PERMEABILITY OF BACTERIAL SPORES

II. Molecular Variables Affecting Solute Permeation

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ABSTRACT

GERHARDT, PHILIPP (University of Michigan, Ann Arbor) AND S. H. BLACK. Permeability of bacterial spores. II. Molecular variables affecting solute permeation. J. Bacteriol. **82**:750–760. 1961.—More than 100 compounds were tested for their uptake by dormant spores of a bacillus. The extent of penetration was found to be dependent on at least three molecular properties: (i) The dissociation of electrolytes usually resulted in high or low uptake predictable from their charge. (ii) Lipid insolubility restricted permeation of small molecules. (iii) The molecular weight of unsubstituted glycol and sugar polymers exponentially limited penetration to eventual exclusion at mol wt above 160,000. The results were plotted as a generalized curve, calculations from which permitted an interpretation that the effective spore surface contains pores varying in diameter from 10 to 200 A.

Dismissal of the hypothesis that dormant spores are completely impermeable has been afforded by a demonstration of glucose penetration occurring as passive diffusion (Black and Gerhardt, 1961). It follows that spores should be permeable also to other molecules similar to glucose; moreover, an examination of unlike materials might reveal the molecular variables governing the penetration of solutes into dormant spores. Accordingly, a survey was made of representative compounds, including sugars and sugar alcohols and polymers, glycols and polyglycols, amino acids and polypeptides, organic acids, dyes, inorganic salts, other selected solutes, and water.

MATERIALS AND METHODS

Details of the experimental procedures have been published before (Black and Gerhardt, 1961). Clean dormant spores of the same test organism, *Bacillus cereus* strain terminalis, were used. The extent of uptake of exogenous solutes again was assessed by the space technique: the resulting uncorrected space value (S^w) indicates the extent of penetration into the spore pack (centrifuged for 30 min at 17,000 × *g*), whereas the corrected space value (R^w) allows for interstitial space ($S_{in}{}^w$) and shows the extent to which the spore itself is penetrated. These indices of total uptake were calculated on a spore weight basis, as indicated by the superscript *w*. Unless otherwise indicated, the permeability determinations were made at neutral pH with freshly prepared solutions.

The dilution of added solute by pack or cell water, which is the basis of the space method, was assayed (at least in duplicate) gravimetrically, isotopically, by carbon combustion, or by the anthrone reaction for carbohydrates (Black and Gerhardt, 1961). When specific analyses were made, polyethylene glycols of high molecular weight were analyzed by the silicotungstic acid precipitation method of Shaffer and Critchfield (1947), inorganic phosphate by the method of Fiske and SubbaRow (1925), and tritium-labeled water by liquid scintillation spectrometry. The method of analysis for a given compound is not specified below, because the measurement of uptake was found to be independent of the assay technique.

RESULTS

The spore permeability determinations with 113 representative compounds are compiled in Table 1. The experimental conditions were comparable for different compounds, although some variations were employed; for example, materials of low molecular weight that might be metabolized were used at 4 C instead of 25 C, and large molecules that required a longer equilibration period were allowed to react for at least 60 min to insure complete and reproducible uptake (see below). The number of determinations and the range in values for S^w, the solute

TABLE 1. *Permeability of dormant spores of Bacillus cereus strain terminalis to different molecular species varying in charge, lipid solubility, and molecular weight*

