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# Macromolecular Sieving by the Dormant Spore of *Bacillus cereus*[1]

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The threshold surface porosity in the dormant spore of *Bacillus cereus* strain T was assessed by measuring passive permeabilities to a series of polydisperse polyethylene glycol samples which increased in average molecular size. The apparent exclusion threshold at diffusional equilibrium corresponded to a polymer of number-average molecular weight ($\bar{M}_n$) = 150,000 and equivalent hydrodynamic radius ($\bar{r}_{ES}$) = 16 nm, which confirmed a previous report. However, analytical gel chromatography before and after uptake by the spores revealed that only the low molecular weight fractions in a polymer sample distribution were taken up. From graphical analyses of the changes in molecular weight distributions, a quasi-monodisperse exclusion threshold was determined corresponding to $M_n$ = 8,000 and $r_{ES}$ = 3.2 nm. Thus, the equivalent porosity in the limiting outer integument appeared much more restrictive than heretofore shown for spores, although still more open than the monodisperse equivalent for the cell wall of vegetative bacilli.

A new uptake-distribution method has been described by Scherrer and Gerhardt (14) for accurately determining the equivalent porosity threshold of bacterial cell integuments from their sieving properties for probing macromolecules. A polymer sample typically is not monodisperse, like a monomer or oligomer, but rather polydisperse, i.e., the molecular size fractions in the sample are distributed in amount around a mean, such as a Poisson distribution for polyethylene glycols (6). Consequently, the apparent uptake of a given polymer may reflect the sieving of only the smaller molecules in the distribution. From graphical analyses of changes in the molecular weight distribution of a sample before and after uptake, however, a quasi-monodisperse exclusion threshold, and consequently the equivalent porosity, is obtained (14). The results with this new method have indicated that the maximum radial dimension in porosity of the *Bacillus megaterium* cell wall is much more restrictive than previously estimated from determinations of the polydisperse exclusion threshold (9, 13).

Previous studies of molecular sieving by dormant bacterial spores have employed the latter determination, which is based on uptake measurements with a series of polydisperse samples of polymers increasing in molecular size (4, 7, 8). Eventually, the largest samples are completely excluded, and the end point is designated as the apparent exclusion threshold. The equivalent porosity in the effective surface of the *B. cereus* spore corresponds to a polydisperse dextran sample of number-average molecular weight ($\bar{M}_n$) = 160,000 and equivalent hydrodynamic radius ($\bar{r}_{ES}$) = 9 nm (8).

The present experiments were undertaken to reassess the surface porosity of this representative spore. The results confirmed the polydisperse exclusion threshold previously reported, but application of the uptake-distribution method revealed a much smaller quasi-monodisperse limit corresponding to $M_n$ = 8,000, $r_{ES}$ = 3.2 nm.

## MATERIALS AND METHODS

**Spores.** The terminalis (T) strain of *B. cereus* was grown in modified G medium, and the free spores were cleaned by differential centrifugation in 45% (w/w) sucrose solutions and repeated washing in distilled water, as described previously (12).

**Solutes.** All of the solutes used are listed in Table 1 with identification numbers. Suppliers of the polyethylene glycol samples are given in the footnotes to Table 1. Dextrans were purchased from Pharmacia Fine Chemicals, Inc. Solute concentrations were determined by refractometry (14) or gravimetrically (3) with equal results.

Determinations of $\bar{M}_n$ and computations of $\bar{r}_{ES}$ have been published previously (14).

