Biochem. J. (1984) **220**, 117–123
Printed in Great Britain

Quasi-elastic light scattering studies on dormant and germinating *Bacillus subtilis* spores

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(*Received 1 December 1983/Accepted 31 January 1984*)

Spores of *Bacillus subtilis* in suspension, both dormant and germinating, have been examined by light-scattering methods, both integrated intensity and correlation versions. When intensity of scatter at constant volume was plotted against angle, curves possessing a maximum at about 20° were regularly obtained but without any noticeable features at higher angles. This indicated polydispersity and/or asymmetry among the population. Curves of the intensity correlation function for both dormant and germinating spores at different angles, θ, did not superimpose at the lower angles when plotted appropriately, but did so for $\theta > 35°$. This was considered to arise from asymmetry of the spores. By using the high-angle data the apparent diffusion coefficient was determined for both dormant spores and for germinating spores from 1 min after germinant addition. No appreciable difference was observed, from which volume changes greater than 6% during germination could be excluded. The occurrence of germination was confirmed by both absorbance and phase-contrast-microscopy observations.

The structural changes which occur during the germination of bacterial spores are of great interest to the bacteriologist. Knowledge of these changes is of great relevance in attempting to explain the dormant state. It seems generally agreed that in a spore the protoplast is in a dehydrated state, but opposing theories have been proposed in explanation. On the one hand, Lewis *et al.* (1965) have suggested that contraction of the cortex is responsible; on the other, Gould & Dring (1975) have suggested that the peptidoglycan is rather in an expanded state due to repulsion of uncrosslinked acidic groups. This structure is osmotically highly active and dehydration of the protoplast occurs as a result (see also Algie, 1980). Finally, Ellar (1978) has proposed that dehydration through osmosis from the spore to the mother cell occurs throughout sporulation and the rigid cortex acts merely to maintain the dehydrated state.

Germination can be triggered either chemically (using 'germinants', e.g. L-alanine) or physically (e.g. by pressure). Gould & Dring (1975) have proposed that the initial triggering event involves the contraction of the expanded cortex after cross-linking of the peptidoglycan acid groups via Ca^{2+} ions released by the protoplasm. The outer membrane becomes simultaneously permeable to the displaced counterions which are excreted. The osmotic pressure of the cortex would be vastly reduced and water would be able to enter the protoplast. On the other hand, hydrolytic products of the cortex peptidoglycan have been detected (Scott & Ellar, 1978; Johnstone & Ellar, 1982) minutes after the triggering event: the problem is whether this is preceded either by a contraction of the cortex (Gould & Dring, 1975) or by the activation of a hydrolytic enzyme whose target is the cortex peptidoglycan. It is conceivable that inflow of water to the protoplast causes activation of an enzyme. The most direct evidence for a contraction appears to be some unpublished data of M. Izard & P. A. Willis quoted in a review article by Murrell (1980): a reduction in the packed cell volume of *Bacillus cereus* spores of up to 20% is reported for approx. 8s after adding the germinant. However, the details of this technique are not reported and it is not clear how the disturbing effects of incomplete mixing at short times are avoided. Further, the mercuric chloride used to arrest germination could be responsible for other effects. Gould (1969) also, on the basis of phase contrast and light field photomicrographs, reported an increase in the breadth of the spores after 30min but no contraction at shorter times. His observations did not, however, extend to times as short as a few seconds.

In this study we have re-examined the problem

using the technique of quasi-elastic laser light scattering on spore suspensions; a change in volume of the spores could manifest itself as a change in translational diffusion coefficient. To facilitate such observations, the germination has been slowed down some 10-fold by choice of organism and conditions. Further, consideration has been given to accomplishing rapid mixing and to the reliability of measurements at relatively short times after mixing.

