ml samples after the addition of sodium hypochlorite or peracetic acid. Stock disinfectant solutions were added by pipette, and the final concentration was 1.2% (w/v) for sodium hypochlorite and 3% (w/v) for peracetic acid. Samples were mixed by manual agitation and data collection started 2 min after disinfectant addition, to ensure both homogeneous mixing and thermal equilibrium. The protocol was similar to our earlier germination studies on *B. subtilis* (Harding & Johnson, 1984*a*,*b*) and *B. megaterium* KM (Harding & Johnson, 1986), except that measurements were performed at 25.0 °C. Q.l.s. measurements were taken automatically every 5 min, and turbidity readings every 2 s.

RESULTS AND DISCUSSION

Q.l.s. measurements were performed on native spores for all three cultures (BC20, BC30 and BC40). As expected, the spore suspensions proved to be strongly light-scattering, and highly reproducible curves of the intensity correlation function against channel number b ($b = t/\tau$, where τ is the sample time) were obtained. Unlike the case for *B. subtilis* and *B. megaterium* (Harding & Johnson, 1984*a*,*b*, 1986) for *B. cereus* more curved plots of the logarithm of the normalized autocorrelation function $g^{(2)}(t)$ versus time, t, were produced (Fig. 1), indicative of somewhat higher polydispersities of spore preparations of this species. Polydispersity factor (PF) values were correspondingly higher [0.3 ± 0.2 compared with $\sim 0.10 \pm .05$ for *B. subtilis* (Harding & Johnson, 1984*b*) or 0.15 ± 0.05 for *B. megaterium* (Harding & Johnson, 1986)].

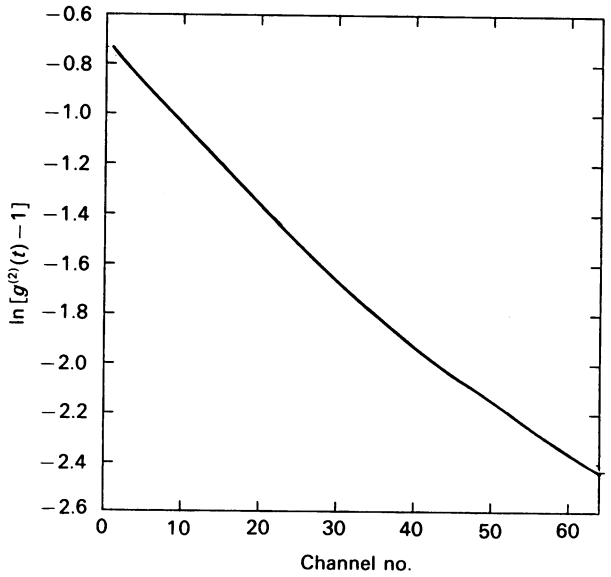


Fig. 1. Plot of $\ln[g^{(2)}(t)-1]$ [where $g^{(2)}(t)$ is the normalized intensity autocorrelation function] against channel number for *Bacillus cereus* spores (BC40)

The scattering angle was 90°, the temperature was 25.0 °C, the spore concentration was $\approx 4.0 \times 10^7$ particles/ml, an He/Ne laser was used ($\lambda = 632.8$ nm, 25 mW) and the sample time, τ, was 200 μs. Time (t) = channel number $\times \tau$.

Table 1. Apparent translational diffusion coefficient and corresponding PF and Stokes-radii data for native *B. cereus* spores

Symbols: D_z, z-average translational diffusion coefficient at 25.0 °C; r_H, equivalent Stokes radius.

Culture	$10^9 \times D_z$ (cm²/s)	PF	r_H (μm)
BC20	2.7 ± 0.1	0.4	0.91
BC30	2.9 ± 0.2	0.6	0.85
BC40	3.6 ± 0.2	0.4	0.68

From the results in Table 1 it is clear that one of the cultures (BC40) appears somewhat different from the others. BC20 and BC30 have similar (z-average) Stokes radii (0.91 ± 0.06 and 0.85 ± 0.06 μm respectively), whereas that for BC40 is smaller (0.68 ± 0.05 μm). The smaller value for BC40 correlates with its lower measured heat resistance compared (see below) with the other two spore cultures and also with observations of their apparent size by s.e.m. (Figs. 2*a*, 2*b* and 2*c*), although caution has to be exercised when treating s.e.m. data on these substances (owing to changes in size which may occur during preparation).

The values we have obtained for the hydrodynamic radii are all larger than those for *B. subtilis* (0.59 ± 0.01 μm) and *B. megaterium* KM (0.60 ± 0.01 μm); these higher values, together with the larger polydispersity factors, appear consistent with the presence of exosporia in *B. cereus* species and their absence in *B. subtilis* and the particular *B. megaterium* strain studied.

