PERMEABILITY OF BACTERIAL SPORES

III. Permeation Relative to Germination[1]

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ABSTRACT

Black, S. H. (The University of Michigan, Ann Arbor) and Philipp Gerhardt. Permeability of bacterial spores. III. Permeation relative to germination. J. Bacteriol. **83**:301–308. 1962.—The passive diffusion of solutes into dormant spores, characterized previously with the test organism *Bacillus cereus* strain terminalis, has now been examined in relation to germination. Dormant spores did not take up specific germinants differently than they did other compounds, under conditions optimal for germination. Germinated spores, viable but prevented from growing out, displayed some changes in permeability, evidenced by increased total uptake of glucose and water and by observable penetration of a fluorescigenic dye. Heat-killed spores were as permeable to glucose and the dye as germinated ones.

How dormancy in bacterial spores is maintained, and then on chemical or physical signal broken, has long been subject to speculation. One view has been that inertness is a result of isolation from the environment—that is, a spore is impermeable. Results of experiments testing this view proved the opposite (Black and Gerhardt, 1961; Gerhardt and Black, 1961). Moreover, the fact that a spore can germinate in an appropriate chemical environment would seem to make permeability indispensable. But does the dormant spore take up specific chemical germinants differently than it does other compounds? Is germination accompanied by a change in permeability? If so, will disruptive treatments that might be expected to simulate the effects of chemical germinants also affect solute uptake? Such points were examined in the experiments reported below.

[1] A preliminary account of this study was presented at the VIIth International Congress for Microbiology, 1958.

MATERIALS AND METHODS

Details of the materials and methods have been described previously (Black and Gerhardt, 1961; Gerhardt and Black, 1961). The test species again was *Bacillus cereus* strain terminalis, for which procedures have been developed to obtain hectogram masses of clean, dormant spores. Although the chemical requirements for germination of these spores were known (Church, 1955), the procedures employed for the usual dilute suspensions, even with proportional increases, did not effect rapid and complete germination when dense suspensions (about 10^{11} spores per ml) were used.

Eventually, the following procedure proved successful: Clean dormant spores in water suspension (approximately 50%, w/v) were heated at 65 C for 2 hr. After the spores were centrifuged and the supernatant water decanted, packs of about 10 g wet weight were resuspended in 50 ml of Trypticase soy broth (Baltimore Biological Laboratories) supplemented with 300 mg of L-alanine and 200 mg of adenosine. These suspensions were placed in 250-ml Erlenmeyer flasks and incubated at 30 C on a rotary shaker for 30 min. Germination of the spores was evidenced by their darkness under the phase microscope, stainability, and heat sensitivity; the completeness of germination was judged from the fact that repeated examination of heavy smears on slides did not reveal any ungerminated spores. The spores were separated from the germination broth by centrifugation and then washed twice with deionized water. During final resuspension of the germinated spores, care was exercised not to disturb the tightly packed pellet of crystalline dipicolinic acid, which was released during germination and collected at the bottom of the centrifuge tube. The spores were distributed in 3-g samples into tubes and centrifuged for 30 min at 17,000 $\times$ g; after draining, drying, and weighing the tubes, the washed germinated spores were ready for use. The germinated spores pre-

pared in this way were stable, did not develop into vegetative cells, and remained fully viable for about 3 days if refrigerated. The principle has since been extended to arresting stages of outgrowth (Goldman and Blumenthal, 1961).

As before (Black and Gerhardt, 1961), the uptake of exogenous materials by spores was assayed by the space technique: the resulting space value (R^w), which is corrected for interstitial space and is calculated on a spore weight basis, indicates the percentage of the spore itself that is permeated. The antecedent values for S^w represent the pack space; these are included in one of the tables (Table 4) below.

