Molecular Sieving by the *Bacillus megaterium* Cell Wall and Protoplast

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Passive permeabilities of the cell wall and protoplast of *Bacillus megaterium* strain KM were characterized by use of 50 hydrophilic probing molecules (tritiated water, sugars, dextrans, glycols, and polyglycols) which varied widely in size. Weight per cent uptake values ($R^w$) were measured at diffusional equilibrium under conditions that negated the influences of adsorption or active transport. Plots of $R^w$ for intact cells as a function of number-average molecular weight ($\bar{M}_n$) or Einstein-Stokes hydrodynamic radius ($R_{ES}$) of the solutes showed three phases: a protoplast uptake phase with a polydispersity exclusion threshold of $\bar{M}_n = 0.6 \times 10^4$ to $1.1 \times 10^4$, $R_{ES} = 0.6$ to 1.1 nm; a cell wall uptake phase with a polydispersity exclusion threshold of $\bar{M}_n = 0.7 \times 10^4$ to $1.2 \times 10^4$, $R_{ES} \approx 8.3$ nm; and a total exclusion phase. Isolated cell walls showed only the latter two phases. However, it became evident that the cell wall selectively passed only the smallest molecules in a heterodisperse polymer sample. When the molecular-weight distributions of polyglycol samples ($\bar{M}_n = 1,000, 1,450, \text{ and } 3,350$) were determined by analytical gel chromatography before and after uptake by intact cells or isolated cell walls, a quasi-monodisperse exclusion threshold was obtained corresponding to $\bar{M}_n = 1,200$, $R_{ES} = 1.1$ nm. The permeability of isolated protoplasts was assessed by the relative ability of solutes to effect osmotic stabilization. An indefinite exclusion threshold, evident even with monodisperse sugars, was attributed to lengthwise orientation of the penetrating rod-shaped molecules. Altogether, the best estimate of the limiting equivalent porosity of the protoplast was 0.4 to 0.6 nm in radius and of the cell wall, 1.1 nm.

The bacterial cell wall and protoplast membrane, in addition to other functions, act to sieve molecules that diffuse through the aqueous channels or pores that are an integral part of their structure. The ability of a porous integument to exclude or pass a compound is governed mainly by the relative sizes and shapes of the openings and the molecules. However, the relative geometries cannot be viewed as fixed properties (43), and the total relationship is complex. In the cell wall, the openings apparently exist not as simple, uniformly sized perforations but rather as tortuous, heterogeneously sized pathways through a three-dimensional, fenestrated matrix (16, 19). It has also been demonstrated that bacterial walls flex and change volume in response to a changed ionic environment, possibly with dilation and contraction of the openings in their matrix (29).

In the protoplast membrane, the openings may be more ordered in array and uniform in size, but apparently they are more restrictive than in the wall (40). With both structures, heterogeneity of the cell population may add a further complication. Chemical interaction may occur between the sieving matrix and a permeating solute—for example, electrostatic charge and hydrophobic substituent groups are known to affect diffusional penetration. Furthermore, the size and shape even of small molecules is complicated by a surrounding hydration shell. Consequently, the limiting porosity of an integument must be expressed as an apparent value relative to an equivalent parameter of molecular size, such as the effective hydrodynamic diffusion radius (33, 43).

However, if the experimental conditions are defined and the probing molecules are selected with a view toward minimizing such complicating effects, it appeared feasible to characterize the apparent porosity of cell integuments by a study of their sieving properties with compounds of increasing molecular size under conditions that preclude interference from adsorptive or active uptake. This approach has been applied in microbiology to spores of *Bacillus cereus* by Black and Gerhardt (4, 15), to vegetative cells of
**B. megaterium** by Scherrer and Gerhardt (40), to isolated walls of *B. megaterium* and *Saccharomyces cerevisiae* by Gerhardt and Judge (16), and to isolated walls of *Neurospora crassa* by Trevithick, Metzenberg, and Costello (45).

In the present work, we sought first to characterize the cytostructural permeability of a model bacterium by employing a wide array in size of probing molecules with greater analytical precisio
than before, and by using isolated walls and protoplasts in comparison with intact cells. Two series of hydrophilic, relatively inert, nonelectrolyte compounds were employed: monosaccharides, oligosaccharides, and dextran; and ethylene glycols, propylene glycols, and polyglycols. *B. megaterium* strain KM was selected as the test organism because it is widely used and well characterized. The cell wall of *B. megaterium* can be isolated by mechanical disruption, appears generally uniform in section by electron microscopy, and consists mainly of a teichoic acid-peptidoglycan complex without lipids (10, 20, 37). Also, the protoplast of *B. megaterium* can be isolated readily by use of lysozyme (48), and its membrane can be isolated and is comprised of typical lipids and proteins (38, 49). The relative permeabilities of the two structures to monomers and polymers was shown by use of the primary experimental approach. However, it became apparent that the true dimensions of the maximum openings were overestimated by such experiments.

An accurate measurement of an uptake threshold is complicated by the fact that preparations of polymeric compounds typically are not monodisperse like monomers or oligomers but instead are polydisperse. The molecular-weight fractions in a polydisperse preparation vary around a mean, such as a Poisson distribution for the glycols (12). Furthermore, the higher the mean molecular weight usually the more polydisperse the polymer preparation (Fig. 1). Consequently, the apparent uptake of a given large polymer in fact may reflect the uptake of only the smaller molecules in the distribution.

Consequently, a second objective was to compare the molecular-weight profile of a polymer preparation both before and after uptake, as determined in fractions obtained by means of gel permeation chromatography. The polymer distribution curve after uptake proved to be relatively skewed and intercepted the original curve at a point corresponding to the monodisperse exclusion threshold. The results obtained by use of this new uptake-distribution method indicated that the limiting porosity of the cell wall is much more restrictive than heretofore shown.

A third objective stemmed from the fact that neither of the direct methods for measuring solute uptake could be applied to isolated protoplasts. Consequently, their permeability was assessed by measuring their stability against osmotic bursting as a function of solute molecular size. The results suggested that, as an exclusion limit is approached, rod-shaped molecules penetrate protoplasts with lengthwise orientation. The limiting radial dimension in porosity of the isolated protoplast approximated 0.4 nm, compared to about 1.1 nm in the cell wall.

**MATERIALS AND METHODS**

**Organism.** Cells of strain KM of *B. megaterium* were grown in 10-liter batches of Trypticase Soy Broth (BBL, 15 g/liter) in an aerated and agitated fermentor at 30 C, harvested at the end of the exponential growth phase by centrifugation at 25 C, and washed twice in distilled water (40). Viable cell counts were made by plating on Trypticase Soy Agar (BBL), and total cell counts were made by use of a Petroff-Hauser chamber.

![Fig. 1. Differential distribution of the number of repeating units and molecular weights in polyethylene glycols. Whereas ethylene glycol is monodisperse, the polymer samples are increasingly polydisperse as they increase in mean molecular weight. In a polyethylene glycol of about 1,000 mean molecular weight, for example, only 8% of the total weight is this size, and the range extends from about 500 to 1,800. The graph was reproduced with minor modifications from illustrative data of the Union Carbide Corp., by permission.](http://jb.asm.org/)
Cell walls were obtained by disruption of aqueous cell suspensions in a refrigerated pressure cell and by subsequent differential centrifugation at 4 °C (16). Homogeneity of the cell wall preparations was assessed by transmission electron microscopy of metal-shadowed specimens and by centrifugation in density gradients of sucrose or Ficoll (Pharmacia Fine Chemicals, Inc.). Cell wall suspensions (pH 6.5) were kept at 4 °C or stored at −20 °C.