Materials and methods

Bacterial spores

Bacillus subtilis spores (a generous gift from Dr. G. J. Dring, Unilever Research Laboratories, Sharnbrook, Beds., U.K.) were chosen as most suitable because (i) they have no exosporia, (ii) they germinate relatively slowly and (iii) they are spheroidal, of low asymmetry. Native spores were incubated in 0.03M-HCl at 0°C for 45min. They were then washed several times by centrifugation at 2000*g* and resuspension in distilled water. The purified spores were finally resuspended in 20mM-KCl/18mM-Tris/HCl buffer, pH8.2. The same buffer was used throughout this work. The germinant used was L-alanine. A stock solution of 1.0M-L-alanine in the same solvent was diluted to 20mM to initiate germination. All solvents used, including those used for resuspending the spores after centrifugation, had been degassed to reduce the tendency of the spores to aggregate (D. J. Ellar, personal communication).

Quasi-elastic light scattering

The apparatus has been previously described (Godfrey *et al.*, 1982). A 15mW beam from a He–Ne laser (Spectra Physics) of wavelength (λ) 632.8nm was focused onto the centre of a 1cm square fluorimeter cuvette. The cuvette was placed at the centre of a goniometer so that the scattering angle θ could be varied from 3 to 120°. Stray light was reduced by a system of 'baffles' which screened off the path of the incident and emergent beams from the collecting system. The scattered light was collected (homodyne detection) by well-collimated slits to reach an EMI 9863 photomultiplier. Output from the multiplier was led to a Malvern Amplifier Discriminator (RR63) which gave output pulses of width 30ns and −2V amplitude, suitable for processing in a 128-channel Malvern Correlator (K7025) and for display of the un-normalized correlation function products against channel number (or time) on an oscilloscope. Digital output from the correlator was directed to the memory in a BBC microcomputer which could store 10 runs. These data were then passed on to the main university IBM/370/165 computer for processing. The routine used produced an accurate plot of $\ln[g^{(2)}(\tau)-1]$ against time where $g^{(2)}(\tau)$ 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' (Pusey, 1974), which is merely the normalized z-averaged variance of the distribution diffusion coefficients. A guide to the best fit was provided by the ε function (see, e.g., Teller, 1973, p. 375).

Integrated light scattering

Conventional light scattering measurements (integrated over time) were also performed on dormant spore suspensions with essentially the same apparatus but with a PSL (air-cooled; model 82) argon ion laser at 24mW as the light source. This laser had been fitted with an intensity feedback mechanism so that the intensity was constant to $\pm 2.5\%$ within the longer periods required for scanning over a wide range of angles (3–120°). The prism was adjusted to a wavelength of 488.0nm.

The total number of counts, as recorded using the correlator, for time intervals of 20s was averaged over several measurements for each angle. Measurements were made repeatedly for particular angles to confirm that within the limits of experimental error there was negligible fall-off in intensity caused by any settling of the spores during the time course of the full angular scan. Settling was only observed over much longer time periods ($\geqslant$12h).

Spectrophotometry

Absorbance measurements for spore concentration determinations were performed at a wavelength of 580.0nm in a single-beam Pye–Unicam SP.500 spectrophotometer. Germination experiments at 35°C were followed in a double beam Pye–Unicam SP.800b spectrophotometer. With a wavelength of 580.0nm, the absorbance was plotted against time on a Servoscribe chart recorder. Absorption measurements were also recorded over the range 325–700nm.

Mixing procedures for the germination experiments

Since observations within the first few minutes after mixing are of importance, it was desirable to perform the mixing rapidly and to ensure that it was complete. All solutions, pipettes, etc. were equilibrated at the temperature of the experiment (35°C) so that thermal equilibration was not involved. Two methods of mixing were used, both performed with appropriate controls using the normal suspending buffer. The first method was to add the small volume of germinant (approx. 0.03ml) solution rapidly to the suspension in the