**Refractometry.** An Abbé precision refractometer and a Brice-Phoenix recording differential refractometer

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TABLE 1. *Permeability of dormant spores of Bacillus cereus to polyethylene glycols of increasing size*

| Compound | | | | | Uptake determinations | | |
|---|---|---|---|---|---|---|---|
| Identification no. | Sample | Mol wt[c] | $M_n$ | $\bar{r}_{ES}$ (nm) | No. of determinations | $S^w \pm \sigma$ (%) | $R^w \pm \sigma$ (%) |
| | *Reference Substances* | | | | | | |
| 1 | Glucose | 180 | | 0.3 | 4 | 52.9 ± 6.8 | 43.0 ± 8.8 |
| 2 | Dextran 500 | | 200,000 | 14.0 | 12 | 17.3 ± 4.7 | 0 ± 4.7 |
| | *Polyglycols* | | | | | | |
| 3 | Polyethylene glycol 1,000[a] | | 1,000 | 1.0 | 4 | 48.5 ± 0.7 | 37.7 ± 1.5 |
| 4 | Polyethylene glycol 1,540[a] | | 1,450 | 1.2 | 9 | 43.5 ± 3.3 | 31.7 ± 4.2 |
| 5 | Polyethylene glycol 4,000[a] | | 3,350 | 1.9 | 4 | 41.5 ± 7.7 | 29.3 ± 8.9 |
| 6 | Polyethylene glycol 6,000[a] | | 8,000 | 3.2 | 6 | 34.9 ± 3.5 | 21.3 ± 4.4 |
| 7 | Polyethylene glycol 9,000[b] | | 9,500 | 3.6 | 4 | 35.5 ± 3.7 | 22.0 ± 4.5 |
| 8 | Polyethylene glycol 20,000[a] | | 17,500 | 4.9 | 6 | 31.8 ± 2.5 | 17.5 ± 3.9 |
| 9 | Polyethylene glycol 70,000[b] | | 70,000 | 12.0 | 4 | 19.3 ± 1.2 | 2.4 ± 1.9 |

[a] From Union Carbide Chemicals Co., New York (Carbowax series).
[b] From Dow Chemical Co., Midland, Mich. (Polyglycol E series).
[c] Molecular weight.

were employed. Their sensitivities and calibrations have been described previously (2, 14). Refractive indices ($n$) of the solutes in aqueous solutions were determined at 25 ± 0.02 C at 589-nm wavelength. At the concentrations used, the refractive index increment ($\Delta n$) was linearly proportional to the concentration of polyethylene glycols (12, 14).

**Solute uptake.** The space or thick-suspension technique was used to determine equilibrium solute uptake under conditions (e.g., high concentration of solute, 4 C) which nullify or minimize adsorptive phenomena (3, 8, 14). A sample of spore suspension was sedimented in a refrigerated centrifuge in 50-ml tubes. The firm spore pellet [about 3 g (wet weight)] was weighed after removing the supernatant fluid, a known amount of solute [usually 3 ml of 3% (w/v)] was added to the pellet and mixed thoroughly, and the suspension was allowed to reach uptake equilibrium at 4 C for 2 hr. Exposure of spores to solutes for 2 hr did not cause the release of ultraviolet-absorbing substances into the supernatant solutions, which could have influenced the refractive index determinations.

An uptake value based on the weight of the spores was calculated as follows: $S^w = (V_s/W_p)(C_0/C_f - 1) \times 100$, where $S^w$ is the weight-percentage uptake (space) value, $V_s$ is the solution volume added to the cells, $W_p$ is the pellet weight, and $C_0$ is the initial and $C_f$ the final solute concentration.

Because of the presence of interstitial water in the pellet, it was necessary to obtain a corrected weight-percentage uptake value ($R^w$) as follows: $R^w = (S_{sol}{}^w - S_{in}{}^w)/100 - S_{in}{}^w$. $S_{in}{}^w$ is the percentage space value for the interstitium measured with a polymer sample which does not penetrate into spores. $S_{sol}{}^w$ is the percentage uptake value for a penetrating solute. Therefore, $R^w = 0$ would indicate that the test solute was completely excluded from entering the spores, and $R^w > 0$ would mean that the test solute was taken up to a fractional extent.