A possible change in permeability with spore germination was also assessed microscopically by observing uptake of the ultraviolet-fluorescing dye, *n*-tolyl-α-naphthylamine-8-sulfonic acid (National Aniline Division, Allied Chemical Corporation). For fluorescence microscopy, a Reichert 'Fluorex' illuminator and a conventional dark-field microscope were employed. Light from a 200-w maximum-pressure mercury-vapor source (Osram HBO-200) in the illuminator was directed through two pass-filters (Schott UG1-2mm and UG1-1mm) with an optimal passage at 365 mμ. Contact between the microscope condenser and the specimen slide was maintained with immersion oil. The ocular contained an excluding filter (Schott GG 9) barring light below 500 mμ. For spores, it was necessary to use a 20 × objective (0.65 NA) and a 12.5 × ocular. The observations were recorded on Eastman Tri-X film.

RESULTS

Uptake of germination compounds by dormant spores. Spores of *B. cereus* strain terminalis can germinate with adenosine plus L-alanine or glucose (Church, 1955); the rate of germination increases with prior heating at 65 C for 15 min for dilute suspensions. The space in dormant spores available to glucose has been determined to be about 40% and to be essentially independent of environmental variables, including temperature (Black and Gerhardt, 1961); other sugars, ineffective as germination agents, were taken up by dormant spores to about the same degree (Gerhardt and Black, 1961). L-Alanine also failed to penetrate to an unusually high degree and, in fact, was found to be admitted into dormant spores to a consistently lesser extent (32%) than most small molecules. As shown in Fig. 1, both glucose and alanine could be water-

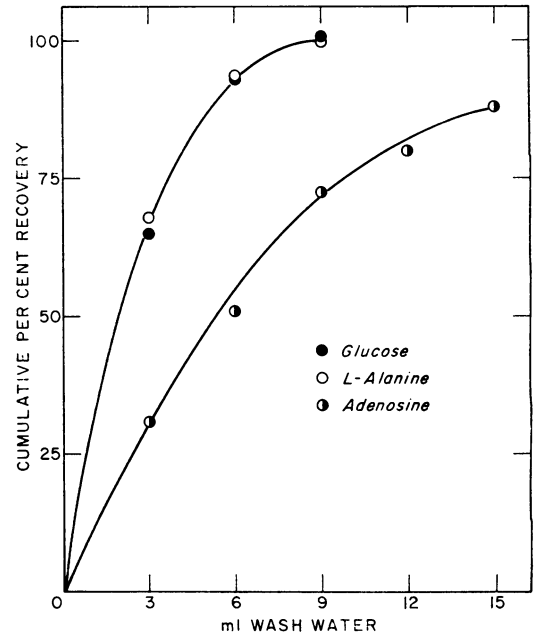

FIG. 1. *Elution of germination compounds from dormant spores. Each tube contained about 3 g of wet spores which were washed with 3-ml increments of water.*

eluted completely and readily. Both L- and DL-alanine were taken up to an equal degree, a finding at variance with the stereospecificity in binding of L-alanine reported by Harrell and Halvorson (1955). However, the amount they reported bound is so small (about 5×10^{-21} moles per spore) that it would be obscured by the predominantly diffusional uptake occurring with the high concentrations used in the present experiments.

In contrast to alanine and glucose, adenosine was taken up by dormant spores to a high degree. As shown by the results in Table 1, however, several lines of evidence suggest that the high but variable adenosine space was attributable to nonspecific adsorption: (i) The amount of adenosine taken up was a function of the quantity of cells available for binding. (ii) The adenosine taken up was resistant to removal by water washing (Fig. 1) unless the pH was reduced near the pK_a (3.45) for this riboside. (iii) The similarly basic adenine component, but not ribose, gave a comparably high uptake value; neither moiety, however, substitutes for adenosine as a germinant.