cell from a 'Pipetteman' and then to mix the contents by rapidly aspirating and expelling with a pipette. The second method was to use a rapid mixing device constructed from a small square of perspex (P in Fig. 1), loosely fitting into the fluorimeter cell (C). Conical holes were drilled in the upper surface by tapered drills of a diameter determined by the volume of liquid to be held in them and each communicated with the liquid below through a small cylindrical hole of 0.05cm diameter. A short rod of perspex or plastic covered stainless steel syringe tubing was used as a handle (H) to plunge the disc once into and out of the spore suspension. This caused homogenization as judged by observation of the mixing of a small quantity of highly coloured solution in a time which could be as low as a few seconds. This latter method was used for the germination experiment followed in the quasi-elastic light scattering apparatus and reported in Fig. 6.

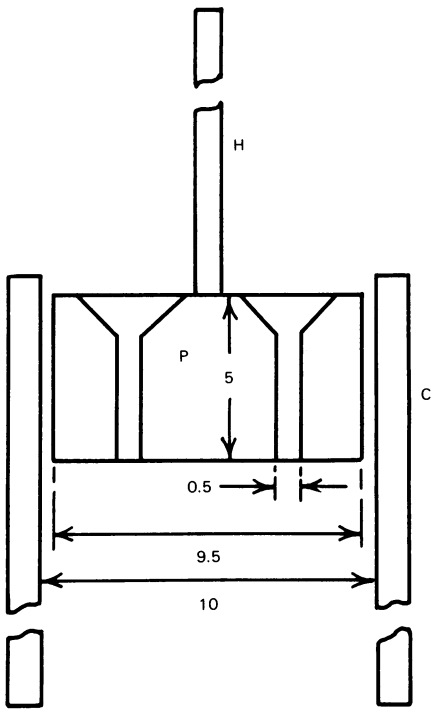

Fig. 1. *Rapid mixing plunger device used for introducing the germinant (cf. run 3 of Fig. 6)*
All dimensions are in mm.

Control experiments with both methods were performed, in which the spore suspensions were mixed with solvent. In both cases irregular correlation curves were obtained immediately after mixing, probably a result of residual convection in the cell. However after 30s such disturbances were no longer evident and quite normal results were obtained after 1 min.

Ultrasonication

A Dawe 500W sonicator incorporating a 38.73kHz transducer at the base of a water tank was used for times of up to 60s.

Results and discussion

Dormant spores

As expected, the spore suspensions proved to be strongly scattering, and highly reproducible curves of the intensity correlation function against channel number (or time) were obtained for angles ranging from 5 to 90°. Typical curves are shown in Fig. 2. While the curve at 90° appears to be the

