IRCS Medical Science: Biochemistry; Biomedical Technology; Cell and Membrane Biology; Environmental Biology and Medicine; Metabolism and Nutrition; Microbiology, Parasitology and Infectious Diseases. [*IRCS Med. Sci.*, **12**, 200-201 (1984)]

Photon correlation spectroscopy as a probe for bacterial spore germination

Stephen E. Harding and Paley Johnson

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England, UK

Paper received: 9th January, 1984; amended 13th February, 1984

Several different theories have been put forward to explain the stability of the dormant spore, its formation and its germination. On the one hand, Lewis, Snell and Burr (1) have proposed that contraction of the peptidoglycan cortex is responsible for the dehydration of the protoplast, whilst Gould and Dring (2) believe that this occurs in response to the high osmotic activity of the cortex and its expansion brought about by the presence of many unshielded acidic groups. On the latter view, germination occurs when cross-linking of the acidic groups occurs, leading to loss of osmotic activity and contraction of the cortex. Ellar (3) however believes that dehydration of the protoplast occurred at an earlier stage by osmosis to the mother cell and that the rigid cortex merely maintains the dehydrated state. In his view germination occurs on the activation of a hydrolytic enzyme which attacks the peptidoglycan.

Evidence for a contraction in the early stages of germination of *B. cereus* spores was provided by Izard and Willis, and reported by Murrell (4). These results were based upon packed cell volume measurements taken when germination was arrested at various times (including a few seconds) by mercuric chloride addition. For times up to 8 s, a contraction of up to 20% was reported, followed by expansion to and beyond the original volume. Details of the mixing technique by which the germinant (L-alanine) or the mercuric chloride was added were not provided. In both cases, unless special mixing devices were used, it would be expected that complete mixing itself would require at least a few seconds so that readings taken of almost any property at around these times might be erratic. There is evidence from microscopy of an increase in the breadth of the spores on germination after 30 min (5), but no contraction at shorter times.

Materials and methods: To avoid the problems of very short times, a slower germinating organism, *B. subtilis,* has been studied at 35°C in degassed 18 mM Tris-20 mM KCl buffer, at pH 8.2. Addition of germinant (L-alanine at 35°C in the same solvent) was performed either by a calibrated "pipetteman" pipette followed by rapid aspiration and expiration, or by means of a plunger device carrying the small volume of alanine solution, which also produced rapid mixing. In both cases, reliable readings could be taken 1 min after germination. The properties of stable spores and the progress of germination were studied by photon correlation spectroscopy (PCS) by which the apparent translational diffusion coefficient (D_T) and therefore the equivalent Stokes particle radius (r), could be determined: $r = (kT)/(6\pi\eta D_T)$ where k is the Boltzmann constant, η the solvent viscosity and T the absolute temperature. Spores scatter light very strongly so that ultrafiltration of suspensions was unnecessary, and at a concentration of 6×10^7 particles/ml, sufficient photon counts were recorded in about 50 s to allow an accurate D_T value to be evaluated.

B. subtilis spores approximate prolate ellipsoids of revolution of dimensions 1.2×0.6 μm so that rotational as well as translational motion might be expected to contribute to the correlation function. However, Chen *et al.* (6) have shown that for such a particle size range, rotational motion is so slow as to be negligible, particularly at high scattering angles. High scattering angles are also desirable so as to minimize the effects of any possible traces of dust (7). Accordingly, a scattering angle of 90° was used and diffusion coefficients were obtained from a plot of $\ln(g^{(2)}(t) - 1)$ vs channel number (b), where $g^{(2)}(t)$ is the normalized intensity correlation function and b is related to time, t by $t = b\tau$, τ being the chosen sample time. The basic equation may be written (8) $\ln[g^{(2)}(t) - 1]^{1/2} = -D_T k^2 t$ where $k = [4\pi n/\lambda_0]\sin(\theta/2)$, n being the refractive index of the solution, λ_0 the wavelength *in vacuo*, and θ the scattering angle. A He-Ne laser was used, with $\lambda_0 = 632.8$ nm. Figure 1 shows a typical plot for dormant spores.

Results and discussion: For the dormant spore, D_T was $(5.10 \pm 0.09) \times 10^{-9}$ cm^{2}sec^{-1}. This correponds to a Stokes radius of 0.59 μm consistent with the dimensions as visualized from optical and electron microscopy (5, 9, 10). After the addition of the germinant (L-alanine) little if any change in the diffusion coefficient (to 4.91 ± 0.10) was manifested during the next 120 min (Figure 2) by which time observation by phase contrast microscopy and optical density measurements confirmed that 99% of the spores had germinated (phase dark). The averaged Stokes radius had risen slowly from 0.59 μm (dormant) to only 0.61 μm after 80 min. A decrease in volume of $\sim$ 20% would correspond to an increase in diffusion coefficient of $\sim$ 7%: this appears to be excluded for the case studied. Optical density measurements

performed in parallel with the PCS revealed a 10 min lag period before a fall in absorbance (with first order characteristics) from 0.45 to 0.25 over the 120 min period: D_T measurements extended well into this lag period. Figure 2 contains points (×) for which ultrasonication of a suspension was performed before the addition of germinant; microscopy confirmed the absence of aggregates in this case and germination proceeded normally.