Compound			Experimental conditions			S^w			
Species	Mol wt	K_e	Solute concn	Temp	Equil time	No. of dets	Range	Avg	R^w, avg
			g/100 ml	*C*	*min*		%	%	%
Sugars and derivatives									
1. Ribose	150		3	4	15	4	45–48	45	41
2. Glucose	180	0.0$_5$45	1 or 3	4	15	87	31–51	45	40
3. Sucrose	342	0.0$_5$11	3	4	15	2	46–46	46	39
4. Melibiose	342		3	4	120	2	44–50	47	44
5. Maltose	360	insol	3	4	15	2	47–47	47	44
6. Raffinose	504	insol	3	4	120	2	45–45	45	42
7. Melezitose	540	insol	3	4	120	2	41–42	41	38
8. Stachyose	667	insol	3	4	120	2	45–46	45	39
9. Glucosamine	179	0.0$_3$1	3	4	15	2	54–61	58	53
10. Glucuronate	194		3	4	15	2	41–43	42	35
11. Acetylglucosamine	221		3	4	15	2	45–45	45	38
12. Erythritol	122	0.0$_3$11	3	4	15	4	46–54	50	42
13. Sorbitol	191	insol	3	4	15	4	55–55	55	48
14. Glycerol	92	0.0$_2$66	1 or 3	4	15	9	44–53	48	43
15. Glycerol monoacetate	134	0.041	3	4	120	2	42–48	45	38
16. Glycerol diacetate	176	0.22	3	4	120	2	46–52	49	43
17. Glycerol triacetate	218	1.4	3	4	120	2	49–51	50	44
18. Inulin	5,000		3	4 or 25	15 or 120	7	34–38	37	31
19. Dextran[a]	1,290[b]	insol	3	25	15	2	40–40	40	34
20. Dextran[a]	1,490[b]	insol	3	25	15	2	41–42	41	35
21. Dextran[a]	1,680[b]	insol	3	25	15	2	40–42	41	35
22. Dextran[a]	1,800[b]	insol	3	25	15	2	39–40	39	35
23. Dextran[a]	2,190[b]	insol	3	25	15	2	40–40	40	36
24. Dextran[a]	2,680[b]	insol	3	25	15	2	37–38	37	33
25. Dextran[a]	3,650	insol	3	25	15 or 120	6	29–34	32	28
26. Dextran[a]	9,600	insol	3	25	120	2	25–27	26	22
27. Dextran[a]	10,000	insol	3	25	120	4	29–33	32	25
28. Dextran[a]	16,000	insol	3	25	120	2	20–21	20	16
29. Dextran[a]	19,000	insol	3	25	120	7	20–24	22	12
30. Dextran[c]	38,000	insol	3	25	120	2	21–22	22	13
31. Dextran[a]	40,000	insol	3	25	120	2	19–20	20	11
32. Dextran[a,c]	80,000	insol	3	25	120	6	7–16	13	4
33. Dextran[c]	100,000	insol	3	25	120	4	11–15	13	3
34. Dextran[a]	150,000	insol	3	25	120	2	11–11	11	2
35. Dextran[c]	175,000	insol	3	25	120	4	6–13	9	2
36. Dextran[a]	500,000	insol	3	25	120	2	9–9	9	0
37. Dextran[c]	700,000	insol	3	4 or 25	15 or 120	78	2–14	9	0
38. Dextran[c]	2,000,000	insol	3	25	120	4	5–14	9	0
39. Polysucrose (Ficoll)[a]	100,000	insol	3	25	120	2	22–24	23	15
Glycols and derivatives[d]									
40. Ethylene	62	0.0053	3	25	120	4	53–57	56	53
41. Diethylene	106	0.0040	3	25	120	2	56–57	57	52
42. Triethylene	150	0.0030	3	25	15 or 120	6	52–57	53	49
43. Tetraethylene	194	0.0024	3	25	120	3	43–51	47	43
44. Polyethylene	200	0.0024	3	25	120	6	42–51	47	42
45. Polyethylene	300		3	25	120	6	41–54	47	40
46. Polyethylene	400		3	25	120	2	42–46	44	38
47. Polyethylene	600		3	4 or 25	15 or 180	4	44–48	46	39
48. Polyethylene	1,000		3	4 or 25	120	8	35–44	41	35
49. Polyethylene	1,450		3	25	120	2	41–41	41	35
50. Polyethylene	3,350		3	25	120	2	34–35	35	28
51. Polyethylene	6,750		3	4 or 25	120 or 180	8	24–31	28	20
52. Polyethylene	17,500		3	4 or 25	120 or 180	10	21–25	24	15
53. Polyethylene	70,000		3	25	120	4	10–16	13	4
54. Ethylene diacetate	146	2.0	3	25	120	2	64–65	65	60
55. Diethylene monomethyl ether	120	0.037	3	25	120	2	46–47	46	40
56. Diethylene monoethyl ether	134	0.064	3	25	120	2	52–54	53	47
57. Diethylene monobutyl ether	162	1.1	3	25	120	2	49–51	50	44
58. Propylene	76	0.018	3	25	120	4	49–59	54	50
59. Dipropylene	134	0.038	3	25	120	4	42–57	50	46
60. Polypropylene	150		3	25	120	4	41–54	48	44
61. Polypropylene	425	0.88	3	4 or 25	120	4	41–47	44	39
62. Polypropylene	1,025	30	3	4	120	2	41–43	42	38