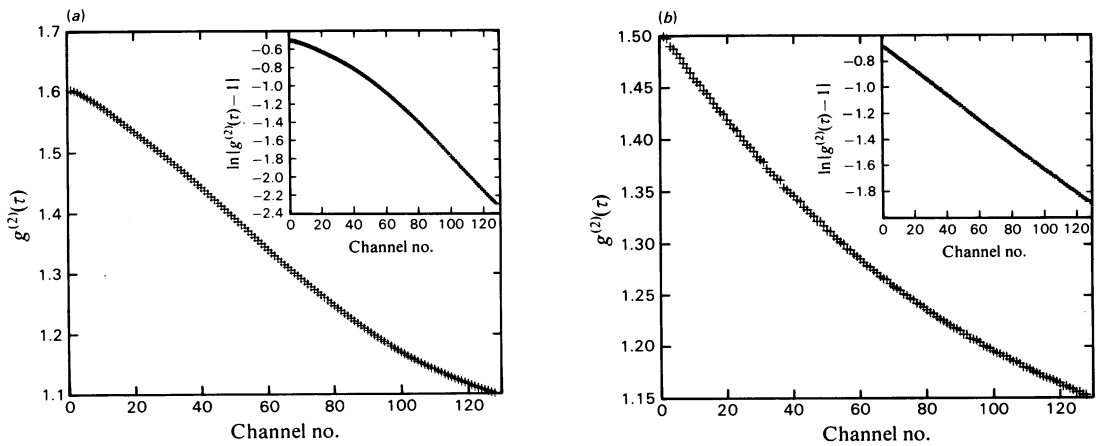

Fig. 2. *Plot of the normalized autocorrelation function, $g^{(2)}(\tau)$, as a function of channel number*
(*a*) $\theta = 5°$, sample time = 1.50ms; (*b*) $\theta = 90°$, sample time = 0.025ms. The temperature was 34.6°C and the spore concentration was 6×10^7/ml. Insets show the corresponding logarithmic plots.

expected exponential decay for Brownian motion, the appearance at low angles of opposite curvature at low channel numbers indicates the presence of other features. Directed motion by the spores themselves can be ruled out, as was confirmed by phase contrast microscopy. Furthermore, the possibility of directed motion in the cell arising from sedimentation under gravity and resulting convection (see Godfrey *et al.*, 1982) was considered but also ruled out as the spores did not settle out significantly.

Fig. 3 illustrates an attempt to superimpose plots of the normalized autocorrelation function for several angles plotted as a function of q^2t. Here, q, the Bragg wave vector, is defined by:

$$q = \frac{4\pi n}{\lambda_0} \cdot \sin(\theta/2) \qquad (1)$$

where n is the refractive index of the solvent, λ_0 the wavelength *in vacuo* (cm), and t the time in seconds. These curves should superimpose upon one another, i.e. 'scale' for spherical isotropic scattering particles (Chen *et al.*, 1977), even if their dimensions are comparable with the wavelength of the incident radiation. The curves of Fig. 3 clearly do not, and in fact show an oscillatory behaviour with changing scattering angle, particularly at low angles. From optical (Gould, 1969) and electron (Robinow, 1951; Santo & Doi, 1974) microscopy the spores are estimated to be prolate ellipsoids of dimensions $1.2\mu m \times 0.6\mu m$, and it would seem that the 'non-scaling' property may be ascribed to their departure from spherical symmetry (or the presence of significant polydispersity or aggregates; see below). Chen *et al.* (1977) demonstrated this property theoretically in a simplified case for particles of similar dimensions to the spores. It was further pointed out that the ratio of the relaxation time for rotation to that for translation is given approximately by $(qd)^2$, d being a characteristic dimension of the scattering particles. Putting $d = 1.2\mu m$ and $\theta = 90°$, $(qd)^2 = 260$. Thus the orientation of a spore remains apparently constant during the relaxation time for translation and this will remain true for those angles for which $(qd)^2 \gg 1$. This would account for the near superposition of the curves for 90°, 60°, and 35° [for the latter, $(qd)^2 = 46.5$]. However, for lower angles the oscillatory behaviour is marked.

At high angles, therefore, the rotational motion of the spores merely provides a flat background for the translational decay, and it is therefore permissible to derive an apparent diffusion coefficient, $D_{Z(app.)}$, by a plot of $\ln[g^{(2)}(\tau) - 1]$ versus time (t). This was performed for several runs on dormant spores and the mean value obtained was $(5.10 \pm 0.09) \times 10^{-9} cm^2 \cdot s^{-1}$ at 35°C in the KCl/Tris buffer at pH 8.2 (corrected to water at 25°C this becomes $4.2 \times 10^{-9} cm^2 \cdot s^{-1}$). The corresponding "Stokes' radius" is $0.59\mu m$. No significant change occurred with ultrasonication of the spores suspension.