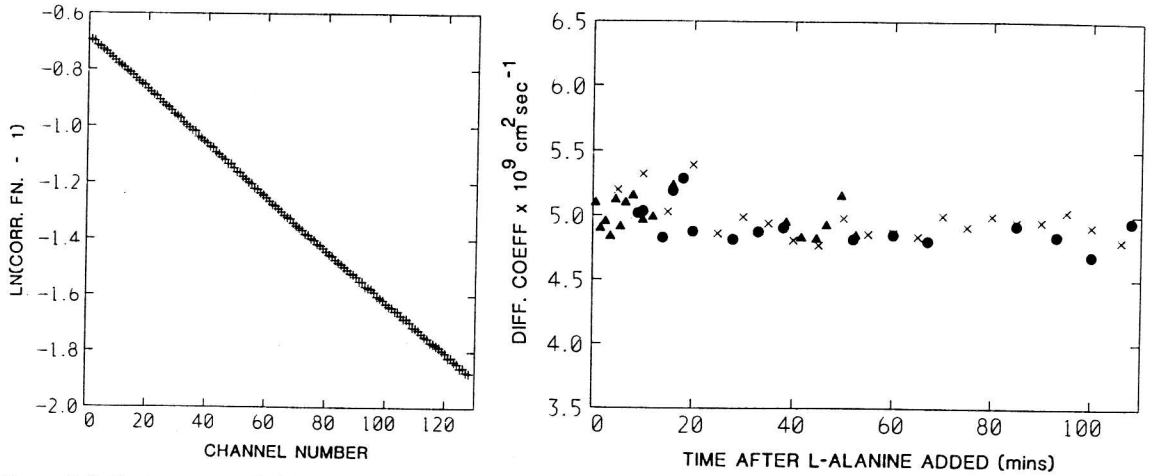

Figure 1 (left): Plot of the $\ln[g^{(2)}(t)-1]$, *where* $g^{(2)}(t)$ *is the normalised intensity autocorrelation function, versus channel number for dormant* B. subtilis *spores. A Malvern multibit correlator (K7025) at a sample time of 25* μs *was used. The scattered light was examined at an angle of 90°. The* B. subtilis *spores, at a particle concentration of* 6×10^7/*ml were incubated at 35 °C in degassed 18 mM Tris- 20 mM KCl buffer, pH = 8.2. Figure 2 (right): Plot of the z-average apparent diffusion coefficient* D_T *measured at an angle of 90° as a function of time after the addition of germinant (L-alanine diluted to 20 mM). Spore concentration =* 6×10^7*/ml; temperature = 35 °C; buffer as above;* ● *and* ▲ *fresh preparations, mixing by pipette;* × *ultrasonicated preparation, mixing by plunger. All solutions, plungers and pipettes were equilibrated at the temperature of the experiment (35 °C) so that thermal re-equilibration upon mixing was not involved. Control experiments on the mixing procedures revealed no disturbance to the results after a 1 min period.*

Thus a cortex contraction at short times cannot be confirmed for this spore. It is conceivable that a volume decrease of 6% (equivalent to an increase in D of ~ 2%) or less occurred or even that a larger change (later reversed) occurred within the first minute after addition of germinant. More rapid mixing and more accurate detection methods would be required to investigate such possibilities. The fall off in absorbance at almost constant volume suggests loss of cellular material, lowered refractive index and scattering power during the germination process, consistent with the observations of others (11, 12).

1. Lewis, J.C., Snell, N.S. and Burr, H.K. (1960) *Science,* **132**, 544-545
2. Gould, G.W. and Dring, G.J. (1975) *Nature,* **258**, 402-405
3. Ellar, D.J. (1978) *Symp. Soc. Gen. Microbiol.,* **28**, 295-325
4. Murrell, W.G. (1980) *Proceedings of the International Spore Conference: Sporulation and Germination,* Vol. 8, pp. 64-77, American Society of Microbiology, Washington D.C.
5. Gould, G.W. (1969) in *The Bacterial Spore,* (Gould, G.W. and Hurst, A., eds), p. 397, Academic Press, New York
6. Chen, S.H. *et al.* (1977) *Appl. Opt.,* **16**, 187-194
7. Godfrey, R.E., Johnson, P. and Stanley, C.J. (1982) in *Biomedical Applications of Laser Light Scattering,* (Satelle, D.B., Lee, W.I. and Ware, B.R., eds), pp. 373-389, Elsevier Biomedical Press, Amsterdam
8. Pusey, P. (1974) in *Photon Correlation and Light Beating Spectroscopy,* (Cummins, H.Z. and Pike, E.R., eds), p. 387, Plenum Press, New York
9. Robinow, C.F. (1951) *J. Gen. Microbiol.,* **5**, 439-457
10. Santo, L.Y. and Doi, R.H. (1974) *J. Bacteriol.,* **120**, 475-483
11. Scott, I.R. and Ellar, D.J. (1978) *Biochem. J.,* **174**, 627-635
12. Johnstone, K. and Ellar, D.J. (1982) *Biochim. Biophys. Acta,* **714**, 185-191