**Gel chromatography.** Molecular weight distributions of the polyethylene glycols were studied by analytical gel permeation chromatography (1, 5). Polyacrylamide gels (Bio-Gel P series, 100 to 200 mesh) and agarose gel (Bio-Gel A-15 m, 100 to 200 mesh) were purchased from Bio-Rad Laboratories. The dry materials were hydrated in distilled water and poured into glass columns (1.5 by 30 cm) with a Teflon mesh support to obtain stable hydrophilic gels. The eluent (distilled water) was pumped through the gel columns at a constant flow rate and pressure head by means of a positive displacement micropump (Technicon Chromatography Corp.). The effluent was constantly monitored with a differential refractometer. A sequence of 2-ml fractions was collected with a fraction collector. The polymer solution (0.5 ml containing less than 15 mg of polymer) was injected at the top of the gel column by means of a sample injection valve (model 1 SV-8031, Chromatronix, Inc.). Column void volumes ($V_0$) were determined separately by use of Blue Dextran 2000 (5). The polymers eluted according to their molecular weights. For each gel type, a calibration curve was made by plotting the peak elution volume versus the logarithm of $M_n$ (1). From the calibration curves, the $M_n$ of each elution fraction was interpolated. The mass of polymer in each elution fraction was determined from its $\Delta n$ and the known specific refractive index increment ($\Delta n/\Delta_c$) for each molecular weight. Differential and cumulative molecular weight distributions were then calculated (1). The effects of flow and pressure on $\Delta n$ were very slight under the experimental conditions used, and therefore were neglected (2).

## RESULTS

**Uptake measurements based on mean molecular size.** The spore mass after centrifugation consists of an intercellular (interstitial) aqueous space and intracellular spaces in which probing

solute molecules are distributed after diffusional equilibrium has been reached. Expressed as the percentage of the total weight of the spore pellet, the interstitial space ($S_{in}^{w}$) was measured with dextran $\bar{M}_n$ = 200,000, which was selected because even the smallest molecules in the sample distribution were not taken up by the spores (8). The $S_{in}^{w}$ value of 17.3% was significantly higher than that of 9% obtained previously by Gerhardt and Black (8). The discrepancy was attributed to different spore-packing conditions.

Table 1 summarizes the uptake determinations and the molecular sizes for a limited number of polyethylene glycol samples, plus glucose as a reference compound. The average reference value for interstitial space ($S_{in}^{w}$ = 17.3%) was used to obtain the cellular uptake value ($R^{w}$) for each probing solute. Probing solutes of increasing average molecular size were taken up to a decreasing extent by the dormant spores.

Routinely, an equilibration time of 2 hr was allowed for uptake, as in previous experiments (8). However, the possibility was examined that a 2-hr uptake period might not be enough for the largest molecules in the polymer distribution to reach diffusional equilibrium. The results in Table 2 show that no significant increase in the uptake values for selected polymer samples was obtained by extending the uptake duration to 24 hr. The experimental variations in Table 2 were all within the limits of standard error.

Figure 1A shows the relationship between $R^{w}$ and log $\bar{M}_n$; Fig. 1B shows the relationship between $R^{w}$ and $\bar{r}_{es}$. The extrapolated polydisperse solute exclusion threshold corresponded to $\bar{M}_n$ = 150,000 and $\bar{r}_{es}$ = 16 nm. Thus, the results reasonably confirmed the findings with a larger number of samples previously reported by Gerhardt and Black (8).

**Uptake measurements based on molecular size distribution.** After preliminary determinations similar to those described previously by Scherrer and Gerhardt (14), elution chromatograms for the distribution of polyethylene glycols were compared before and after uptake by dormant spores. Graphical analyses of the results yielded a quasi-monodisperse solute exclusion threshold, as summarized in Table 3.

Figure 2 shows elution chromatograms of polyethylene glycol, $\bar{M}_n$ = 1,450 before and after uptake. The overall aspects of the two chromatograms were the same. That is, all of the polymer fractions in the sample distribution apparently were taken up by the spores. Similar results were obtained by gel chromatographic analysis of polymer samples $\bar{M}_n$ = 1,000 and $\bar{M}_n$ = 3,350.