The polydispersity factor was 0.10 ± 0.05, indicating significant polydispersity whether from variability among single spore particles or the presence of aggregates. No detectable improvement occurred on sonication, however. Further, a plot of intensity versus scattering angle, θ, normalized to $\theta = 27°$ showed no features other than the maximum below 20° (Fig. 4). Wyatt (1972, 1973) has shown the presence of highly specific maxima

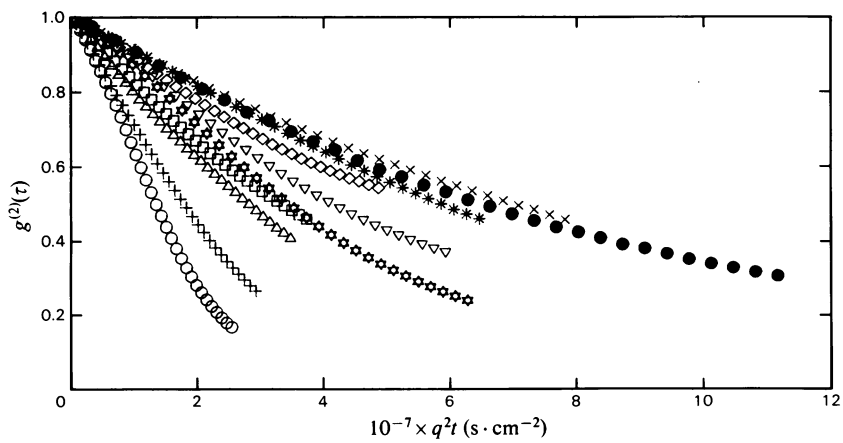

Fig. 3. *Plot of $g^{(2)}(\tau)$ against q^2 t for different angles*
Spore concentration was 6×10^7/ml; temperature was 35.0°C. ○, 5°; +, 6°; △, 8°; □, 10°; ◇, 15°; ▽, 20°; *, 35°; ✱, 25°; ×, 60°; ●, 90°.

and minima for single spores of *B. sphaericus*: for spore suspensions he has demonstrated by simulation the effect of different degrees of polydispersity on such curves. He defined a '% distribution' as the full width at half maximum divided by the average size, × 100. A factor of approx. 50–70% would be required to produce a curve of similar structure to our observed curve for *B. subtilis* (Fig. 4). It should be noted, however, that Wyatt's calculations were made on the basis of spherical particles of structure similar to *Staphylococcus aureus*. Clearly *B. subtilis* spores are asymmetric with higher and probably non-uniform refractive index, for which the Rayleigh–Gans–Debye approximation:

$$\frac{4\pi nd}{\lambda_0}\left(\frac{n}{n_0}-1\right) \ll 1$$

is not valid. Asano (1979) and Barber (Barber, 1973; Wang *et al.*, 1979; Barber & Massoudi, 1982) have provided theories for non-spherical particles based on the general Lorenz–Mie theory. It is apparent that asymmetry can also contribute to the damping of the detailed features in the intensity profiles (see, for example, Fig. 7 of Wang *et al.*, 1979). Another contribution to the absence of any observed secondary maxima or minima could arise from the presence of aggregates, although ultrasonicated dispersions gave virtually identical curves to those of Fig. 4.

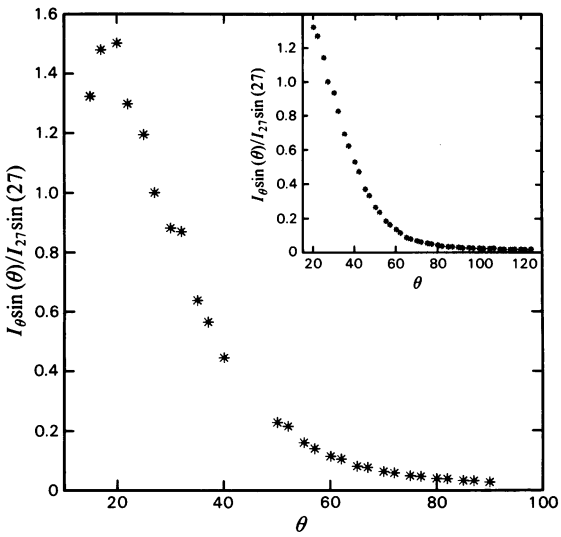

Fig. 4. *Ratio of the intensity of light scattered at a particular angle* (θ) *to that at* $\theta = 27°$, *as a function of* θ, *all corrected to constant volume by the factor* $\sin(\theta)$
$\lambda = 488.0$nm; a square fluorimeter cell was used. The missing points at 40–50° correspond to a corner of the curvette. Inset: corresponding plot down to $\theta = 20°$, but with a cylindrical cell.