**Solutions.** All of the solutes used are listed in Table 1 with identification numbers, which also are referred to parenthetically in the text. Suppliers of the polyethylene glycols and dextrans are given in the footnotes of Table 1. Tritiated water was purchased from the Volk Radiochemical Co., and its radioactivity was assayed by the Bray (5) method in liquid scintillation spectrometers (Tri-Carb, models of 2000 series, Packard Instrument Co.). Mono- and oligosaccharides, except glucose and sucrose, were obtained from Pfanstiehl Laboratories. All other chemicals were obtained from standard sources and were of reagent quality.

**Refractometry.** The concentration of all solutes except triitated water was determined with an Abbé precision refractometer (model 33-45-01, sodium light source, Bausch & Lomb Optical Co.) having a sensitivity of 2 × 10⁻⁴ refractive index units (n), or with a Brice-Phoenix, high-sensitivity, automatic recording, differential refractometer (model R-2000-T, Phoenix Precision Instrument Co.) having a sensitivity of 2 × 10⁻⁷ n. Refractive indices were determined at 20 or 25 ± 0.02 °C at a wavelength of 589 nm. Standard test pieces, aqueous KCl solution, and doubly glass-distilled water were used for instrument calibration (3, 22).

Pure distilled water was considered to have the following n values at 20 and 25 °C with sodium D lines (589 nm; reference 44): n₀ = 1.3329877 and n₀ = 1.3325026. At low concentrations, the refractive index increment (Δn) was linearly proportional to concentration (c), but the proportionality depended on the solute (3). Calibration curves with Δn versus c were constructed for all the solutes. For the polyethylene glycols, the specific refractive index increment (dn/dc)₂₀,₅,₅ increased from 0.100 ml/g for polyethylene glycol M₀ = 200 (no. 35) to 0.159 ml/g for polyethylene glycol M₀ = 8,000 (no. 49) (reference 11).

**Nonspecific leakage.** The intact cells used for uptake studies released a considerable amount of ultraviolet-absorbing material, which interfered with refractive index determinations of the added solute. The leaked material reached a peak in the absorption spectrum at 257 nm (8), which was proportional in amount to the cell mass, and increased with time. For example, 1 mg of cell dry weight yielded 0.02 A₂₅₇ optical density in a Beckman DU spectrophotometer in 2 hr. The leaked material caused a slight refractive index increment (i.e., dn/dc) of 18.5 × 10⁻⁴ for the same experimental conditions. The nature of the solute employed in the uptake study appeared to influence neither the amount nor the nature of the leaked material. The refractive index increment produced by leakage was therefore subtracted from the total Δn in determining the net n of the solute. In gel chromatography of polyethylene glycols, the gels were selected so that elution peaks produced by the leaked material were different from those of the glycols.

**Polymers.** Two series of polymers were used, polyethylene glycols and dextrans. The polyglycols are linear, flexible, unbranched nonelectrolyte polymers with an oxyethylene repeating unit and two hydroxyl end groups (14). The dextrans are partially branched, nonelectrolyte polyglycoses of biological origin, which are partially degraded by chemical means.

In both series of polymers, a sample consists of a grouping of homologous molecular species with an identifying average and a characteristic distribution in molecular weight (13), as illustrated in Fig. 1. The type of distribution is determined by the mode of polymerization or partial degradation. Polyethylene glycols have a Poisson type of molecular-weight distribution (12).

The average molecular weight (M) of a polymer sample depends on the mode of analysis. The number-average molecular weight (Mₙ) is determined from coligative properties or by chemical end-group analysis, as follows:

\[ Mₙ = \Sigma N_i M_i/\Sigma N_i \]

The weight-average molecular weight (Mₚ) is usually determined by light scattering, and is defined as follows:

\[ Mₚ = (\Sigma N_i M_i^2)/\Sigma N_i M_i \]

In both equations, Nᵢ is number of moles of polymer species i, and Mᵢ = molecular weight of species i (13). Mₙ and Mₚ are usually different in a polymer sample. The ratio Mₚ/Mₙ indicates the degree of polydispersity. Polyethylene glycols show a degree of polydispersity between 1.1 and 1.3, whereas dextrans are much more polydisperse.

For a homologous series of polymers, such as the polyethylene glycols or dextrans, refractometry can be considered to reflect the molecular mass and therefore Mₙ.

**Molecular-weight determinations.** The Mₙ values for inulin and polyethylene glycols were determined by using a vapor pressure osmometer (model 301A, Hewlett-Packard Co.) at 25 °C with an aqueous probe, with sucrose solutions for calibration and with least-square linear regression analysis of the data (11). All other Mₙ or Mₚ values were taken from manufacturer’s or published data (1, 39).

**Molecular sizes.** The sizes of solute molecules in aqueous solution were calculated directly or by graphical interpolation from published data and were expressed in nanometers (1 nm = 10 Ångstrom units). Equivalent hydrodynamic diffusion radii, which usually are called Einstein-Stokes radii (rₑ), for the sugars were taken from Schultz and Solomon (41), for dextrans from Wallenius (47), and for the polyethylene glycols from Rempp (36). It was necessary to estimate some rₑ values by interpolation from other values in the series.

The statistical radii of gyration (P₀) for the polyethylene glycols were calculated according to Mark and Flory (26).

**Solute uptake.** The space or thick-suspension technique (4, 25, 30) was used to determine equilibrium solute uptake under conditions (i.e., high solute and cell concentrations, 4 C) which nullify or minimize the effects of adsorptive and energy-requiring solute uptake (30). The uptake values for representative solutes were
<table>
<thead>
<tr>
<th>Identification no.</th>
<th>Sample</th>
<th>Molecular wt</th>
<th>$\bar{M}_w^a$</th>
<th>$\bar{M}_x^a$</th>
<th>$r_{ea}$ (nm)</th>
<th>$r_{e0}$ (nm)</th>
<th>$S^* \pm \sigma$ (%)</th>
<th>$R^* \pm \sigma$ (%)</th>
<th>$10^5 K_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0. Trinitiated water</td>
<td>20</td>
<td>450,000</td>
<td>200,000</td>
<td>0.15</td>
<td>18</td>
<td>84.4 ± 5.6</td>
<td>80.1 ± 1.8</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>25. Dextran 500$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Reference substances**