With polyethylene glycol $\bar{M}_n$ = 9,500, however, the shapes of the elution chromatograms before and after uptake were quite different (Fig. 3A). Instead of all fractions in the sample being taken up, only the fractions with smaller molecular sizes penetrated the spores. Also, the distribution curves extended over a much wider range; the higher the mean molecular size, typically the more polydisperse the polymer preparation. The two distribution curves intercepted at a point between fractions 15 and 16, which represented the point beyond which larger molecules were completely excluded and which corresponded to $M_n \cong 8{,}000$.

Figure 3B depicts the differential molecular weight distributions of the same polymer before and after spore uptake. Here, the differences between the two distribution curves were graphically exaggerated with a dimensionless ordinate, and the intercept was confirmed more precisely.

Figure 3C shows a third graphical step in which the data were converted into the incremental percentage difference for the two determinations in each fraction, i.e., the percentage uptake. Extrapolation of the linear regression to an intercept with the zero abscissa yielded the same value of $M_n$ = 8,000 for the exclusion threshold.

Below $M_n$ = 4,500, a plateau in the uptake curve was established at about 40% (Fig. 3C). This finding was consistent with the uptake of glucose at $R^{w}$ = 43% (Table 1) and previously

TABLE 2. *Influence of equilibration time on the uptake of heterodisperse polymers by dormant spores*

| Compound | | | Uptake determinations, $S^{w}$ (%)[a] | | | |
|---|---|---|---|---|---|---|
| Identification no. | Sample | $\bar{M}_n$ | 2 hr[b] | 6 hr | 12 hr | 24 hr |
| 2 | Dextran 500 | 200,000 | 11.6 | 12.3 | 12.9 | 15.5 |
| 4 | Polyethylene glycol 1,540 | 1,450 | 40.1 | 44.6 | 44.6 | 43.9 |
| 6 | Polyethylene glycol 6,000 | 8,000 | 33.2 | 34.6 | 34.6 | 33.4 |
| 8 | Polyethylene glycol 20,000 | 17,500 | 33.1 | 37.5 | 43.5 | 35.8 |

[a] Gravimetric determination of solute concentrations. Each figure represents the average of three determinations.

[b] Equilibration time.

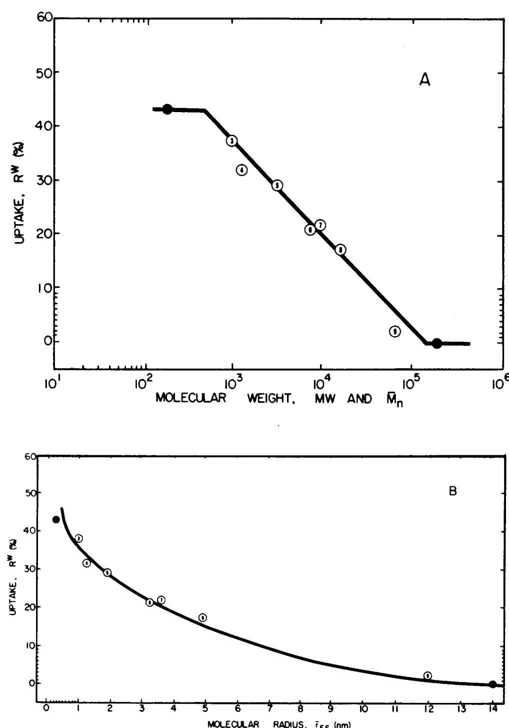


FIG. 1. *Percentage uptake by dormant spores of a series of polyethylene glycols as a function of (A) the logarithm of molecular weight ($\bar{M}_n$) and (B) the equivalent hydrodynamic diffusion radius ($\bar{r}_{ES}$). The point identification numbers correspond to the compounds listed in Table 1. Dextran $\bar{M}_n$ = 200,000 (no. 2) is a reference compound for complete exclusion and glucose (no. 1) for maximum uptake by the integuments. The lines were drawn by visual best fit with interpretations based on previous findings by Gerhardt and Black (8). The statistical (least squares) slope of the linear regression in A was −17.7.*

reported observations that permeation exceeds 40% of the spore weight only if a molecule has a molecular weight of less than about 200 and if it also has appreciable solubility in lipid (8). These properties suggest that a secondary permeability barrier exists in the membrane on the surface of the spore protoplast.