TABLE 1—*Continued*

Compound			Experimental conditions			S^w			
Species	Mol wt	K_e	Solute concn	Temp	Equil time	No. of dets	Range	Avg	R^w, avg
			g/100 ml	*C*	*min*		%	%	%
Other nonelectrolytes									
63. Methylcellulose,[e] 10 cps	25,500		0.5	37	15	2	13–14	14	6
64. Methylcellulose,[e] 50 cps	47,300		0.5	37	15	2	10–14	12	4
65. Methylcellulose,[e] 400 cps	85,000		0.5	37	15	2	3–6	5	−4
66. Methylcellulose,[e] 4,000 cps	142,000		0.5	37	15	2	8–15	12	4
67. Methylcellulose,[e] 7,000 cps			0.5	37	15	1	6–6	6	−3
68. Polyvinyl pyrollidone,[f] k-30	40,000		1	25	120	2	12–16	14	6
69. Polyvinyl pyrollidone,[f] k-60	160,000		1	25	120	4	7–9	8	−1
70. Polyvinyl pyrollidone,[f] k-90	360,000		1	25	120	4	12–15	13	4
Amino acids and derivatives									
71. L- or DL-Alanine	89	0.0514	1	4	15	21	33–39	38	32
72. DL-Valine	117	insol	1	4	15	2	42–48	43	40
73. L-Aspartate	133	insol	0.3	25	120	3	17–23	21	12
74. L-Glutamate	147	insol	0.4	25	120	3	27–35	30	22
75. α-Amino-isobutyrate	103	insol	1	4	15	2	45–47	46	41
76. L-Lysine	146	insol	1	4 or 25	15 or 120	4	44–50	47	42
77. L-Lysine methyl ester	160	insol	1	25	120	2	52–53	52	47
78. Glycine	75	insol	1 or 3	25	120	5	31–40	35	29
79. Glycine methyl ester	89	insol	3	25	120	2	48–52	50	47
80. Diglycine	132	insol	1	25	120	2	38–40	39	33
81. Triglycine	189	insol	1	25	120	2	34–35	35	30
82. Tetraglycine	246	insol	1	25	120	4	31–40	35	29
83. Ribonuclease	12,700		1 in NaCl	25	15	2	37–39	38	34
84. Lysozyme	15,000		1	25	120	2	130–220	175	192
85. Trypsin	23,800		1 in NaCl	25	15	2	60–60	60	58
86. β-Lactoglobulin	35,000		3 in NaCl	25	15	2	8–11	10	4
87. Egg albumin	35,000		3 in NaCl	25	15	2	19–20	19	16
88. Ovalbumin	38,000		3 in NaCl	25	15	2	14–15	15	9
89. Bovine hemoglobin	70,000		3 in NaCl	25	15	2	22–25	24	19
90. Bovine albumin	75,000		3 in NaCl	25	15	2	6–8	7	3
91. γ-Globulin	160,000		1 in NaCl	25	15	2	16–22	19	11
92. Fibrinogen	340,000		3 in NaCl	25	15	2	15–18	17	11
93. Bovine γ-globulin	900,000		3 in NaCl	25	15	6	16–31	24	19
Organic acids									
94. Pyruvate	88	0.16	1	4	15	21	41–55	47	42
95. Succinate	118	0.15	1	4	15	8	48–53	50	44
96. Citrate	192	0.0086	1	4	15	10	45–56	46	41
Dyes									
97. Methylene blue	320		3	25	120	2	70–75	73	70
98. Orange G	452		3	25	120	2	57–63	60	56
99. Eosin Y	692		3	25	120	2	49–53	51	46
Inorganic salts									
100. Phosphate	95		0.1M	4	15	2	42–46	44	40
101. Lanthanum nitrate	325		3	4	15	2	78–80	79	76
102. Sodium chloride	58		0.15M	4	15				30
Other compounds									
103. Urea	60	0.0347	3	4	15	20	54–65	60	56
104. Methyl urea	74	0.0012	3	4	15	2	57–61	59	54
105. Ethyl urea	88	0.0041	3	4	15	2	47–53	50	44
106. *N,N*-Dimethyl urea	88	0.0029	3	4	15	2	63–65	64	59
107. *N,N'*-Dimethyl urea	88	0.0031	3	4	15	2	54–54	54	48
108. Urethane	89	0.64	3	4	15	2	53–60	56	51
109. Paraldehyde	132	8.9	3	4	15	2	63–68	66	62
110. Antipyrine	188	0.073	3	4	15	2	59–61	60	56
111. Adenine	135	insol	0.1	4	15	4	107–111	110	112
112. Adenosine	267		0.3	4	15	14	64–121	79	76
113. Water (^2H_2O)	18	0.018		4	15	20	57–77	70	67

[a] Contributed by or purchased from Pharmacia, Uppsala, Sweden.
[b] Estimated from a probable $M_w/M_n = 1.25$ and from end group analysis for M_n, both provided by Pharmacia.
[c] Contributed by Commercial Solvents Corporation, Terre Haute, Ind.
[d] Contributed by Carbon and Carbide Company, New York.
[e] Contributed by Dow Chemical Company, Midland, Mich.
[f] Contributed by General Aniline and Film Corporation, New York.

space of the spore pack, allow judgment of reliability and variability. The values for R^w, the solute space of the spore itself, were derived by averaging individual $S_{sol}{}^w$ values and correcting for an $S_{in}{}^w$ value determined in each group of experiments.

Molecular species. The results shown in Table 1 reveal that all of the small molecules penetrated dormant spores. The R^w value found for glucose (Black and Gerhardt, 1961) was approximated by other sugars and also by many dissimilar compounds. It appears, therefore, that small molecules typically permeate these spores to an extent of about 40% of the spore weight.

The molecular species in itself did not seem to influence the uptake by spores, as would be expected for an inactive, diffusional process. Nor was specificity observed for physiologically significant compounds, for example those metabolizable or required for germination. Contrary to common notion, several dyes were found to penetrate spores, a fact which also was observed microscopically in spores stained but left unwashed. The uniform distribution and intensity of staining provided evidence that permeability was equal for all the spores in the population.

As the solvent for other permeating molecules, water is pivotal in a study of spore permeability. Tritium-labeled water had a mean R^w value of 67% in 20 replicate determinations, and is being employed further to determine spore water content and distribution.

Molecular dissociation and charge. The survey of permeability determinations revealed a general difference in uptake between electrolytes and nonelectrolytes, the former often showing unusually high (in comparison to glucose uptake) or low space values attributable to the electrostatic charge of ionized radicals, with resultant attraction or repulsion depending on the prevalence of charges in the molecule and on the spore surface. Douglas' (1959) electrophoresis studies led him to suggest that both carboxyl and amino groups are present on the surface of a dormant spore and that they are isoelectric at pH 7; at low pH, the surface amino groups tend to ionize, producing a net positive charge. If this is the case with spores of *B. cereus* strain terminalis, acidic molecules should be taken up less than their neutral counterparts at the usual neutral pH of permeability determinations. Examples were found among the substituted sugars (e.g., glucuronate) and the amino acids (e.g., glutamate and aspartate), although unexplained exceptions occurred (e.g., glycerol triacetate and organic acids); similarly, esterification of carboxyl groups was observed to cause an increase in the uptake of amino acids (e.g., glycine and lysine methyl esters). On the other hand, if the pH is lowered, acidic molecules should be taken up to a greater extent and be held more firmly. A confirmation of this prediction is illustrated in Table 2. With a pH change from 7 to 2.3, the R^w values for citric and succinic acid rose and the extent of recovery from water-washes fell.