1. Erythritol | 122 | ~0.28 | 6 | 71.5 ± 2.0 | 63.7 ± 2.0 | 79.5 |
2. Xylose | 150 | ~0.31 | 4 | 62.7 ± 0.5 | 52.4 ± 2.5 | 65.4 |
3. Arabinose | 150 | 0.31 | 5 | 64.9 ± 1.3 | 55.3 ± 2.2 | 69.0 |
4. Glucose | 180 | ~0.36 | 6 | 61.6 ± 2.0 | 50.1 ± 2.1 | 63.7 |
5. Inositol | 180 | 0.36 | 4 | 53.5 ± 4.6 | 40.7 ± 2.5 | 50.8 |
6. Sucrose | 342 | 0.46 | 7 | 50.5 ± 2.1 | 36.9 ± 2.0 | 46.0 |
7. Cellobiose | 342 | ~0.46 | 4 | 47.0 ± 0.9 | 32.4 ± 2.5 | 40.4 |
8. Methylbiose | 342 | ~0.46 | 3 | 47.3 ± 2.7 | 32.8 ± 2.9 | 40.9 |
9. Maltose | 342 | ~0.48 | 5 | 49.4 ± 1.3 | 35.5 ± 2.3 | 44.3 |
10. Raffinose | 504 | 0.57 | 5 | 49.5 ± 5.1 | 35.6 ± 2.3 | 44.4 |
11. Melezitose | 504 | ~0.57 | 5 | 49.5 ± 5.1 | 35.6 ± 2.3 | 44.4 |
12. Stachyose | 667 | ~0.66 | 4 | 43.4 ± 1.4 | 27.8 ± 2.5 | 34.7 |
13. Inulin | 3,100 | 1.40 | 8 | 41.2 ± 2.5 | 25.1 ± 1.9 | 31.3 |
14. Dextran$^c$ | 1,500 | 1.15 | 8 | 36.5 ± 4.0 | 19.1 ± 1.9 | 23.8 |
15. Dextran$^c$ | 3,650 | 2.10 | 14 | 38.8 ± 6.8 | 22.0 ± 1.5 | 27.5 |
16. Dextran$^c$ | 5,400 | 4.10 | 17 | 35.2 ± 1.7 | 17.4 ± 2.6 | 21.7 |
17. Dextran 100$^c$ | 9,400 | 6.20 | 17 | 35.8 ± 2.5 | 18.1 ± 1.4 | 22.6 |
18. Dextran$^c$ | 16,000 | 9.60 | 4 | 35.6 ± 2.3 | 17.8 ± 2.6 | 22.2 |
19. Dextran 19$^c$ | ~19,000 | ~12,000 | 10 | 32.1 ± 4.2 | 13.4 ± 1.8 | 16.7 |
20. Dextran 20$^c$ | 22,700 | 17,000 | 6 | 30.5 ± 1.3 | 11.4 ± 2.2 | 14.2 |
21. Dextran 38$^c$ | ~38,000 | ~24,000 | 9 | 34.5 ± 4.5 | 16.5 ± 1.8 | 20.6 |
22. Dextran 40$^c$ | 42,000 | 26,000 | 9 | 29.8 ± 4.9 | 10.5 ± 1.9 | 13.1 |
23. Dextran 50$^c$ | 74,500 | 48,000 | 7 | 23.7 ± 5.8 | 2.7 ± 2.1 | 3.4 |
24. Dextran 150$^c$ | 153,000 | 95,000 | 5 | 23.6 ± 2.0 | 2.7 ± 2.4 | 3.4 |
25. Dextran 500$^c$ | 450,000 | 200,000 | 4 | 21.6 ± 5.0 | 0 ± 5.0 | 0 |
26. Dextran 700$^c$ | 700,000 | ~350,000 | 3 | 22.2 ± 0.6 | 0 ± 3.0 | 0.1 |
27. Dextran 2000$^c$ | 2,000,000 | ~1,000,000 | 6 | 22.2 ± 1.7 | 0.8 ± 2.2 | 0.1 |

**Glycols and polyglycols**

28. Ethylene glycol$^d$ | 62 | 0.33 | 11 | 74.1 ± 5.1 | 67.1 ± 1.7 | 83.4 |
29. Propylene glycol$^d$ | 76 | ~0.36 | 8 | 72.1 ± 1.8 | 64.6 ± 1.9 | 80.6 |
30. Diethylene glycol$^d$ | 106 | ~0.41 | 9 | 74.7 ± 6.6 | 67.9 ± 1.4 | 84.8 |
31. Dipropylene glycol$^d$ | 134 | ~0.44 | 7 | 66.2 ± 1.7 | 57.1 ± 2.0 | 71.3 |
32. Triethylene glycol$^d$ | 150 | ~0.46 | 8 | 63.5 ± 2.1 | 53.6 ± 2.3 | 66.9 |
33. Polypropylene glycol 150$^d$ | 150 | ~0.46 | 7 | 62.0 ± 5.1 | 51.7 ± 2.1 | 64.5 |
34. Tetraethylene glycol$^d$ | 194 | ~0.50 | 7 | 62.0 ± 5.1 | 51.7 ± 2.1 | 64.5 |
35. Polyethylene glycol 200$^d$ | 200 | 0.56 | 11 | 61.2 ± 6.0 | 50.7 ± 1.7 | 63.3 |
36. Polyethylene glycol 300$^d$ | 300 | 0.58 | 11 | 60.2 ± 4.5 | 49.4 ± 1.7 | 61.7 |
37. Polyethylene glycol 350$^d$ | 350 | 0.64 | 10 | 57.6 ± 4.4 | 46.1 ± 1.8 | 57.6 |
38. Polyethylene glycol 400$^d$ | 400 | 0.66 | 13 | 53.7 ± 4.4 | 41.2 ± 1.6 | 51.4 |
39. Polyethylene glycol 425$^d$ | 425 | ~0.69 | 14 | 52.0 ± 2.7 | 39.0 ± 2.1 | 48.7 |
40. Methoxy-polyethylene glycol | 550 | 0.78 | 10 | 51.3 ± 2.8 | 38.1 ± 1.8 | 47.6 |

$^a$ Some of the values for $\bar{M}_w$ or $\bar{M}_x$ are indicated as approximations (~), either because of inexact designation from the source or of estimation by interpolation from other values in the series.


$^c$ Contributed by Kirsti Granath, Pharmacia AB, Uppsala, Sweden.

$^d$ From Commercial Solvents Corp., Terre Haute, Ind.

$^e$ From Union Carbide Chemicals Co., New York (Carbowax series).

Table 1—Continued

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular wt</th>
<th>Equiv. molecular radius</th>
<th>No. of determinations</th>
<th>10^4 K_D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification no.</td>
<td>Sample</td>
<td>MW</td>
<td>( \bar{M}_w )</td>
<td>( \bar{M}_n )</td>
</tr>
<tr>
<td>41. Polyethylene glycol 600°</td>
<td>...</td>
<td>600</td>
<td>0.80</td>
<td>0.78</td>
</tr>
<tr>
<td>42. Methoxy-polyethylene glycol 750°</td>
<td>...</td>
<td>750</td>
<td>0.89</td>
<td>10</td>
</tr>
<tr>
<td>43. Polyethylene glycol 1000°</td>
<td>...</td>
<td>1,000</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>44. Polyethylene glycol E 1450°</td>
<td>...</td>
<td>1,450</td>
<td>1.20</td>
<td>1.20</td>
</tr>
<tr>
<td>45. Polyethylene glycol 1540°</td>
<td>...</td>
<td>1,450</td>
<td>1.20</td>
<td>1.20</td>
</tr>
<tr>
<td>46. Polyethylene glycol 4000°</td>
<td>...</td>
<td>3,350</td>
<td>1.90</td>
<td>1.83</td>
</tr>
<tr>
<td>47. Polyethylene glycol E 4000°</td>
<td>...</td>
<td>4,500</td>
<td>2.30</td>
<td>2.12</td>
</tr>
<tr>
<td>48. Polyethylene glycol E 6000°</td>
<td>...</td>
<td>6,750</td>
<td>2.90</td>
<td>2.60</td>
</tr>
<tr>
<td>49. Polyethylene glycol 6000°</td>
<td>...</td>
<td>8,000</td>
<td>3.20</td>
<td>2.83</td>
</tr>
<tr>
<td>50. Polyethylene glycol E 9000°</td>
<td>...</td>
<td>9,500</td>
<td>3.60</td>
<td>3.08</td>
</tr>
<tr>
<td>51. Polyethylene glycol 20,000°</td>
<td>...</td>
<td>17,500</td>
<td>4.90</td>
<td>4.18</td>
</tr>
<tr>
<td>52. Polyethylene glycol 70,000°</td>
<td>...</td>
<td>70,000</td>
<td>12.0</td>
<td>8.40</td>
</tr>
</tbody>
</table>


demonstrated to be independent of solute concentration over the range used (40), and no indications of concentration uptake were observed.