Although glucose, alanine, or adenosine uptake

TABLE 1. *Evidence for adsorptive uptake of adenosine by dormant spores*

Solute	Equil time	W'_p	R^w	Cumulative recovery in two water washes	
				pH	%
	min	*g*	%		
Adenosine	15	3.00	76	7.0	35
Adenosine	15	3.49	119	3.0	78
Adenosine	60	3.49	121	7.0	58
Ribose	15	3.19	41	7.0	90
Adenine	15	3.16	112		

TABLE 2. *Uptake of germination compounds under various conditions of incubating spores*

Conditions	R^w		
	Glucose	L-Alanine	Adenosine
	%	%	%
0 C for 15 min	40	32	76
30 C for 15 min	42	31	71
30 C for 15 min after heating spores at 65 C for 30 min	40	33	74

TABLE 3. *Uptake of germination compounds, singly and in combination, by dormant spores*

Compound measured	Additional compound present	R^w
		%
Glucose-U-C^{14}	None	39
Glucose-U-C^{14}	Adenosine	39
Adenosine-8-C^{14}	None	78
Adenosine-8-C^{14}	Glucose	81
Adenosine-8-C^{14}	DL-Alanine	78
DL-Alanine-1-C^{14}	None	32
DL-Alanine-1-C^{14}	Adenosine	31

by dormant spores was uninfluenced by incubation at a temperature optimal for germination or by mild heating of the spores (Table 2), it seemed possible that the permeation of these compounds might be different if present in combination, as required for germination. Neither glucose nor alanine in the presence of adenosine, nor adenosine in the presence of glucose or alanine, showed a greater uptake than when presented singly to the spore (Table 3).

Uptake by germinated spores. The first definable event in germination is often considered to be a break in the impermeability of the spore. The evidence that has led to this concept, however, is considerable but only circumstantial. Germinating spores lose their refractility and become dark under phase optics (Pulvertaft and Haynes, 1951; Powell, 1957; Knaysi, 1959; Rode and Foster, 1960), decrease in dry weight by about one-third (Powell and Strange, 1953), exude most of their dipicolinic acid and much of their calcium and a nondialyzable mucopeptide into the germination medium (Powell and Strange, 1953), resume active respiration (Murrell, 1955), and become uniformly stainable with basic dyes (Grethe, 1897; Leifson, 1931; Murrell, 1955). Germinating spores also enlarge (Cohn, 1877; Fischer, 1897; Grethe, 1897; Leifson, 1931; Rode and Foster, 1960), suggesting imbibition of water. Direct evidence for water uptake is lacking, but further indirect evidence is provided by the inability of spores to germinate in environments of low water activity, that is, in concentrated solutions of sucrose (Beers, 1957), triethylene glycol, or sodium chloride (Black and Gerhardt, *unpublished data*).

Permeability determinations on germinated spores are compiled in Table 4. The test compounds were selected from among those used previously with dormant spores (Table 1 in Gerhardt and Black, 1961); the same identifying numbers are retained in Table 4, and the R^w values for dormant spores are reproduced in parentheses for convenience. The antecedent S^w values for germinated spores include figures to allow judgment of the reliability and variability of the permeability determinations.

At once evident from the results shown in Table 4 was the greater uptake of water by germinated spores (R^w = 76%) as compared to dormant spores (R^w = 67%). This immediately suggested the possibility that the increased uptake also observed for some small solutes, glucose especially, was associated with the increased water content. Another possibility was that a lipidlike component of spores is lost during germination, since repeated permeability determinations with glucose showed that its uptake was no longer restricted, as for small lipid-insoluble compounds in dormant spores.