Germinating spores

The addition of L-alanine to a suspension of 6×10^7 spores/ml in KCl/Tris buffer (pH 8.2) at 35°C gave, after a short lag period (approx. 10 min), a fall in absorbance at 580 nm which continued for some 120 min (Fig. 5). A plot in the first-order fashion (inset) confirmed first-order characteristics. To the spore suspension, taken after 120 min germination time, an equal volume of 40% formalin was added prior to examination by phase contrast microscopy. The almost completely germinated (phase dark) condition was thus confirmed. When the correlation function for com-

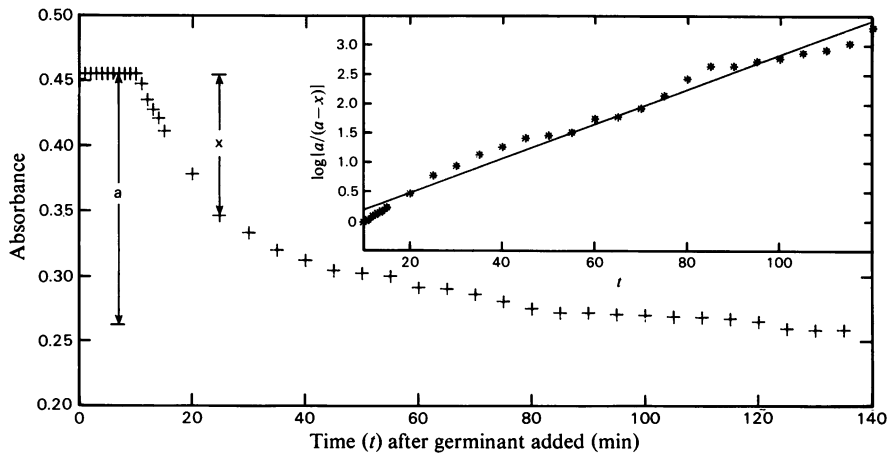

Fig. 5. *Plot of absorbance at 580nm as a function of time after addition of* L-*alanine to 0.02*M
Temperature was 35.0°C, spore concentration was 6×10^7/ml. Inset: first-order reaction plot; rate constant, k, $= 0.029\,s^{-1}$.

pletely germinated spores was plotted againt q^2t at several angles, a set of non-superimposing curves, very similar to those for dormant spores, was obtained. Thus it seems that on germination the asymmetry of the spores is not removed, though as other workers (e.g. Gould, 1969) have suggested, it may be decreased.

Autocorrelation curves at $\theta = 90°$ were also used to investigate possible changes in the apparent diffusion coefficient with time after the addition of L-alanine. Fig. 6, containing results for three different experiments, including an ultrasonicated sample (run 3), demonstrates the absence of any substantial change. Run 1 was performed simultaneously with, and from the same stock suspension as, the absorbance measurements of Fig. 5.

The mean of all the $D_{Z(app.)}$ values was $(4.97 \pm 0.15) \times 10^{-9} cm^2 \cdot s^{-1}$; the mean after 80min was $(4.91 \pm 0.10) \times 10^{-9} cm^2 \cdot s^{-1}$. Thus only a slight increase in the Stokes' radius (from 0.59 to 0.61 μm) is indicated during germination. The experimental points cover the same time scale as the absorbance measurements of Fig. 5, the earliest readings (at 1 min) extending well into the 'lag' period. Whatever mixing device was used, the same $D_{Z(app.)}$ versus time behaviour was observed over the whole range of times investigated. A decrease in volume of approx. 20% as suggested by Murrell (1980) would be expected to cause an increase in the diffusion coefficient of approx. 7%; this seems to be excluded for the case studied.