The quasi-monodisperse solute exclusion

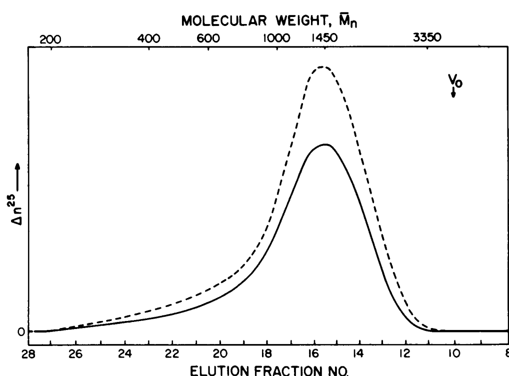


FIG. 2. *Elution chromatograms of polyethylene glycol $\bar{M}_n$ = 1,450 before uptake by dormant spores (- - -) and after (——), without adjustment for differences in polymer mass. Experimental conditions for this and subsequent figures are summarized in Table 3.*

TABLE 3. *Determinations of monodisperse solute exclusion threshold in dormant spores by uptake-distribution method*

| Compound | | | | | Gel chromatography | | | Monodisperse solute exclusion threshold | |
|---|---|---|---|---|---|---|---|---|---|
| Identification no. | Sample | $\bar{M}_n$ | $\bar{r}_{ES}$ (nm) | $R^w$ (%) | Gel type[a] | Gel column dimensions | Eluent flow rate[b] | $M_n$ | $r_{ES}$ (nm) |
| 3 | Polyethylene glycol 1,000 | 1,000 | 1.0 | 33.5 | P-10 | 1.5 by 26.0 cm | 13 | ND[c] | ND |
| 4 | Polyethylene glycol 1,540 | 1,450 | 1.2 | 35.3 | P-10 | 1.5 by 26.0 cm | 13 | ND | ND |
| 5 | Polyethylene glycol 4,000 | 3,350 | 1.9 | 34.8 | P-30 | 1.5 by 26.3 cm | 7 | ND | ND |
| 7 | Polyethylene glycol 9,000 | 9,500 | 3.6 | 20.2 | P-300 | 1.5 by 26.5 cm | 7 | 8,000 | 3.2 |
| 8 | Polyethylene glycol 20,000 | 17,500 | 4.9 | 2.1 | A-15m | 1.5 by 25.3 cm | 9 | 8,000 | 3.2 |

[a] Biogel P series (100 to 200 mesh) or Biogel A-15m (100 to 200 mesh).
[b] Flow rate of eluent (water) calculated per gel cross-sectional ara (1.77 $cm^2$). Values expressed as milliliters per square centimeter per hr.
[c] Not detectable.