Molecular weight principally regulated solute uptake in dormant spores, and a graphical analysis of this factor for germinated spores is presented in Fig. 2. Within the limitations of the number and scatter of points, the graph revealed

TABLE 4. *Permeability of germinated spores to selected compounds**

Compound		Experimental conditions			S^w			R^w, avg
No.	Species	Solute concn	Temp	Equil time	No. of dets	Range	Avg	
		g/100 ml	*C*	*min*		%	%	%
113	Water		4	15	10	76–86	78	76 (67)
2	Glucose	1	4	15	10	55–59	56	51 (40)
25	Dextran 3,650	3	4	60	3	32–42	37	30 (28)
27	Dextran 10,000	3	4	60	4	24–32	26	18 (25)
29	Dextran 19,000	3	4	120	1	17	17	7 (12)
31	Dextran 40,000	3	4	60	4	11–20	16	7 (11)
34	Dextran 150,000	3	4	60	4	12–13	12	2 (2)
36	Dextran 500,000	3	4	60	4	6–11	8	−1 (0)
37	Dextran 700,000	3	4	15	33	7–10	9	0 (0)
38	Dextran 2,000,000	3	4	60	3	6–13	10	1 (0)
40	Ethylene glycol	3	4	60	3	67–71	69	64 (53)
42	Triethylene glycol	3	4	60	2	61–69	66	62 (49)
46	Polyethylene glycol 400	3	4	60	3	45–48	47	41 (38)
48	Glycol 1,000	3	4	60	3	28–38	33	26 (35)
50	Glycol 3,350	3	4	60	3	25–32	29	21 (28)
52	Glycol 17,500	3	4	60	3	21–26	24	16 (15)
53	Glycol 70,000	3	4	60	3	19–22	21	12 (4)
71	DL-Alanine	1	4	15	6	53–58	56	50 (32)

* Numbers of the compounds correspond to those given in Table 1 of Gerhardt and Black (1961). The R^w values in parentheses are for dormant spores, reproduced from the same source.

several trends: (i) An inverse relationship was preserved between uptake and the log of the molecular weight, and the line slope (−17.5) was only slightly steeper than that for dormant spores (−16.4). (ii) The inflexion point in the correlation line was about the same for the two spore types (130,000 mol wt for germinated spores and 160,000 mol wt for dormant spores), indicating that the loss of polymeric constituents during germination did not change the maximal porosity of the spore coat. (iii) The plateau at an S^w value of about 9, which represents the intersporal space and is equivalent to an R^w value of 0, was maintained.

The foregoing results evidenced some changes in permeability, notably to water and glucose, accompanying germination of spores. It also seemed desirable to visualize the changes, if possible. In a sense, one does so with simple stains, but the ease with which germinated spores stain entirely does not distinguish between a change in permeability and a change in affinity. Newton (1954), however, has used a fluorescigenic dye to demonstrate a change in permeability that occurs in polymyxin-treated bacteria. The dye, *n*-tolyl-α-naphthylamine-8-sulfonic acid (TNS) fluoresces in ultraviolet light if conjugated with negatively-charged groups of protein. Intact *Pseudomonas aeruginosa* cells in the presence of TNS could not be detected in a fluorimeter, but addition of polymyxin immediately resulted in fluorescence, indicating that TNS penetrated to intracellular proteins. This dye has been used in our laboratory for fluorescence microscope examination of hexachlorophene-treated cells (Joswick and Gerhardt, *unpublished data*), and its use seemed especially applicable to the present problem in spores.

To test whether penetration of TNS occurred, spores were germinated, washed, and placed in an 0.85% (w/v) saline solution of 0.001 M TNS. Germinated spores (Fig. 3A) were seen as distinct, brightly fluorescing points; by contrast, the dormant spores (Fig. 3C) were identifiable only by an ill-defined blur. The dim fluorescence of dormant spores on this 30-min photographic exposure was surprising, since the same field was uniformly dark to the eye and to film exposed only 1 min, a time sufficient to record fluorescence of germinated spores. The picture of dormant spores suggested halation, that is, scattering of light beyond its proper boundary, which might