Twenty-five milliliters of a cell or wall suspension was sedimented in a refrigerated centrifuge (model RC-2, Sorvall, Inc.) at 17,000 \( \times g \) for 60 min or 23,000 \( \times g \) for 90 min, respectively, in tared 50-ml tubes. The firm cell or wall pellet (about 3 g, wet weight) was weighed after decanting the supernatant fluid and wiping dry the wall of the tube. A known amount of solute (usually 3 ml of 3% concentration, \( w/v \)) was added to the pellet, mixed thoroughly with two wooden applicator sticks, and allowed to reach uptake equilibrium at 4 C. An equilibration period of 2 hr was demonstrated to be sufficient, as evidenced by the attainment of maximum uptake values with representative solutes.

An uptake value based on the weight of the cells was calculated from the relation

\[
S^* = (V_s/\bar{W}_t)(C_\text{p}/C_1 - 1) \times 100
\]

where \( S^* \) is the percentage uptake (space) value, \( V_s \) is the solution volume added to the cells, \( C_\text{p} \) is the initial solute concentration, \( C_1 \) is the final solute concentration, and \( \bar{W}_t \) is the pellet weight.

Because of the presence of interstitial water in the pellet, it was necessary to obtain a corrected weight-percentage uptake value (\( R^* \)) as follows

\[
R^* = (S_{\text{mol}}^* - S_{\text{in}}^*)/(100 - S_{\text{in}}^*)
\]

\( S_{\text{mol}}^* \) is the percentage space value for the interstitium measured with a polymer sample which is completely precluded from penetrating into cells or cell walls (dextran \( M_\text{w} = 200,000 \), no. 25). \( S_{\text{in}}^* \) is the percentage uptake value for a penetrating solute. Therefore, \( R^* = 0 \) would indicate that the test solute was completely excluded from entering the cells or walls. \( R^* < 100 \) would mean that the test solute was taken up to a fractional extent, with the internal concentration equal to the outside concentration. \( R^* > 100 \) would indicate concentration active uptake.

The relative distribution coefficient, \( K_D \), was also used as a parameter of uptake and was defined as follows. \( K_D \) is equal to the concentration of solute in cell or wall water divided by the concentration of solute in interstitial water. \( K_D \) was calculated as follows

\[
K_D = R^* \text{ solute} / R^* \text{ water}
\]

**Gel chromatography.** Molecular-weight averages and molecular-weight distributions of the polyethylene glycols were studied by gel permeation chromatography with Biogel P-6 and P-10 (both 50 to 150 mesh, Biorad Laboratories) and Sephadex G-25 and G-50 (both fine mesh, Pharmacia Fine Chemicals, Inc.). The materials were hydrated in distilled water and poured into glass columns (inner diameter = 2 cm, sintered porous glass disk support) to obtain gel columns having a total volume (\( V_s \)) of 94.2 cm³. The eluant was distilled water at a constant flow rate of 30 ml/hr. The eluent fractions were analyzed by refractometry and ultraviolet absorption spectroscopy. Column void volumes (\( V_v \)) were determined separately by use of Blue Dextran (Pharmacia Fine Chemicals, Inc.) or dextran \( M_\text{w} = 200,000 \) (no. 25), which were excluded by all the gels used. The polymers eluted according to their molecular weights. For each gel type, a calibration curve was made by plotting the peak elution volume (\( V_e \)) versus the logarithm of \( M_\text{w} \) (2, 9).

From the calibration curves, the molecular weight (\( M_\text{w} \)) of each elution fraction was interpolated. The mass of polymer in each elution fraction (1.9 ml) was determined from its refractive index increment and the known dn/dc ratio for each molecular weight. Differential and cumulative molecular-weight distributions were then calculated (2).

**Protoplast stabilization.** The permeability of protoplasts was assessed by determining the concentration of
solute necessary to accomplish 50% osmotic stabilization of the protoplast suspension.

Protoplasts were formed by adding lysozyme (EC 3.2.1.17, Sigma Chemical Co.) in amounts of 10 to 100 μg/ml to cells suspended in 0.03 M phosphate buffer (pH 7.0) in the presence of the stabilizer to be tested. The initial cell concentration gave values from 200 to 250 units in a Klett-Summerson photoelectric colorimeter (model 800-3, filter No. 66, Klett Manufacturing Co.). Addition of a solute in the absence of lysozyme immediately decreased the turbidity. This turbidity decrease depended to a small degree on the nature and to a large degree on the concentration of the solute added. Protoplast formation or cell lysis was usually completed in less than 2 hr. Changes in protoplast suspension turbidity and cellular morphology were followed over a 9-hr period by colorimetry and phase-contrast microscopy. The protoplast suspensions decreased in turbidity only 15 to 30% as compared with the corrected initial Klett readings, whereas total lysis gave a turbidity decrease of 80 to 90%. These readings remained the same for the entire period of observation for intermediate solute concentrations, but at higher solute concentrations the protoplast suspensions showed a secondary increase in turbidity after 3 to 5 hr. The 50% osmotic stabilization point for protoplasts was obtained graphically by plotting the percentage change in turbidity versus solute concentration (molarity, or g per liter per $\bar{M}_n$) at 2 hr.

**Osmotic pressure and osmolarity.** Polymer solutions deviate enormously from ideal solution laws. The osmotic pressure ($\pi$) is usually expressed by the equation (13)

$$\pi/c = \frac{(RT)}{\bar{M}_n}(A + Bc + Cc^2 + \ldots)$$

where $\pi$ is the osmotic pressure, $c$ is the concentration, $R$ is the gas constant, $T$ is the absolute temperature, and $A$, $B$, etc., are the so-called virial coefficients. If $A = 1$ and only the second virial coefficient, $B$, is taken into account, the equation reduces to the following

$$\pi = RT(c/\bar{M}_n)(1 + Bc)$$

The osmotic coefficient $\phi = (1 + Bc)$ represents the correction factor for non-ideality.

Osmolarity in turn is calculated from the following relationship: osmolarity $= \phi$ (molarity).

Virial coefficients, $B$, for the polyethylene glycols were taken from published data (11, 39) and used for calculating the osmotic pressure of the glycol solutions. Those for the sugars were obtained from T. R. Corner (Ph.D. Thesis, University of Rochester, 1968) and that for dextran $\bar{M}_n = 6,200$ (no. 17), from Kirst Granath (*personal communication*). The value of $B$ for inulin was calculated from vapor pressure osmometry measurements. An osmolarity of 1.0 was considered to correspond to an osmotic pressure of 22.4 atm.

**RESULTS**

**Uptake measurements based on mean molecular size: intact cells.** A packed mass of bacterial cells is comprised of intercellular as well as intracellular aqueous spaces in which probing molecules are distributed after diffusional equilibrium is reached. The interstitial space between the cells ($S_{ic}^*$) was measured with dextran $\bar{M}_n = 200,000$ (no. 25 in Table 1), which was selected so that even the smallest molecules in the sample distribution were not measurably taken up by the cells. Under the experimental conditions with cells of *B. megaterium*, the $S_{ic}^*$ averaged 21.6 ± 5.0% (Table 1).