TABLE 2. *Effect of pH on uptake of citrate and succinate*

Solute	pH	R^w	Recovery in 2 water washes
		%	%
Citrate	7.0	44	91
	2.3	175	47
Succinate	7.0	46	95
	2.3	107	42

An opposite effect should occur when basic substances are used. At neutrality, unusually high uptake values were thus expected and frequently did happen with such compounds (e.g., glucosamine, methylene blue, adenine, but not lysine). Furthermore, when the pH was changed from 7 to 2, the R^w value for methylene blue, for example, fell from 70 to 54, and an increased amount of dye was recovered by washing.

Lipid solubility. Quantitative assessment of this molecular variable in spore permeability has precedence in animal, plant, and bacterial studies, notably in the extensive work of Collander (1957) with algae. In view of reports (Yoshida et al., 1957; Salton and Marshall, 1959) that the coat of spores is rich in fatty materials, homologous compounds with a range in lipid solubility were included among the test materials in Table 1. Partition coefficients (K_e) in a diethyl ether-water system (Collander, 1949, 1950, 1954, 1960) were arbitrarily chosen as an index of lipid solubility because the composition of spore lipids is not known. Analysis of this factor was necessarily restricted to nonelectrolytes.

Unsubstituted sugars below mol wt 700 were found to penetrate spores to a limited and con-

stant degree (R^w about 40%). These substances are virtually insoluble in lipid, with K_e values less than 0.00001. Unless influenced by charge, other like materials (e.g., amino acids) usually behaved similarly. The suggestion is thus made that lipid insolubility generally restricts solute permeation into spores.

Beyond this generalization, however, a clear relationship could not be established between lipid solubility of small molecules and spore permeability. That is, small lipid-soluble molecules penetrated spores more than lipid-insoluble ones, but the degree of further penetration was not correlated with increasing K_e values. This was exemplified by the glycerol derivatives: although increased acetate esterification imparts a greatly increased solubility in ether, it did not result in significant changes in uptake. Another example was seen in the series of nonpolar polypropylene glycols, in which a 1,000-fold increase in lipid solubility was accompanied by a *decreasing* trend in penetrability. This trend will be shown below to be directly related to molecular weight.

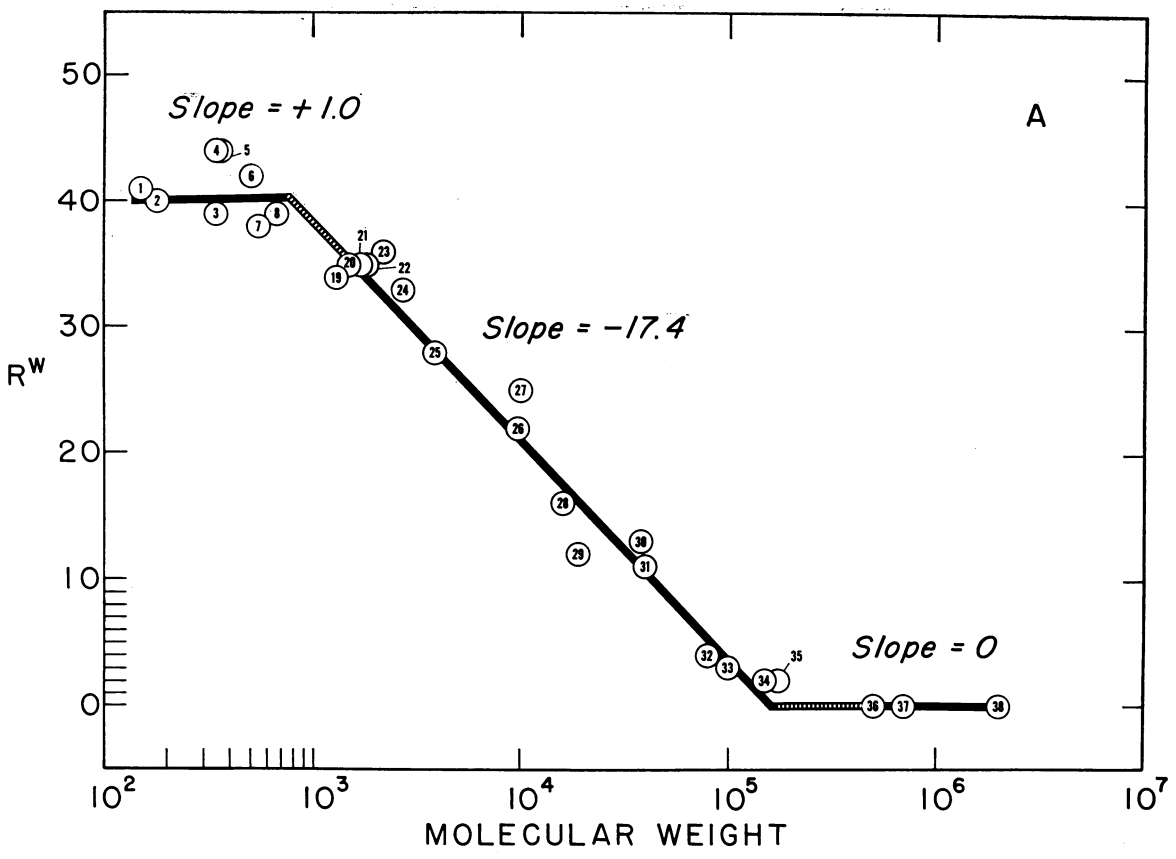
The validity of the results with lipid-soluble materials might be questioned because of the small residuum of sporangial poly-β-hydroxybutyrate granules which contaminated the spore suspensions. This material already was known to affect determinations of intercellular space (Black and Gerhardt, 1961). After completion of most of the experiments, a polypropylene glycol extraction method for separation of these granules became available from A. G. Marr (*personal communication*). Spores so cleaned exhibited unchanged permeability properties when tested with glucose, ethylene glycol, and adenine.