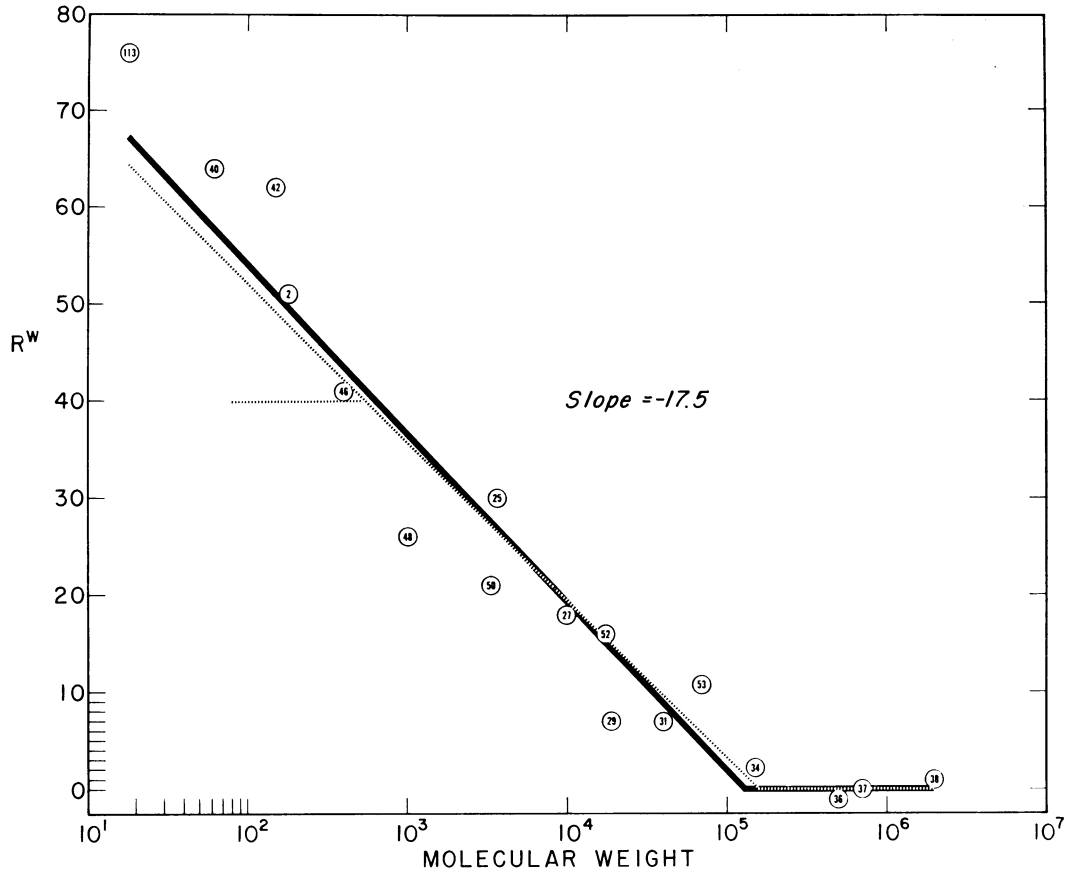

FIG. 2. *Correlation between the molecular weight of dextrans and glycols and the uptake by germinated spores. The numbered points correspond to the compounds listed in Table 4. The lines were plotted by the method of least squares, with the points weighted according to the number of determinations. Points for glucose (no. 2) and water (no. 113) are plotted for reference; if these points are included, the slope becomes −19.2. The broken line represents the corresponding regression line for dormant spores (Fig. 2 in Gerhardt and Black, 1961).*

be produced on the photographic emulsion as an artifact of overexposure or from the dye coupling onto the spore periphery. Since both photographs (Fig. 3A and 3C) were exposed for the same time, it appeared that a halation effect was produced by dye conjugating with spore coat polypeptide. Upon germination, the polypeptide was exuded (Powell and Strange, 1953) and a change in permeability allowed the dye to penetrate to the core, there to couple with intracellular protein. Fluorescence thus originated from a dormant spore over a comparatively large area, whereas the origination of light from a germinated spore, which lacks peripheral peptide, approximated a point source, as observed.