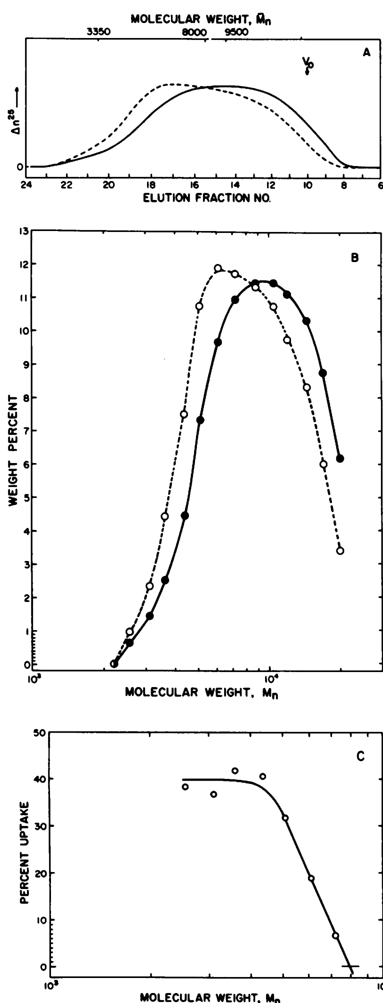


FIG. 3. *The distribution in uptake by dormant spores of polyethylene glycol $M_n$ = 9,500, expressed graphically in three different ways. In each case, the intercept corresponds to a monodisperse exclusion threshold of $M_n$ = 8,000. A, Elution chromatograms of the sample before uptake (- - -) and after (——), with adjustment to the same polymer mass. B, Molecular weight distribution diagrams of the sample before uptake (O) and after (●). C, Incremental percentage uptake of the sample as a function of molecular weight.*

threshold of $M_n$ = 8,000 was confirmed by the intercept in elution chromatograms before and after spore uptake of the next higher sample in the series, polyethylene glycol $\bar{M}_n$ = 17,500, even though this sample proved to be very polydisperse and to have a very irregular distribution pattern (Fig. 4). The composite results with the uptake distribution method thus indicated that monodisperse molecules can permeate the effective surface of the dormant spore only if they are equivalently smaller than a glycol of $M_n$ = 8,000, which is equivalent to $r_{ES}$ = 3.2 nm.

## DISCUSSION

The threshold in surface porosity of the dormant spore appears much more restrictive when based on the uptake limit for monodisperse solutes rather than for polydisperse solutes. The monodisperse limit of 3.2 nm could yet be overestimated because of solute shape (10, 15) and other factors, although to a much lesser degree than before.

This direct measurement of porosity can be compared with a few indirect assessments of sieving that involve biologically or chemically active molecules. A number of solutes relatively small in molecular size apparently permeate the surface with ease, including compounds (such as glucose, alanine, and adenosine) that induce germination (8). At the other extreme, very large macromolecules apparently are excluded, such as antibodies that are specific for a structure located deep in the spore (16). An informative situation exists in regard to lysozyme, which in molecular weight (14,400) and size ($r_{ES}$ ≅ 2.8 nm) falls slightly below the porosity threshold measurement based on monodisperse solutes and considerably below that based on polydisperse solutes. Lysozyme apparently does not penetrate the surface of the intact *B. cereus* spore to react with its peptidoglycan substrate in the cortex. When the spore is treated with agents that break disulfide bonds in the coat, lysozyme then penetrates and reacts (11). This behavior suggests that assessment of porosity, even if based on a monodisperse equivalent of polyglycol, may indeed be slightly overestimated and that factors other than size may contribute.

The equivalent surface porosity of this spore appears more open than that similarly determined for the vegetative cell wall of *B. megaterium*, (3.2 and 1.1 nm, respectively; reference 14).

Gerhardt and Black (8) previously inferred from the gradual slope of the uptake plot for

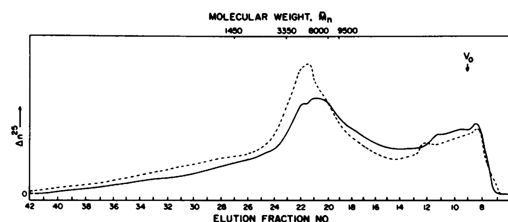


FIG. 4. *Elution chromatograms of polyethylene glycol $\bar{M}_n$ = 17,500 before uptake by dormant spores (- - -) and after (——), with adjustment to the same polymer mass.*

polydisperse solutes that the effective surface of the spore consists of pores or openings that vary in size. This deduction is complicated by the fact that polydispersity increases with increase in $\overline{M}_n$ of polymers, indicating that the slope could reflect heterodispersity in size of the solutes as well as in openings of the spore surface. However, when an uptake plot was determined on the basis of monodisperse fractions, a gradual slope still remained, albeit less so (Fig. 3C and Fig. 1A). Apparently the effective surface of the spore indeed is heteroporous, not homoporous.

## ACKNOWLEDGMENTS

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