Molecular weight. To facilitate analysis of this variable, permeability determinations were plotted against the logarithm of the corresponding molecular weight values for two groups of generally homologous compounds (listed in Table 1). The unsubstituted monosaccharides, oligosaccharides, and dextrans are shown in Fig. 1*A* and the unsubstituted glycols and their polymers in Fig. 1*B*. Each group afforded a graded series extending over a wide range in molecular weight. Although the polymer molecular weight values are averages, the heterogeneity, which in some instances was known, extended over a narrow range, so that the results were not affected appreciably. Both groups of compounds are water-soluble, nonelectrolytic, and nontoxic; neither induces spore germination. All the sugars are insoluble in lipid, but the low molecular weight glycols are soluble, some very highly so.

Consideration first of small molecules (mol wt less than about 700) revealed two general patterns: (i) The lipid-insoluble sugars (Fig. 1*A*) displayed a relatively constant ability to penetrate spores. This independence in uptake from molecular weight also prevailed with other groups of lipid-insoluble materials (e.g., substituted glycerols, glycine peptides) although not always at the same level of uptake. (ii) The lipid-soluble glycols (Fig. 1*B*) showed a decrease in ability to penetrate spores in linear relation to an increase in the logarithm of molecular weight. Points for both the ethylene and propylene glycols fitted the regression line, despite the fact that the latter compounds become extremely lipid-soluble as they increase in molecular weight. Points for some other small lipid-soluble molecules (e.g., urea, methyl urea, urethane) also followed the trend, whereas others (e.g., ethyl urea, paraldehyde) unexplainably did not. Similarly peculiar results with urea derivatives have been observed in plant cells (Collander, 1957).

Uptake of the macromolecules followed the same regression observed for small lipid-compatible molecules, the relationship thus extending

FIG. 1. *Correlation between solute molecular weight and the uptake by dormant spores. The numbered points correspond to the compounds listed in Table 1. The lines were plotted by the method of least squares, with the points weighted according to the number of determinations. A, unsubstituted monosaccharides, oligosaccharides, and dextrans. The positions of the upper and lower plateaus were set principally by the many determinations with glucose and 700,000 mol wt dextran, respectively. Broken lines indicate extrapolations. B, unsubstituted glycols and polyglycols. A straight line was assumed although there possibly was an inflection at about mol wt 1,000, R^w = 40. Below this coordinate, the slope was −15.9 and above, −17.2.*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A
Slope = −17.4
Slope = 0
R^W
50
40
30
20
10
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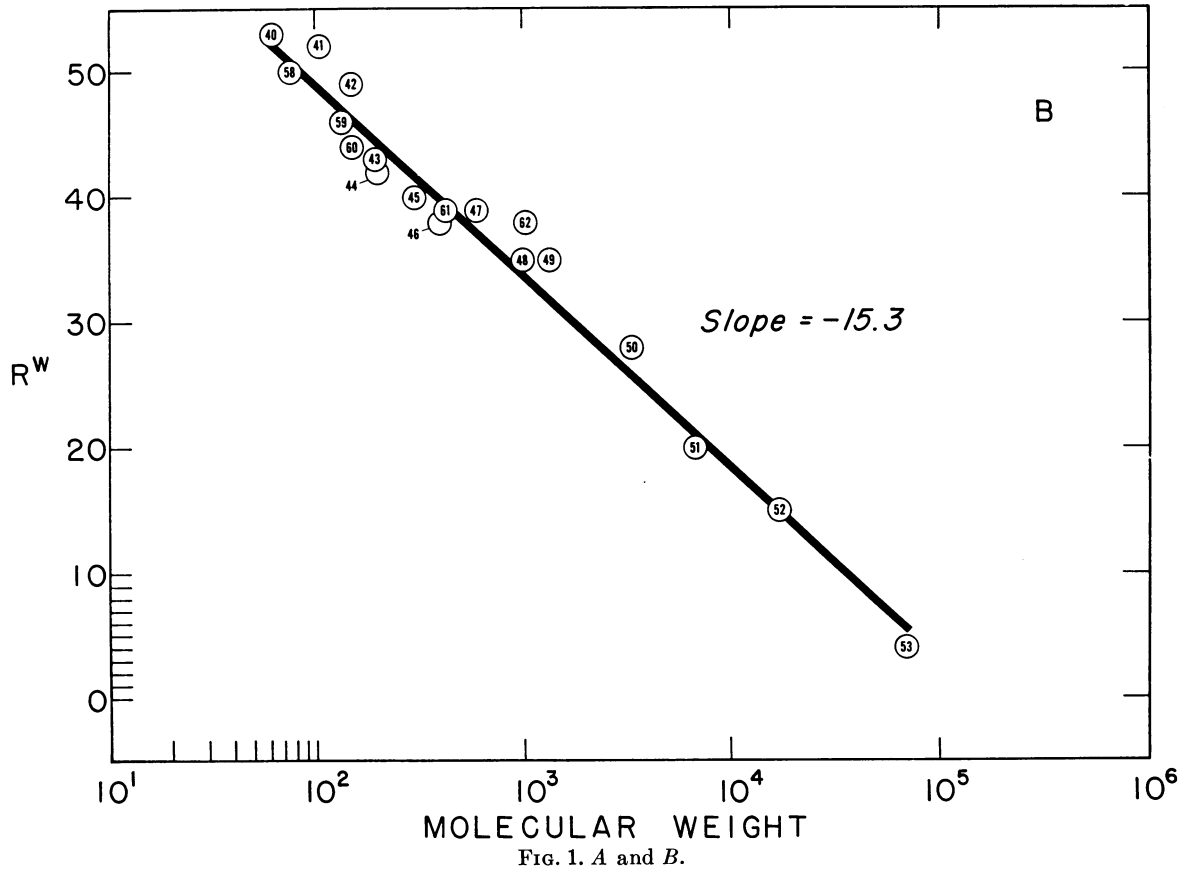
10^2
10^3
10^4
10^5
10^6
10^7
MOLECULAR WEIGHT