It still remains to interpret the fall in absorbance on germination. Electronic transitions normally associated with absorption in the visible are not involved, but rather the summed light scattering in all directions which depletes the transmitted intensity. Nor, in view of their dimensions, can the spores be treated as Rayleigh-type scatterers: thus a plot of log (absorbance) against log λ (Fig. 7) gives a linear plot of slope approx. -0.9 (rather than -4). Jobst (1925) suggested an alternative empirically based equation (see also Sinclair, 1947) for larger particles of restricted refractive index, which Koch (1961) wrote in the form:

$$\text{absorbance} = \text{const.} \left(\frac{dn/dc}{n_0} \right)^2 \cdot \frac{q^2 v}{V^{2/3} \lambda^2} \qquad (2)$$

Here v is the number of particles/ml, q is the anhydrous mass (g/ml), V their volume (ml) and λ the wavelength in the solvent (cm). It seems unlikely that eqn. (2) is obeyed closely by spores, but it is probably a reasonable approximation. If so, the fall in absorbance at almost constant V would suggest a decrease in q, i.e. an expulsion of cellular material during germination, in line with the conclusions of other workers (e.g. Scott & Ellar, 1978; Johnstone & Ellar, 1982).

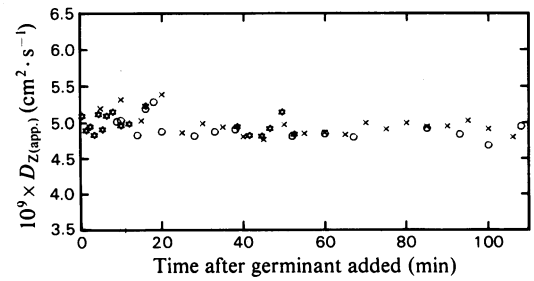

Fig. 6. *Plot of z-average apparent diffusion coefficient $D_{Z(app.)}$ at an angle, θ, of 90°, as a function of time after the addition of germinant*

Spore concentration was 6×10^7/ml; temperature was 35.0°C; buffer was KCl/Tris, pH 8.2. Runs 1 (○) and 2 (☆) were fresh preparations; mixing was by pipette. Run 3 (×) was an ultrasonicated preparation; mixing was by plunger.

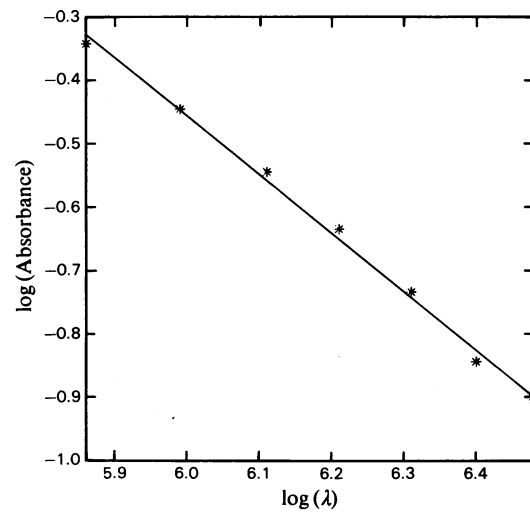

Fig. 7. *Plot of the logarithm of the absorbance versus the logarithm of the wavelength*

The straight line fitted corresponds to a slope of -0.89.

We gratefully acknowledge the help we have received from the preliminary experiments carried out by Miss D. Rees and the gift of spores from Professor G. W. Gould and Dr. G. J. Dring of Unilever Ltd., who, with Dr. M. P. Tombs, also advised us on many occasions. Dr. D. J. Ellar also gave very valuable advice, particularly regarding methods of spore handling. S. E. H. is grateful to the Oppenheimer Foundation for financial assistance during this study.

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