Uptake by chemically and physically treated spores. Treatments that are believed to disrupt membranes might also effect a rise in solute uptake by spores. Organic solvents, for example, often have been thought to enhance cellular permeability, presumably by dissolving a lipid component in the membrane. Killing agents are also generally assumed to cause increased penetration of exogenous substances. Such treatments might be expected to simulate some of the effects brought about by spore germination. Several experiments with treated spores are summarized in Table 5. Dormant spores exposed either to a 5% (w/v) solution of phenol for 150 min or to a saturated solution of *n*-butanol for 5 min were not appreciably altered in their uptake of glucose or in their viability. When the spores were killed

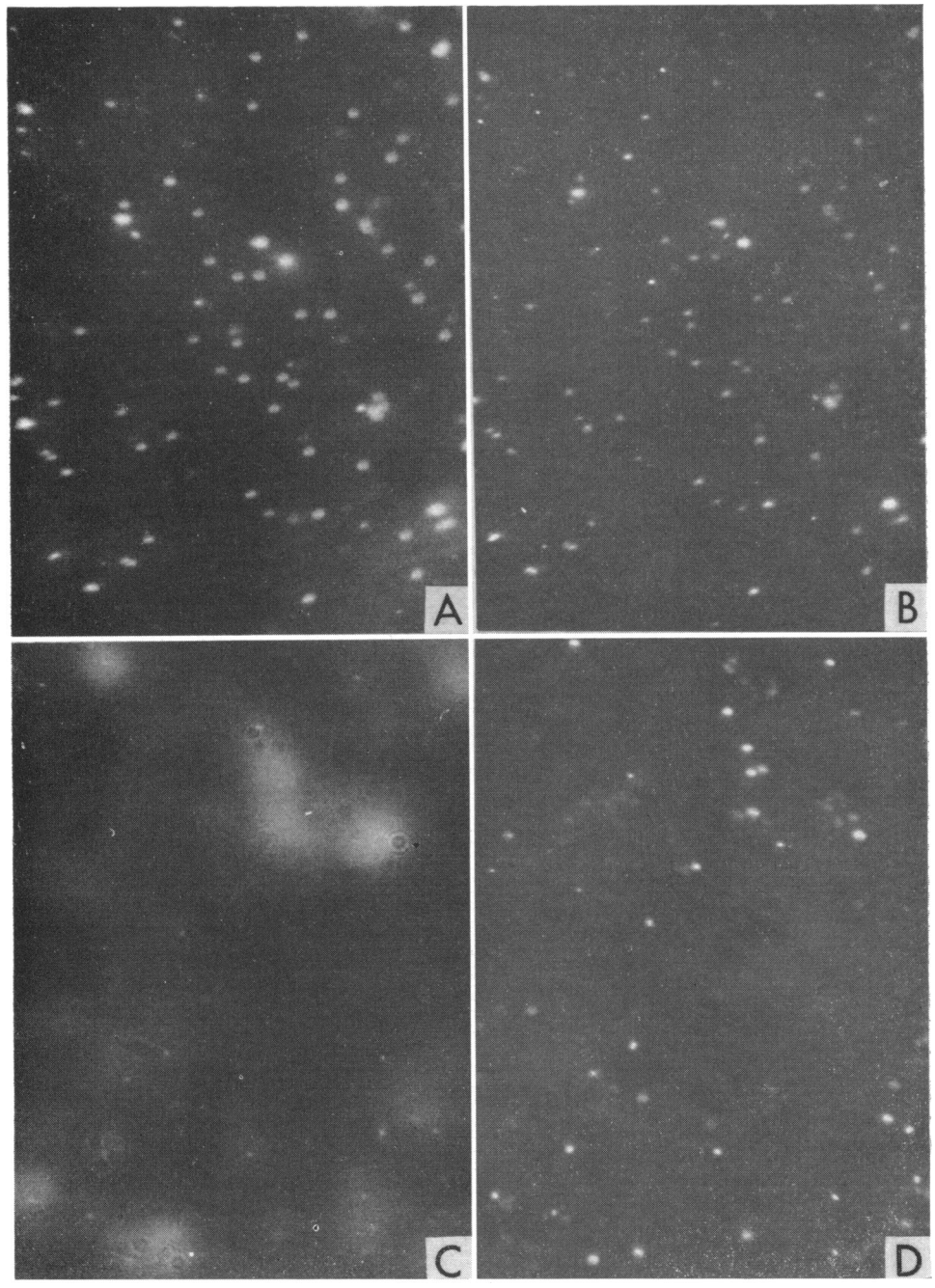

FIG. 3. *Comparison of germinated and dormant spores suspended in a protein-fluorescigenic dye. A. Fluorescence micrograph of germinated spores. B. Reference darkfield micrograph of the same field as A. C. Fluorescence micrograph of dormant spores. D. Reference darkfield micrograph of the same field as C.*

either by mild or extreme heat treatment, however, the uptake of glucose substantially increased and these values were comparable to the R^w value for glucose found for chemically germinated cells (Table 3). Moreover, heat-killed spores fluoresced in the presence of TNS. If killing makes a cell completely permeable, as is usually presumed, then germination of a spore would

TABLE 5. *Glucose uptake by chemically and physically treated spores*

Conditions	Viability after the experiment	Uptake determination	
		S^w Dextran	R^w Glucose
	%	%	%
Standard	100	8	40
Exposed 150 min to 5% (w/v) phenol solution	100	8	45
Autoclaved 60 min at 121 C	0	7	52
Standard	100	9	34
Shaken 5 min in 8% (v/v) butanol solution	100	9	35
Heated 15 min at 100 C	0	9	51

seem from this limited comparison to cause much the same result.

DISCUSSION

Is the dormant spore selectively permeable to germination compounds? No. The experiments reported above indicate that, of the chemical germinants specific for *B. cereus* strain terminalis, glucose penetrated the spores no differently than other sugars, L-alanine was actually admitted to a lesser extent than most small molecules, and adenosine was taken up to a high but explainably nonspecific degree. Nor was the uptake altered when these substances were present in combination, at the temperature optimum for germination, or with spores which had first been mildly heated.