Effects of heating

After heating at 121.1 °C, the culture BC40 again appeared to have different properties when compared with the others, as illustrated in Table 2. The hydrodynamic radius (N.B. a z-average) for BC40 decreased to below 0.5 μm after $\sim$ 20 min autoclaving, with a higher resulting polydispersity. This corresponds with a loss of brightness in the phase-contrast optical microscope and somewhat smaller size from s.e.m. (Figs. 2*d*, 2*e* and 2*f*), although the spores had not been completely disrupted. There is some appearance of cellular debris, however (Fig. 2*f*).

The other spore cultures, BC20 and BC30, did not show loss of phase brightness, and showed less evidence of cellular debris (Figs. 2*d* and 2*e*), even after 80 min autoclaving. Interestingly, the average Stokes radius increased to 1.2 ± 0.2 μm, possibly due to an increased tendency to aggregate.

The heat-resistance test for BC20, BC30 and BC40 also show that BC40 was the least heat-resistant, with an estimated D_{90} of 2.17 ± 0.082 min. The values of D_{90} for BC20 and BC30 were estimated respectively at 14.94 ± 2.25 and 13.13 ± 3.86 min. Details of the D_{90} calculations are available from L. de P. on request.

Effects of disinfectant

The effects of the addition of sodium hypochlorite to suspensions of spores of the three cultures at 4.0×10^7 spores/ml in the phosphate buffer are shown in terms of turbidity (Fig. 3), hydrodynamic diameter (Fig. 4) and polydispersity (Fig. 5).

Turbidity measurements for BC20, BC30 and BC40 all

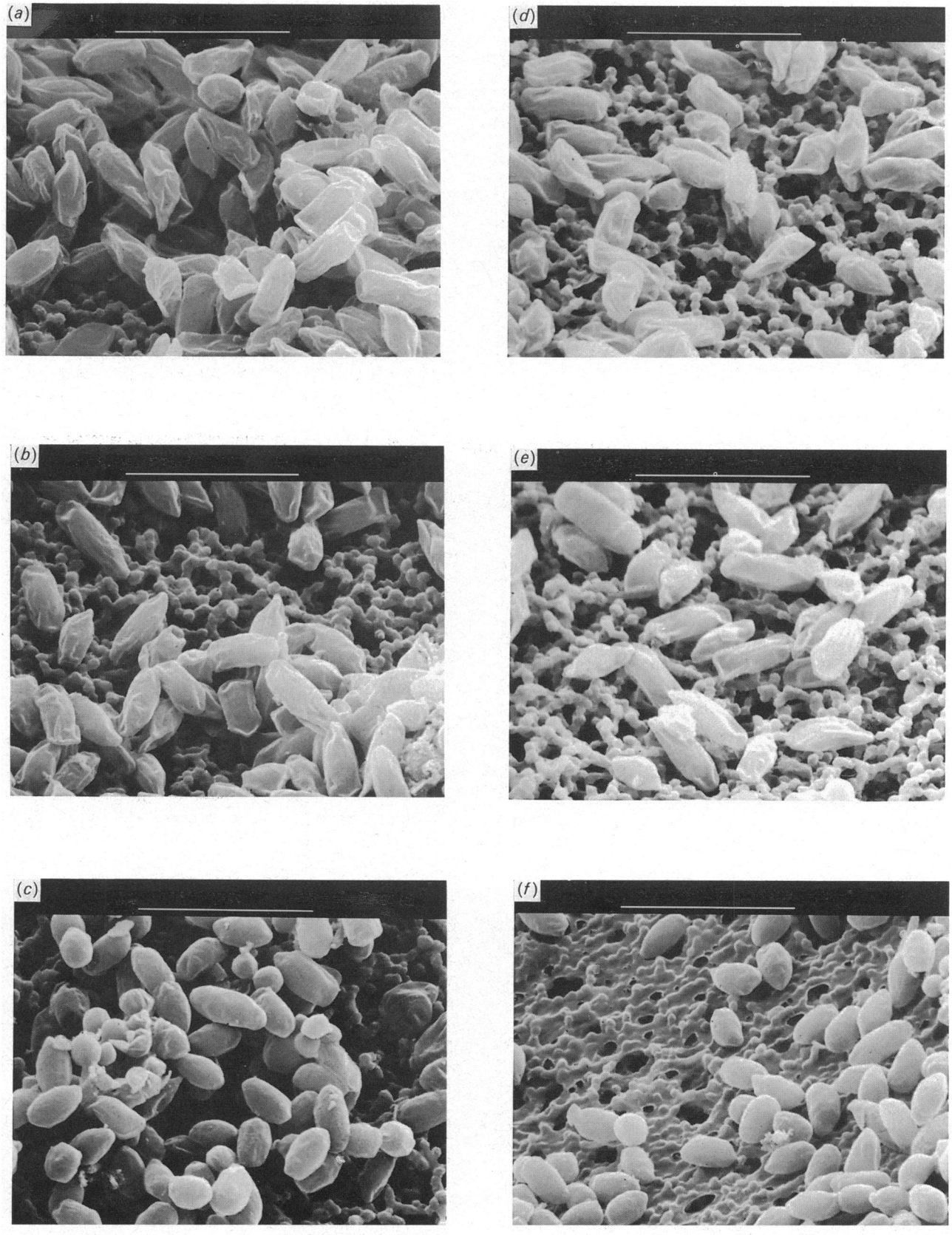


Fig. 2. S.e.m. of native (*a*,*b*,*c*) and heated (*d*,*e*,*f*) *B. cereus* spores