The maximum intracellular space into which solute can penetrate is represented by the total water content of the cell, which for *B. megaterium* has been found to be 78.5% by Gerhardt and Judge (16). The interstitium-corrected, cellular-uptake value ($R*$) for tritiated water averaged 80.1 ± 1.8% (Table 1). The close correspondence between these two measurements indicated that essentially all of the intracellular water could exchange with tritiated water. The uptake of tritiated water did not change significantly in the presence of additional solutes (Table 2).

Between these extremes of complete exclusion and complete uptake, probing molecules of decreasing molecular size were taken up to an increasing extent by intact cells of *B. megaterium* (Table 1). The average reference value for the interstitial space ($S_{ic}^*$ = 21.6%) was used to obtain the cellular uptake value ($R*$) for each probing compound. The average reference value for cell uptake of tritiated water was taken to represent a cell distribution coefficient ($K_D$) of 1.0, so that a relative $K_D$ value also was obtained for each probing compound.

Uptake values for intact cells with the two series of compounds changed alike in general trends as a function of molecular weight but differed quantitatively (Fig. 2). Figure 2A depicts the uptake and distribution values for the series of sugars and dextrans, with the molecular weights ($MW$ or $\bar{M}_n$) plotted in order of increasing size on a logarithmic scale. Two phases of uptake were distinguished. In the first phase, as the size of probing molecules was increased, the respective uptake values decreased sharply in a regular pattern until an inflection occurred at about $MW = 550$. In the second phase, the uptake values decreased with a significantly lesser slope until another inflection occurred at about $\bar{M}_n = 100,000$, which represented the point of total polymer exclusion. Whereas the first uptake phase involved monosaccharides and oligosaccharides, the second phase involved only dextran polymers. This change from monodisperse to polydisperse compounds could influence the general aspect and inflection points of the uptake determinations.

Inulin (no. 13), which is often used for determining interstitial space in animal and yeast
TABLE 2. Influence of additional probing solutes on the uptake of tritiated water by intact cells of Bacillus megaterium

<table>
<thead>
<tr>
<th>Identification no.</th>
<th>Compound added</th>
<th>Molecular wt (MW)</th>
<th>No. of determinations</th>
<th>S* (%)</th>
<th>R* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tritiated water</td>
<td>20</td>
<td>2</td>
<td>84.8</td>
<td>79.5</td>
</tr>
<tr>
<td>28</td>
<td>Ethylene glycol</td>
<td>62</td>
<td>2</td>
<td>93.0</td>
<td>90.7</td>
</tr>
<tr>
<td>35</td>
<td>Polyethylene glycol 200</td>
<td>200</td>
<td>2</td>
<td>88.3</td>
<td>84.4</td>
</tr>
<tr>
<td>40</td>
<td>Methoxy-polyethylene glycol 550</td>
<td>550</td>
<td>2</td>
<td>92.1</td>
<td>90.0</td>
</tr>
<tr>
<td>43</td>
<td>Polyethylene glycol 1000</td>
<td>1,000</td>
<td>2</td>
<td>85.5</td>
<td>80.5</td>
</tr>
<tr>
<td>49</td>
<td>Polyethylene glycol 6000</td>
<td>8,000</td>
<td>2</td>
<td>86.1</td>
<td>82.2</td>
</tr>
<tr>
<td>51</td>
<td>Polyethylene glycol 20,000</td>
<td>17,500</td>
<td>2</td>
<td>90.2</td>
<td>87.5</td>
</tr>
<tr>
<td>25</td>
<td>Dextran 500</td>
<td>200,000</td>
<td>8</td>
<td>88.0</td>
<td>84.6</td>
</tr>
</tbody>
</table>

* See Table 1.

Initial concentration in all cases was 3%, except in tritiated water.

cells, was taken up by B. megaterium cells, albeit to a greater extent than comparable dextrans. The variable molecular weight and polydispersity of inulin samples have been discussed by Phelps (35).

When uptake by intact cells of a series of glycol monomers and polymers was plotted, two phases again were distinguished (Fig. 2B) and the inflections occurred at $M_n$ values not greatly different from those of the sugar-dextran series. Propylene glycol monomers and polymers (no. 29, 31, 33) were taken up about the same as the ethylene glycols of corresponding molecular weight, notwithstanding greatly different lipid solubilities. However, both glycol types are lipid soluble relative to all of the sugars, which are highly insoluble in lipids (15). Polyethylene glycols $M_n = 1,450$ (no. 44) and $M_n = 7,500$ (no. 48) deviated from the general trend. However, they were produced by a supplier different than for most of the other glycols and may have had a different molecular-weight distribution, which could influence their uptake values.

Both the dextrans and polyglycols, although primarily linear polymers, coil when in solution and were assumed to behave approximately as hydrodynamic spheres (18), which in permeability problems provide a useful parameter for relative molecular dimensions (33). Calculated by use of the Einstein-Stokes relationship, the average equivalent hydrodynamic diffusion radius ($R_{DH}$) for each compound is included in Table 1.

Figure 3 depicts the relationship between cell uptake ($R^*$ and $K_D$) and the average size of the respectively. The lines were drawn by visual best fit, with greater weighting of points having greater experimental replication (e.g., no. 25) and with discounting of those known to be aberrant (e.g., no. 13).
probing molecules ($r_{ES}$) in composite pattern for all of the compounds. Two linear regression phases occurred. In each of these phases, however, the slopes for the sugar-dextran series differed slightly but significantly from the slopes for the glycol-polyglycol series.

The inflection point between the first and second uptake phases in Fig. 3, as well as in Fig. 2, indicated the existence inside the cell of a limiting boundary which allows the passage of solutes only if they are smaller than $M_a \cong 0.6 \times 10^3$ to $1.1 \times 10^3$ and $r_{ES} \cong 0.6$ to 1.1 nm. Since the cell wall in *B. megaterium* makes up about 25% of the total cell dry weight (37), the inflection points at comparable $R^*$ values were believed to represent the uptake threshold of the next inner structure, i.e., the limiting membrane of the protoplast.

The observed difference between uptake curves for the sugar-dextran and glycol-polyglycol series in the first uptake phase may be explained by the fact that all of the sugars were monodisperse, whereas the glycols mostly were polydisperse. Consequently, the apparent uptake of glycols would be overestimated. Therefore, the true, monomeric uptake threshold for the protoplast in situ probably may be better estimated by the sugar series, equivalent to $MW = 600$ and $r_{ES} \cong 0.6$ nm.

The observed difference in slopes of the two uptake curves in the second (i.e., cell wall) uptake phase is explained by the facts that dextran are more polydisperse (higher $M_a/M_w$) and more rodshaped than polyglycols. Consequently, the apparent cell wall uptake would be overestimated more with dextrans than with glycols.

The inflection point between the second and third phases in Fig. 2 and 3 indicated the permeability limit for the cell surface, i.e., the cell wall periphery. This uptake threshold apparently was the same for both series of polymers and corresponded to $M_a \cong 10^3$ and $r_{ES} \cong 8.3$ nm.

Still larger molecules behaved alike in the third phase, with $R^* \equiv 0$ and $K_D = 0$ representing complete exclusion of the macromolecules into the interstitium.