B
Slope = −15.3
R^W
50
40
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20
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0
10^1
10^2
10^3
10^4
10^5
10^6
MOLECULAR WEIGHT

FIG. 1. *A* and *B*.

from mol wt 62 to 175,000. Least-squares lines for the polyglycols and the dextrans closely matched each other in slope and position and had a common intercept on the x-axis. Space values for other polymers with similar properties (e.g., inulin and polyvinyl pyrollidones) also approximated the curve, but a series of methylcelluloses gave inconsistent results, probably because of their high viscosity in solution. A series of polypeptides and proteins also gave too scattered a picture to permit reliable conclusions, probably because of their charge.

The factor of molecular weight in uptake of macromolecules is, of course, only an indirect gauge of size and shape. Interpretations of molecular size are made below (*see* Discussion). The influence of shape was assessed in a single comparison between a dextran, a primarily linear polymer of glucose, and a Ficoll, an almost spherical polymer of sucrose, both compounds approximating mol wt 100,000. The S^w value (23%) recorded in Table 1 for Ficoll was significantly higher than that for the comparable dextran (11% in the same experiment). Both molecules act as spheres in solution, but Ficoll apparently is more compact.

The dextran regression line shown in Fig. 1*A* intercepted the abscissa at mol wt 160,000, and permeability measurements with dextrans of mol wt 500,000, 700,000, and 2,000,000 gave a constant R^w value of 0. The corresponding S^w determinations (Table 1) established the interstitial space of the spore pack as 9%. This value represented the average both of the determinations with the three large dextrans and of 78 replications with a single lot of 700,000 mol wt dextran. Proteins of high molecular weight, however, did not provide confirming or even consistent results, and although sometimes used, should not be relied on for measuring interstitium. Inulin also is commonly used but, at least for spores and a coccus (Britt and Gerhardt, 1958), is much too small a molecule. It also should be clear that intercellular space is not a constant value; it varies with the tightness of packing and the presence of extraneous particles (Black and Gerhardt, 1961).

An effort was made to characterize the uptake of dextrans and polyethylene glycols in a manner similar to but more limited than that accomplished for glucose uptake (Black and Gerhardt, 1961). The results were considered increasingly less reliable as materials of increasing molecular weight were studied, principally because the space values diminished while the variability of the space technique remained relatively constant. Nonetheless, several conclusions were drawn: (i) The apparent space value for a given glycol or dextran was independent of the method of analysis, as shown in Table 3. (ii) Negation of metabolic removal, adsorption, and exchange was presumed from the finding that the substances could be recovered with three water washes. (iii) The time factor became measurable with larger molecules. Equilibration was complete in the minimal observable period of 15 min for glucose, triethylene glycol, and mol wt 3,650 dextran. With a polyethylene glycol of intermediate size (mol wt 6,750), however, a longer period was necessary. (In a typical experiment at 4 C, S^w values of 16, 27, and 28% were observed at equilibration intervals of 15, 60, and 180 min, respectively.) Consequently, where there was question of complete equilibration, 120 min was routinely employed. With large molecules that were completely excluded by spores, the time factor was disregarded. If small differences did exist with time (or temperature) in the pack uptake of a molecule as large as dextran mol wt 700,000, they fell within the variability of the determinations. (iv) The variable of solute concentration, found to be influential with glucose in dilute solution only, was also examined with two intermediate-size dextrans (mol wt 10,000 and 19,000). There was a significant (7 to 12%) but unexplained decrease in S^w values when the concentration was reduced from the usual 3 to 0.1%. (v) Two other variables,

TABLE 3. *Determination of polyglycol and dextran uptake by different analytical methods*

Analytical method	S^w		
	Polyethylene glycol		Dextran
	Mol wt 1,000	Mol wt 20,000	Mol wt 700,000
	%	%	%
Gravimetric	39	22	13
Carbon combustion	40	24	14
Silicotungstate ppt of polyglycol	44		
Anthrone reaction with carbohydrate			14

aging of solutions and agitation during equilibration, seemed to affect the extent of uptake of large polymers, but the differences observed were too small to identify with certainty and, from a practical standpoint, were obviated by standardization.