(*a*,*d*), BC20; (*b*,*e*), BC30; (*c*,*f*), BC40. Spore images (*d*,*e*,*f*) correspond to suspensions that had been autoclaved for 80 min at 121.1 °C. The bars represent 5 μm.

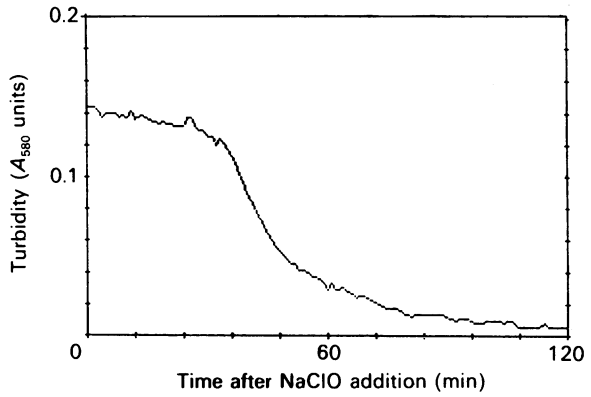
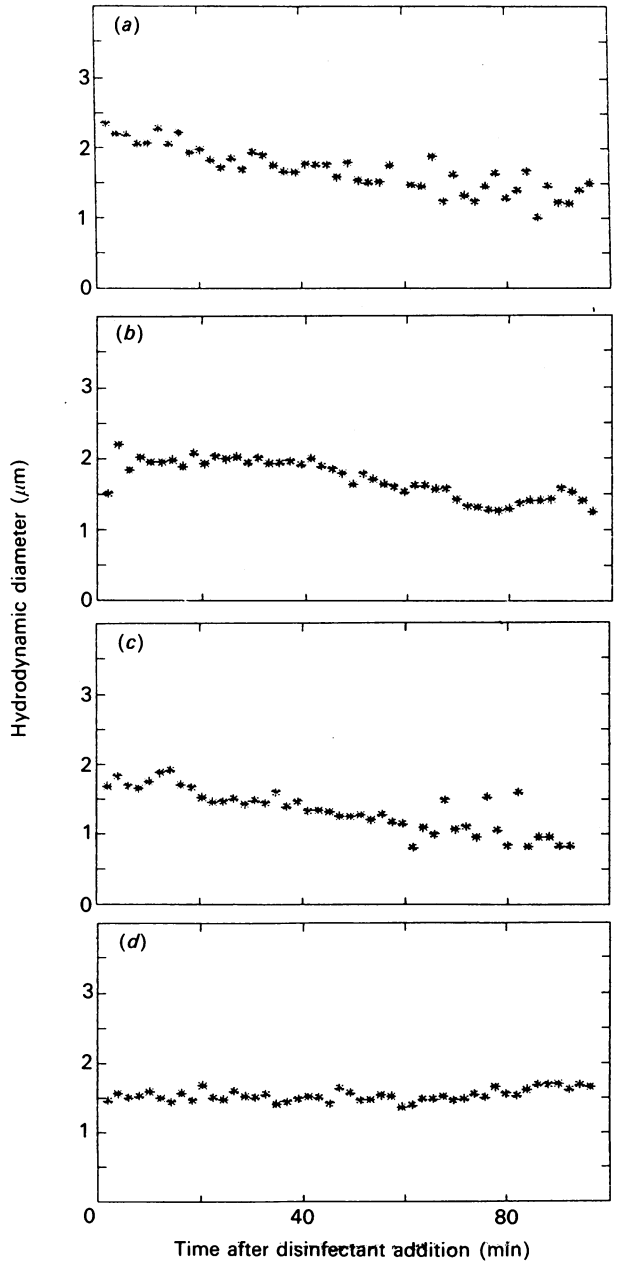


Fig. 3. Turbidity at 580 nm as a function of time after addition of sodium hypochlorite (final concn. 1.2%, w/v) to *Bacillus cereus* BC30 spores

The spore concentration was $\approx 4.0 \times 10^7$ particles/ml.