**Uptake measurements based on mean molecular size: isolated cell walls.** The above interpretations about differential sieving by cell walls in situ were substantiated by uptake determinations made with gram masses of isolated cell walls (Table 3). The regression curves for values of $R^*$ and $K_D$ by isolated walls as a function of molecular weight (Fig. 4) and of molecular size

![Fig. 3. Uptake and distribution by intact cells of the sugars and dextrans and the glycols and polyglycols as a function of the equivalent hydrodynamic diffusion radius.](image-url)
**Table 3.** Permeability of isolated cell walls of Bacillus megaterium to hydrophilic probing molecules of increasing size

<table>
<thead>
<tr>
<th>Identification no.</th>
<th>Sample</th>
<th>Molecular wt</th>
<th>Equivalent molecular radius $\tilde{M}$</th>
<th>Uptake determinations</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>$\tilde{M}$</td>
<td>$\tilde{M}$</td>
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<td>0.</td>
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<td>25.</td>
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<tr>
<td>Dextran 500</td>
<td></td>
<td>450,000</td>
<td>200,000</td>
<td>14.0</td>
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<td>3.</td>
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<td>52.</td>
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*See Table 1.*

(5) can be compared with the second phase of uptake by intact cells shown in Fig. 2 and 3, respectively.

With isolated cell walls (Fig. 4 and 5), the uptake curves originated from the point for tritiated water ($R^* = 84.8\%$), which was slightly lower than the relational value for total water content (92.1%) reported previously by Gerhardt and Judge (16). In contrast to the seemingly linear regression curves observed for cell walls in situ, those for isolated walls definitely were hyperbolic and approached a limit asymptotically. Consequently, the point of inflection between the two phases was difficult to define exactly, but appeared to occur in approximate agreement with the permeability limits observed for cell walls in situ (Fig. 2 and 3).

Moreover, with isolated cell walls, a phase of complete exclusion did not occur. Instead, as the cell wall mass was exposed to polymers of increasing $\tilde{M}$, up to 1,000,000 (Fig. 4) and $\tilde{M}$ up to 27.5 nm (Fig. 5), a slight but definite linear regression occurred. Even very large macromolecules may penetrate the broken cell wall. Possibly, the openings between sublayers or lamellae in the lateral fine structure, which would
be exposed when the wall was broken, may be larger than the openings in the surface structure of the intact wall (16).

**Uptake measurements based on molecular size distribution: uptake of separated polymer fractions by intact cells.** It became apparent that permeability thresholds determined by the use of the mean in molecular weight are overestimated, to an extent depending on the polydispersity of the polymers. This error would especially apply to the cell wall, either isolated or in situ, because it sieves relatively large polymer samples, which are more widely dispersed in size (Fig. 1).

To assess the magnitude of the error and the feasibility of employing less dispersed polymer samples, the molecular-weight distribution profile of polyethylene glycol $M_n = 1,450$ (no. 45) was obtained by polyacrylamide gel column chromatography, with analysis of the eluate fractions by precision refractometry (Fig. 6A). When the fractions were collected and pooled in four subsamples and rechromatographed, polydispersity was still evident in each subsample although over a smaller range than in the primary sample (Fig. 6B). The apparent uptake by intact cells was $R^* = 21\%$, based on the mean molecular weight of the primary polymer sample. However, with the four subsamples, the corresponding $R^*$ values were 2, 11, 33, and 50%, and the highest uptake value corresponded to subsample $M_n = 550$ (Fig. 6C). Attempts at large-scale preparation of narrowly dispersed polymer subsamples proved excessively tedious, and attempts to label a primary polymer sample by Wilzbach initiation only increased its polydispersity.

**Uptake measurements based on molecular size distribution: uptake distribution by isolated cell walls.** Efforts were directed toward obtaining molecular-weight distribution profiles of probing polymer samples both before and after uptake, initially by use of isolated cell wall preparations. A series of preliminary experiments was necessary before this result was obtainable. First, polymer samples had to be selected in which the mean of the molecular-weight distribution would approximate the monodisperse uptake threshold (e.g., polyethylene glycol $M_n = 1,450$). Optimal conditions for resolution of the particular polymer sample by gel permeation chromatography had to be determined (e.g., polyacrylamide gel P-10 in a 2 by 30 cm column with flow rate of 30 ml/hr). Next, the molecular-weight distribution had to be determined from the primary elution distribution, either by use of calibration curves or from separate determinations by vapor pressure osmometry (2, 9). Moreover, the concentration of polymer used in obtaining the primary elution diagram had to be reduced to equal the concentration exposed to uptake by the cell structure alone, by correction for the amount of polymer in the interstitium.

After these preliminary determinations were made, elution diagrams for the distribution of polyethylene glycol $M_n = 1,450$ (no. 45) were compared both before and after uptake by isolated cell walls (Fig. 7A). It was apparent that the polymer was not taken up uniformly. Instead, only the smaller molecular size fractions penetrated the cell walls. The two distribution curves intercepted at about fraction no. 34, which represented the point beyond which larger molecules were completely excluded and which corresponded to $M_n \approx 1,000$.

Figure 7B depicts the differential molecular-weight distributions of the same polymer before and after cell wall uptake. Here the differences in the two distribution curves are more apparent, and the intercept can be determined more precisely. This point corresponded to a quasi-monodisperse exclusion threshold of $M_n = 1,200$.

Further refinement in graphical analysis was attained by measuring the incremental percentage difference for the determinations in each fraction, i.e., the per cent uptake, the positive values of which yielded a straight line regression when plotted as a function of the logarithm of the molecular weight (Fig. 7C). The intercept of this line with the zero abscissa represented a quasi-
monodisperse exclusion threshold of $M_n = 1,200$. The composite results of the three graphical analyses thus indicated that monodisperse molecules actually can permeate the isolated cell wall only if equivalently smaller than a glycol of $M_n = 1,200$, which is equivalent to $r_{ES} = 1.1$ nm.

Uptake measurements based on molecular size distribution: uptake distribution by intact cells. The new uptake distribution method was then applied to the sieving of polyethylene glycol $\bar{M}_n = 1,450$ (no. 45) by intact cells of B. megaterium, and the determinations were repeated with polymer samples of both larger and smaller $\bar{M}_n$ values. Three primary elution diagrams were obtained and then transformed into the corresponding differential molecular-weight distribution curves (Fig. 8). As expected, the point of crossing moved in position toward higher molecular weight, and the difference between the curves before and after uptake became less pronounced, as the mean size of the test polymer increased. With all three polymers, however, the point of intersection corresponding to the monodisperse uptake exclusion threshold remained approximately the same, at $M_n \approx 1,000$ to 2,000. Furthermore, when the results with the three polymers were graphically analyzed by plotting the incremental per cent uptake versus the log molecular weight as in Fig. 7C, the resulting lines converged to a common intercept at $M_n \approx 1,000$. Thus, good agreement in results occurred with cell walls for the isolated and in situ states. Taken together, the results with the new uptake distribution method indicated that monodisperse molecules can penetrate the cell wall only if equivalently smaller than a glycol of $M_n = 1,200$ and $r_{ES} = 1.1$ nm.

**Fig. 5.** Uptake and distribution by isolated cell walls of the sugars and dextrans and the glycols and polyglycols as a function of the equivalent hydrodynamic diffusion radius.
Measurements of protoplast sieving based on osmotic stabilization. The thick-suspension technique to measure uptake could not be used with isolated protoplasts, because even low-speed centrifugation produced bursting and leakage. Therefore, substantiating determinations on the permeability of protoplasts were made by measuring the ability of solutes to stabilize protoplasts against osmotic bursting. It has been shown that the stabilizing effect of a homologous series of carbohydrates or amino acids depends on the solute molecular size (27, 28). The larger the solute size, the lower the osmotic concentration needed to stabilize protoplasts. Also, solutes that penetrate into organisms must be present at much higher osmolality than nonpenetrating solutes (21).