Does permeability change after the spore is germinated? Yes. A number of indirect signs and results of this change are commonly known and were listed above. Direct permeability measurements indicated that the germination of a spore results in some changes in permeability, notably to glucose and water, but the necessarily limited number of determinations permitted only a partial explanation in terms of the molecular variables previously found to govern solute uptake in dormant spores (Gerhardt and Black, 1961). Further evidence was provided by the penetration of a fluorescigenic dye into germinated spores. The occurrence of permeability changes attending germination should not, however, be taken as something unique to spores of bacteria. Pulvertaft and Haynes (1951) have likened the breaking of bacterial spore dormancy to the hatching of amoebic cysts, and others (Fischer, 1897; Burke, 1923) have compared it to the moisture-induced germination of "impermeable seeds" in plants. Beyond qualitative analogy, however, are the detailed experiments of Sussman (1954), who has given quantitative evidence for a change in permeability of ascospores of *Neurospora tetrasperma* during germination.

The third question remains: does killing, which is generally presumed to make a cell completely permeable, simulate the permeability changes brought about by chemical germination? Yes. Dormant spores killed by heat were as permeable to glucose as chemically germinated spores.

The crux for understanding the observed changes in permeability may pertain to a greater *water content* in germinated than in dormant spores, since, to a considerable extent, the larger water-space value obtained for germinated spores accounts for the greater uptake of low mol wt solutes. A problem related to water content, moreover, is that of *water localization*. It may be that a central region is kept anhydrous in the dormant but not the germinated spore (Lewith, 1890; Rode and Foster, 1960). An effort to determine the exact content and location of the water in dormant and germinated spores now seems especially appropriate, not only for resolving the permeability problem but perhaps also for broaching the more general one of heat resistance.

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