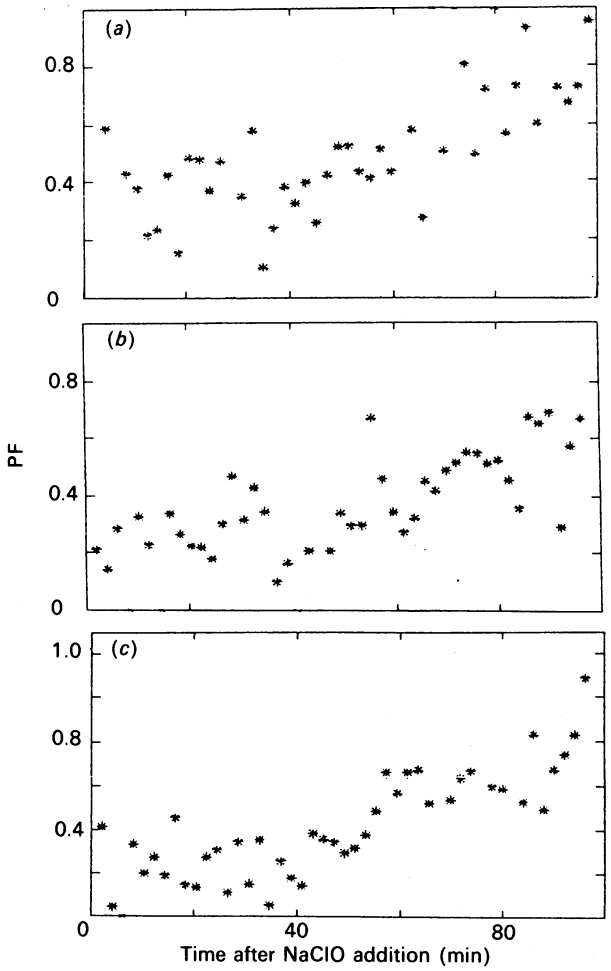


Fig. 5. PF (from q.l.s.) as a function of time after addition of sodium hypochlorite (final concn. 1.2%, w/v)

(*a*) BC20; (*b*) BC30; (*c*) BC40. Other details were as for Fig. 4.

showed a steady decrease (of approximately a first-order nature) after an apparent lag period (Fig. 3). Cuvettes were removed, inverted two or three times and replaced at periodic intervals to check for sedimentation, which was not found to be significant within these time periods. After 4 h the turbidity of the suspensions was totally lost, suggesting complete destruction.

The hydrodynamic diameter of the particles also showed a steady decrease on addition of NaClO, as monitored by q.l.s. (Figs. 4*a* and 4*b*), at least in the first 100 min. However, if peracetic acid was used instead, no change in the hydrodynamic diameter was observed (see, e.g., Fig. 4*d* for BC40; similar results were obtained for BC20 and BC30).

The loss in turbidity of the spore suspension is an

Fig. 4. Hydrodynamic diameter (from q.l.s.) as a function of time after addition of sodium hypochlorite (final concn. 1.2%, w/v) (*a*, *b* and *c*) or peracetic acid (final concn. 3%, (w/v) (*d*)

(*a*) BC20; (*b*) BC30; (*c*) and (*d*) BC40. The scattering angle was 90°, the temperature was 25.0 °C, the initial spore concentration was 4.0×10^7 particles/ml, an He/Ne laser ($\lambda = 632.8$ nm, 25 mW) was used, and the sample time was 200 μs.

Table 2. Effect of autoclaving (121.1 °C) *B. cereus* spores on their physical parameters

Culture...		BC20			BC30			BC40		
Temp. (°C)...		20			30			40		
Autoclave time (min)		$10^9 \times D_z$ ($cm^2 \cdot s^{-1}$)	PF	r_H (μm)	$10^9 \times D_z$ ($cm^2 \cdot s^{-1}$)	PF	r_H (μm)	$10^9 \times D_z$ ($cm^2 \cdot s^{-1}$)	PF	r_H (μm)
0		2.7	0.4	0.91	2.9	0.6	0.85	3.6	0.4	0.68
20		2.4	0.4	1.0	2.8	0.5	0.88	5.1	0.7	0.48
60		2.1	0.8	1.2	2.3	0.7	1.1	–	–	–
80		2.1	1.1	1.2	2.1	0.6	1.2	–	–	–

indication of either a decrease in the numbers of intact spores or, perhaps more likely, loss of cellular material, since turbidity is generally a more sensitive function of spore mass rather than number (Koch, 1961; see also Harding, 1986). Loss of mass is consistent with the fall in hydrodynamic diameter observed using q.l.s.

From these results it is evident that the BC40 *B. cereus* spores, which were produced under conditions identical with those used for the others (BC20 and BC30) but by growth at 40 °C, appear smaller and less resistant to heat than the others. Such a result confirms earlier work suggesting that variation in sporulation conditions changes the size and resistance of spores (Waites *et al.*, 1979).

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