A number of the low-molecular-weight compounds were examined for relative ability to stabilize isolated protoplasts (Table 4). Compounds higher in molecular weight were precluded from use because of relative insolubility. Glycols of $M_w \approx 550$ did not stabilize protoplasts at concentrations up to 100% of the liquid compound.

The effective results were plotted with the osmotic concentration necessary for 50% stabilization as a function of the logarithm of molecular weight (Fig. 9A) and of the Einstein-Stokes radius (Fig. 9B). The response curves were hyperbolic and approached an exclusion limit asymptotically. Although poorly defined, the stabilization threshold for the glycols (plus inulin and a comparable dextran) appeared to occur at $M_w \approx 10^4$ and $r_{ES} \approx 3.0 \, \text{nm}$. These values are greater than those observed in the uptake curves for protoplasts in situ (Fig. 2 and 3).

However, the protoplast stabilization curves obtained with the sugars occurred in a range of molecular weight and size decidedly smaller than those with the glycols, and the apparent protoplast stabilization threshold more closely approximated the exclusion threshold in situ. Similar but not directly comparable results on the ability of sugars to stabilize isolated protoplasts have been reported by Marquis and Corner (7, 27). The observed difference in the responses of protoplasts to sugars and to glycols may be explained in part by the lipid solubility of the glycols, whereas the sugars are lipid insoluble (15). Moreover, the sugars were all monodisperse, whereas the effective glycols, as well as the dextran and inulin, were polydisperse in molecular-weight distribution. These two factors are believed to account for the lower range of molecular dimensions in which the sugars were effective in stabilizing protoplasts.

Even though monodisperse, the series of sugars did not reach a finite size threshold ($r_{ES}$) in their relative ability to stabilize protoplasts—instead, the response curve approached a limit asymptotically (Fig. 9). A sugar molecule may be assumed to retain a finite rod shape and rotate in solution. Furthermore, experiments on the permeability of erythrocytes to small molecules have indicated that the penetration proceeds preferably with a lengthwise orientation of the diffusing molecule; i.e., the transverse diameter of the solute molecule is of primary significance (42).

Accordingly, the sugars were assumed to permeate the protoplast membrane openings with a lengthwise molecular orientation. As the length increased, the probability of proper orientation and penetration then would decrease within the finite experimental time (2 hr). This predicted relationship was reflected in an asymptotic approach to a protoplast stabilization limit (Fig. 9).

An approximation was made of the limiting equivalent dimensions of openings in the protoplast membrane by extrapolating to the zero abscissa the linear regression between the pentoses (no. 2 and 3) and the hexose (no. 4). The intercept at $r_{ES} = 0.4 \, \text{nm}$ was believed to represent the equivalent transverse radius of a sugar molecule just excluded from the protoplast membrane (Fig. 9B). This value of 0.4 nm is consistent with molecular dimensions—e.g., direct measurement from a Fisher-Hirschfelder model indicates that the arabinose molecule averages 0.395 nm in

![Fig. 6. Elution chromatograms of polyethylene glycol $M_w = 1,450$ (no. 45) before pooling of the fractions (A) and after (B). The uptake by intact cells of the four pooled fractions is indicated by bar graphs (C). The reference uptake of the unfraccionated polymer was $R^N = 21$. Biorad gel P-10 was employed, with the void volume ($V_o$) indicated.](http://jb.asm.org/Downloaded from http://jb.asm.org/ on November 2, 2017 by guest)
FIG. 7. Distribution in uptake by isolated cell walls of polyethylene glycol $M_n = 1,450$ (no. 45), expressed graphically in three different ways. In each case, the intercept corresponds to a monodisperse exclusion threshold of $M_n = 1,200$. A, Elution chromatograms of the glycol sample in original concentration ($\Delta$), with correction for the interstitium but before uptake ($\bigcirc$), and after ($\bullet$). B, Molecular-weight distribution diagrams of the glycol sample, before uptake ($\bigcirc$) and after ($\bullet$). C, Incremental per cent uptake of the glycol sample as a function of molecular-weight distribution.

FIG. 8. Molecular-weight distribution of three polyethylene glycol samples before uptake by intact cells ($\bullet$) and after ($\bigcirc$). A, Polyethylene glycol $M_n = 1,000$ (no. 43) distributed by use of Biorad gel P-6. B, Polyethylene glycol $M_n = 1,450$ (no. 45), Biorad gel P-10. C, Polyethylene glycol $M_n = 3,350$ (no. 46), Biorad gel P-10.
<table>
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<th>Identification no. *&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sample</th>
<th>Molecular wt</th>
<th>Equivalent molecular radius</th>
<th>Range of concn tested (% w/v)</th>
<th>Per cent (w/v)</th>
<th>Gram per liter per M&lt;sub&gt;n&lt;/sub&gt;</th>
<th>Molarity</th>
<th>Osmotic coefficient</th>
<th>Osmolality</th>
<th>Osmotic pressure (atm)</th>
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<td>~0.31</td>
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<td>3. Arabinose</td>
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<td>0.31</td>
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<td>1.25</td>
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<td>8. Melibiose</td>
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<td>3.4-14</td>
<td>7.9</td>
<td>0.23</td>
<td>~1.04</td>
<td>0.24</td>
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<td>9. Maltose</td>
<td>342</td>
<td>~0.48</td>
<td>3.6-14</td>
<td>7.6</td>
<td>0.21</td>
<td>~1.04</td>
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<td>4.88</td>
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<td>10. Raffinose</td>
<td>504</td>
<td>0.57</td>
<td>5.0-15</td>
<td>7.6</td>
<td>0.15</td>
<td>1.02</td>
<td>0.15</td>
<td>3.43</td>
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<td>11. Melezitose</td>
<td>504</td>
<td>~0.57</td>
<td>5.0-15</td>
<td>7.1</td>
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<td>~1.02</td>
<td>0.14</td>
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<td>12. Stachyose</td>
<td>667</td>
<td>~0.66</td>
<td>3.3-20</td>
<td>6.0</td>
<td>0.09</td>
<td>1.18</td>
<td>0.11</td>
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<td>13. Inulin</td>
<td>3,100</td>
<td>1.40</td>
<td>6.2-31</td>
<td>12.4</td>
<td>0.040</td>
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<td>17. Dextran 10</td>
<td>6,200</td>
<td>2.30</td>
<td>6.2-31</td>
<td>27.9</td>
<td>0.045</td>
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<td>0.22</td>
<td>4.95</td>
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<td>28. Ethylene glycol</td>
<td>62</td>
<td>0.33</td>
<td>0.6-6.2</td>
<td>&gt;6.2&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>35. Polyethylene glycol 200</td>
<td>200</td>
<td>0.51</td>
<td>0.5-20</td>
<td>&gt;20&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>36. Polyethylene glycol 300</td>
<td>300</td>
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<td>0.55</td>
<td>1.0-30&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>38. Polyethylene glycol 400</td>
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<td>0.63</td>
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<td>40. Methoxy-polyethylene glycol 550</td>
<td>550</td>
<td>0.78</td>
<td>1.3-55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;55&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>42. Methoxy-polyethylene glycol 750</td>
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<td>1.1-45</td>
<td>45</td>
<td>0.600</td>
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<td>0.75-50</td>
<td>20.0</td>
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<td>0.75-29</td>
<td>14.5</td>
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<td>18.98</td>
<td>1.90</td>
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<td>49. Polyethylene glycol 6000</td>
<td>8,000</td>
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<td>0.017</td>
<td>4.99</td>
<td>0.18</td>
<td>1.08</td>
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</table>

* See Table 1.