DISCUSSION

The experimental results demonstrated that dormant bacterial spores were permeable to all types of small molecules. The extent of uptake varied and seemed to reflect more the chemical nature of the molecule than active selectivity of the cell. As in glucose uptake by spores (Black and Gerhardt, 1961), the substances apparently entered by a process of passive diffusion. In general, the permeability resembled that usually attributed to a cell "surface" or "outer region" rather than to a plasma membrane. The conclusion seems inescapable that the effective spore surface is porous. Its penetration was found to be governed by at least three molecular variables: molecular charge, lipid solubility, and molecular weight.

Molecular charge. Attraction of basic and repulsion of acidic molecules, whether small or large, usually occurred. Adsorption of cations is a widespread occurrence in cells, and Sussman, von Böventer-Heidenhain, and Lowry (1957) have extensively studied the phenomenon in ascospores of *Neurospora.* Employing bacteria, McCalla (1940) and Harris (1951) have shown that the cell surface behaves like a base exchange system, and Britt and Gerhardt (1958) have specifically identified the cell wall of *Micrococcus lysodeikticus* with adsorptive uptake of lysine.

Lipid solubility. This factor became limiting only for sugars and other small molecules that are virtually insoluble in lipid or lipid-compatible solvents. Collander (1954) was able to establish

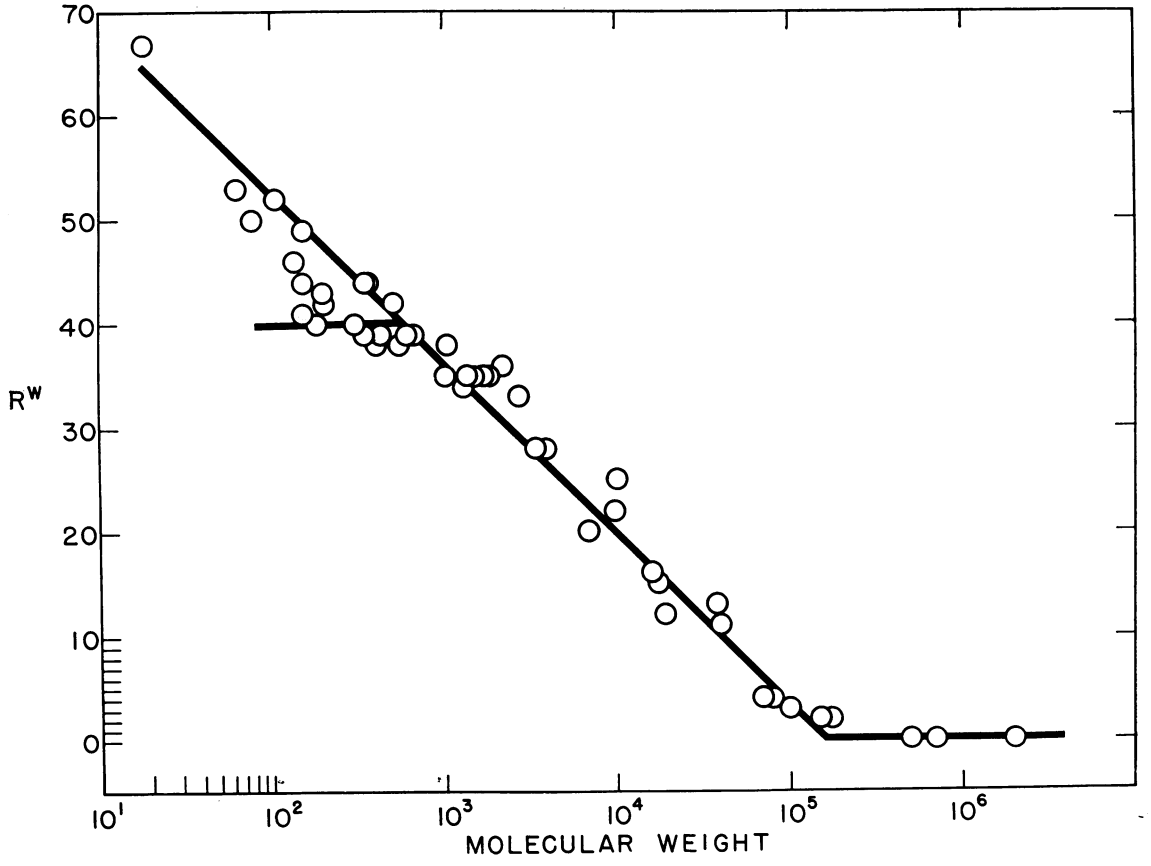

FIG. 2. *Generalized curve for the relationship between molecular weight of linear nonelectrolytes and the uptake by dormant spores. The uppermost point represents water, included for reference.*

that in *Nitella* cells the permeation of substances is proportional to the 1.3 power of the diethyl ether-water partition coefficient. Such a correlation among lipid-soluble compounds clearly did not exist for bacterial spores. In the case of *Nitella* and a number of other cells, the lipid solubility factor seems associated with penetration regulated by a thin, lipoprotein plasma membrane. In spores, the apparent restriction in uptake of lipid-insoluble molecules seems better explained by an internal layer of lipid or lipid-like material (e.g., conceivably the cortex); further penetration into such a layer by lipid-soluble molecules to a degree governed by their size would be expected and was observed.