*<sup>a</sup> No lysozyme action occurred at higher concentrations.

*<sup>c</sup> Lysis occurred at the highest concentration possible to use.
half-length and 0.3025 nm in transverse radius (42).

**DISCUSSION**

The above results distinguished the relative abilities of the *B. megaterium* cell wall and protoplast to sieve probing molecules, and provided new and more accurate measures of permeability limits. Other cell structures or substructures may well be functional, but their contribution was not apparent with the methods employed. However, it would be quite possible, for example, to assess the sieving properties of the capsule on a cell.

**Protoplast permeability.** A distinct phase attributable to permeability of the protoplast was evident in each of the biphasic uptake curves for intact cells of *B. megaterium*, with an inflection point that corresponded to the apparent exclusion threshold. Of the two series of test compounds, the monodisperse oligosaccharides were believed to give the most reliable protoplast threshold value in situ, whereas the polyglycols gave higher estimates because of their polydispersity and lipid solubility. Direct measurements of uptake by isolated protoplasts might be accomplished if the amounts of monodisperse sugars taken up were analyzed directly, or if the distribution of a meticulously fractionated polydisperse glycol were determined before and after uptake. However, the value of 0.6 nm for the radius of a just-excluded monodisperse molecule appeared to provide a good first approximation of the permeability limit for the *B. megaterium* protoplast in situ.

An estimate of the protoplast exclusion threshold also was obtained from stabilization experiments with isolated protoplasts. This method introduced the influence of another molecular parameter governing diffusion, namely lengthwise orientation. When this was taken into account, albeit with limited data, a value of about 0.4 nm was obtained as the transverse radius of a just-excluded molecule. It is not clear whether a real difference in limiting porosity exists between protoplasts in situ and isolated, nor which of the two measurements is the more accurate.

Extensive investigations with other cell membranes, especially in erythrocytes (34), have strongly indicated that the passive movement of
water and hydrophilic molecules occurs either by molecular diffusion or by bulk-flow convection through water-filled channels or "pores" in the membrane. The above results with protoplasts, both in situ and isolated, are compatible with this concept as it relates to diffusion. The influence of convection remains to be examined, for example by studying osmotically induced volume changes in isolated protoplasts. Although it is suggested that the dimensions of the membrane openings are comparable to those of the penetrating small molecules, it should again be emphasized that the relationship is complex and the numerical values are relative rather than absolute. It should also be reiterated that the size limitations on passive sieving may not necessarily apply to active transport of small molecules.

It is generally assumed that the limiting barrier for diffusion of solutes into the protoplast is the protoplast membrane and that the internal cytoplasm is not restrictive once the membrane is passed. If both were restrictive, one might expect to detect two separate phases in the region corresponding to protoplast uptake. Although accurate data are not available on the weight (or volume) fraction of the entire cell occupied by the hydrated membrane, a value of 5 to 15% seems likely from estimates of the dry-weight fraction (48, 49). In a plot of uptake data for intact cells such as in Fig. 2 and 3, if this were the case, the protoplast uptake phase would consist of a membrane subphase extending from about $R_w = 25\%$ (corresponding approximately to the cell wall weight fraction) to about $R_w = 35\%$ and of a cytoplasm subphase extending from this second inflection point to the $R_w$ value for water. However, the results did not disclose such a pattern, possibly because of insufficient accuracy in the determinations. Alternatively, the possibility should be considered that the cytoplasm also contributes to molecular sieving, as suggested occasionally for animal cells (24, 46).

A similar question can be raised about the occurrence of uniform openings in the protoplast membrane (iso- or homoporosity) versus size-distributed openings (heteroporosity). If the former prevails, a very steep slope would be expected in the protoplast uptake phase; if the latter occurs, a more gradual slope would be anticipated. However, the results were not sufficiently precise to distinguish between the two models.

**Cell wall permeability.** Another distinct phase, attributable to permeability of the cell wall, was also evident in the biphasic uptake curves for intact cells. This phase extended from the inflection point that corresponded to the apparent protoplast exclusion threshold to the point that corresponded to the apparent cell wall exclusion threshold. Because the latter was determined from the mean in size of polydisperse polymers, overestimation seemed probable. The extent of this error became evident with application of the new uptake distribution method to both in situ and isolated cell walls.

The value of about 1.1 nm for the radius of a just-excluded monodisperse molecule is substantially smaller than the present and previously reported estimates based on polydisperse compounds. Mitchell (31) summarized the fragmentary evidence in 1959 and concluded that "the cell wall of most bacteria is a fairly porous structure which ... acts as a molecular sieve, preventing hydrophilic solutes of mol wt 10,000 or above from leaving the protoplast or reaching its surface from outside the cell wall." A typical hydrophilic solute with a molecular weight of 10,000 is equivalent to $R_{w9} = 2.5$ nm. Subsequent studies by Gerhardt et al. (6, 15, 16, 40) yielded the following approximations of polymeric exclusion thresholds for microbial cell walls: for *B. megaterium*, $\bar{M}_n = 30,000$ to 57,000 and $R_{w9} = 5.4$ to 6.0 nm; for *S. cerevisiae*, $\bar{M}_n = 4,500$ and $R_{w9} = 1.8$ nm; for spores of *B. cereus*, $\bar{M}_n = 160,000$ and $R_{w9} = 9.0$ nm; and for *Micrococcus lysodeikticus*, $\bar{M}_n = 25,000$ and $R_{w9} = 3.6$ nm. (These values are corrected from those previously reported by Gerhardt et al. to correspond with the equivalent $\bar{M}_n$ and $R_{w9}$ values listed in Table 1.) Clearly, cell walls are much more restrictive in their permeability limits for monomers than for polymers.

The question arises whether lengthwise orientation of penetrating molecules occurs in cell walls as it appears to in protoplasts. Giddings et al. (17) have postulated that the width to length ratios of penetrating solutes in a porous network structure would be the determining factor. Although statistically probable for a molecule shaped like a short rod, such linear orientation becomes increasingly improbable as the relative length of the molecule increases. In the absence of accurate molecular dimensions, one can only estimate whether a polyethylene glycol of $M_n \geq 1,200$ might longitudinally diffuse through the cell wall. Ethylene glycol itself measures 0.701 nm in length and 0.475 nm in diameter (42). Polyethylene glycol $M_n = 1,200$ is comprised of about 27 repeating units (Fig. 1). If the polymer chain is considered as linear, its dimensions would be about 0.5 nm in diameter and 15.0 nm in length. The odds seem remote that such a long molecule would chance to diffuse longitudinally through a tortuous channel in the bacterial cell wall within a 2-hr equilibrium period.

Laurent and Killander (23), Ogston (32), and
Giddings et al. (17) have developed theories of porous gel networks by application of statistical and mechanical principles, and have applied them to gels used in gel filtration. Their calculations of the dimensions and total length of the fibrous network making up such gels agrees well with the chemical nature of the gel studied. Application of these theories to isolated cell walls suggests that the total fiber length is about 10^4 μm per μm² of cell wall, and the average fiber radius of cell walls is 0.5 to 1.0 nm. Such a small fiber radius further suggests that the fibrous network in the cell wall exists as single strands of peptido-glycan-teichoic acid rather than as bundles of fibers, as occur in some plant cell walls.

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LITERATURE CITED