Molecular weight. Quantitative relationships were established between the molecular weight of unsubstituted glycol and sugar polymers and the uptake by spores. The mutually confirmatory results permitted consolidation into a generalized curve (Fig. 2), which should be applicable to other essentially linear nonelectrolytes entering a spore by diffusion. Four features of this curve were evident: total exclusion of molecules larger than mol wt 160,000; progressively increasing penetration of molecules down to mol wt 550; limited penetration of some (lipid-insoluble) molecules independent of mol wt below 550; and further increasing penetration of other (lipid-soluble) molecules. The principal slope of the curve in fact extrapolates to a point near that established for water, included in Fig. 2 for reference.

Useful interpretations may be placed on this generalized picture if it is kept in mind that the permeability determinations represent equilibrium rather than rate values. If the effective surface of the spore contained pores of equal size, one would expect to see an abrupt transition from a zone of small molecule penetration to another of large molecule exclusion. If such isoporosity existed but were impaired by the presence of wall leaks, one would anticipate a gradual transition markedly influenced by the time required for large molecule equilibration. Such a situation has been described, for example, in blood-lymph capillary exchange (Grotte, 1956). In spores there was a gradual regression in uptake with increasing molecular weight, but equilibrium was reached relatively quickly even with large molecules. The conclusion was thus reached that the effective surface of the spore functions as a molecular sieve and that it consists of a mesh-work of pores varying in diameter.

But what are the pore sizes restricting penetration? Grotte (1956) has reviewed evidence that dextran molecules, which are primarily linear polymers, behave in solution as hydrodynamic spheres. Pappenheimer, Renkin, and Borrero (1951) have shown that in biological permeability problems the effective diffusion diameter (i.e., the diameter of a sphere with equivalent diffusion coefficient), rather than the longest dimension of a long-chain molecule, provides a suitable parameter for molecular dimensions. This so-called Einstein-Stokes diameter is calculated from the formula

$$D_{ES} = \frac{RT}{3\pi\eta DN}$$

where R is the gas constant, T the absolute temperature, η the viscosity of water, D the diffusion coefficient, N Avogadro's number, and D_{ES} the molecular diameter in Ångstrom units. Grotte (1956) concluded that the properties of dextran molecules justify the use of this formula. For spores, the upper limit for penetration was mol wt 160,000, corresponding to a diffusion coefficient of 2.4 (20 C), as obtained by slight extrapolation from a plot of experimentally determined values by Wallenius (1954). In turn, a molecular diameter (D_{ES}) was calculated to be 180 A. A similar estimate of a lower threshold for exclusion was made by employing the point at which the regression line in Fig. 1*A* intercepted the plateau in sugar uptake, at mol wt 550. The corresponding molecular diameter was determined to be 11.2 A, by interpolation from values for glucose, sucrose, and raffinose (Pappenheimer et al., 1951). Thus, in the effective surface of the spore, the smallest pores that restrict entrance of lipid-insoluble sugars approximate a diameter of 10 A and the largest ones, a diameter of 200 A.

The generalization is commonly expressed that large molecules are excluded by bacteria, but little exact information has been available on wall porosity. Mitchell (1959) summarized much of the fragmentary evidence 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 (or diameter above 2.5 mμ) from leaving the protoplast or reaching its surface from out-

side the cell wall." Clearly, the pores in the effective surface of spores are much larger than those in vegetative bacteria, although not greatly different from those in human red cells during hemolysis (Marsden and Östling, 1959). The availability of suitable methods and materials invites a needed study comparing wall porosity among different bacterial cells.

Heretofore we referred to the "effective surface" of the spore without attempting to define its location in the spore structure. Logically, the outermost envelope, or exosporium, would be suspect. Yet, inspection of electron micrographs of intact or isolated exosporium failed to reveal pores of the predicted size (up to 200 A). Moreover, there is evidence for crystalline regularity in the exosporium ultrastructure (Gerhardt, Bannan, and Ribi, 1961), which would result in isoporosity rather than the heteroporosity indicated by the permeability determinations. Physiological evidence against the exosporium functioning as the "effective surface" was suggested by the failure to demonstrate that the exosporium acted as an osmometer. Instead it seems plausible that breaks or rents occur in the exosporium, allowing even macromolecules to pass. Unfortunately, such leaks would not easily be found (and in fact were not found) by examination of electron micrographs, and the permeability determinations were not sufficiently sensitive to distinguish uptake into this layer, estimated to be about 30 mμ in thickness. If the exosporium actually is leaky, it would seem that the heteroporous "effective surface" is represented by the second layer of the spore wall, the so-called outer coat. It remains to be seen which is nearer the truth—a hole in the exosporium or a hole in the theory.

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