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Response of *Bacillus* Spores to Combinations of Germinative Compounds

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ABSTRACT

FOERSTER, HAROLD F. (University of Texas, Austin), AND J. W. FOSTER. Response of *Bacillus* spores to combinations of germinative compounds. J. Bacteriol. 91:1168-1177. 1966.—Spores of 21 strains of *Bacillus megaterium* and 25 other strains representing 13 species of *Bacillus* were produced under standardized conditions. The germination of a washed spore suspension of each strain was measured as a response to various combinations of 30 different germinative compounds. The strains were first typed with respect to their response to "primary" germination compounds, i.e., glucose, L-alanine, inosine, and L-alanine-inosine mixture, and also Na^+ and K^+. The second stage was the determination of the response to various organic and inorganic anions and cations, each strain being supplied with the "primary" compounds best for it. Marked differences in germination patterns were observed among species and strains of the same species. No relation to established taxonomic lines was evident. A nonspecific requirement for ions was found for all strains, but not all ions were effective. A striking degree of interchangeability of germinative chemicals was found. "Fractional germination" was very common. A mixture of L-alanine and inosine and various ions was the best germinative solution for most strains. Some anomalous germination patterns were encountered. Those studied included a strain whose cells lysed spontaneously upon germination and other strains for which L-leucine had striking germinative powers.

Hills' (5, 6) discovery that the germinative powers of yeast extract for bacterial spores is attributable to specific substances marks an epoch in spore science. It triggered inquiry, still going on, that has brought forth an impressively large list of compounds germinative for one or another spore strain. Such compounds have been grouped as "physiological" and "chemical" (19). Realization that assorted, unrelated chemicals induce the conversion of a spore cell to a vegetative cell, while complicating any attempt to arrive at a stereotyped formula, has lent a new dimension to germination theory. Different agents offer clues from the mechanisms of their actions. They certainly disabuse notions that germination is contingent on a few, highly specific compounds, that the mechanisms of action of the various compounds are necessarily the same, that their potencies are the same, and that substances active in one organism work in others. Information regarding germinative chemicals and spore species has accumulated rather haphazardly; rarely has an extensive array of different spores been compared for germinative responses to a battery of compounds for determination of the minimal requirements for germination and, particularly, the extent of interchangeability of germinative compounds. Thus, a perspective is lacking for a pattern of germinative responses of bacterial spores compared under identical conditions and for an appreciation of the flexibility of spores with regard to germination inducers. This work undertakes to remedy that lack. Forty-six strains comprising 14 species, including 21 strains of *Bacillus megaterium*, were used; each was exposed to some 30 germinative compounds in various combinations.

MATERIALS AND METHODS

Bacteria. The organisms were obtained as pure cultures through the courtesy of George Savage, The Upjohn Co., Kalamazoo, Mich.; P. C. Fitz-James, University of Western Ontario, Ottawa, Ontario, Canada; Homer Walker, Iowa State University, Ames; J. Stárka, Charles University, Prague, Czechoslovakia; W. A. Clark, American Type Culture Collection, Rockville, Md.; B. Church, University of

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Spores. The following agar medium was sporogenic for the organisms studied: glucose, 1.0 g; sodium L-glutamate, 1.0 g; yeast autolysate (Basamine, Anheuser-Busch, St. Louis, Mo.), 0.5 g; KH_2PO_4, 5.0 g; $(NH_4)_2HPO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 50 mg; $MnSO_4 \cdot 5H_2O$, 7 mg; $ZnSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4$, 10 mg; agar (Difco), 20 g; deionized water, 1 liter. The agar medium in petri plates was inoculated by spreading uniformly over the agar surface, with a sterile, bent glass rod, 0.1 ml of a bacterial suspension prepared from 8- to 12-hr nutrient agar slants. Sporulation took place in 3 days or less at 30 C. The spores were rinsed from the agar surface and washed six to eight times by centrifugation in about 25 volumes of cold, demineralized water. The final stock suspensions in demineralized water were stored at 4 C; they remained stable indefinitely.

Chemicals. All chemicals used were of analytical or reagent grade. Stock solutions of each germinative compound were prepared with deionized water and stored frozen in polyethylene bottles. Prior to each day's preparation of test solutions, the stocks were melted, a portion was removed, and the remainder was refrozen.

Germination procedures. For each experiment, a fresh portion of the spore suspension was preheated for 60 min at 60 C. This "heat activation," although apparently not applicable to all species of bacterial spores, is sufficiently common to warrant routine use in comparative studies with different organisms, to ensure maximal germination rates (2, 14). The cooled spore suspensions in test tubes in an ice bath were supplemented with ice-cold solutions of the desired compounds to a final volume of 5 ml. Germination was measured as the reduction in optical density (OD), by use of a photoelectric colorimeter. The initial OD was measured on the suspension at 0 C and again after 60 min in a water bath at 40 C; longer incubation did not change the results significantly. Each tube was quickly wiped dry before insertion in the colorimeter. Only a few seconds were required for each measurement, and germinating suspensions could be studied kinetically. The starting suspensions were adjusted to approximately the same density, corresponding to about 100 units in a Klett-Summerson colorimeter. The data are presented as the per cent reduction in OD, with the initial reading at 0 C taken as 100%. The higher the value, the greater the extent of germination was. Fully germinated suspensions of *B. megaterium* and *B. cereus* display a 50 to 70% reduction in OD (23, 24, 25). Microscopy verified that OD reduction was a reliable indication of the percentages of spores which germinated, judging from fully refractile and fully darkened spores.

Classification of germination responses. A formidable problem in tactics is confronted in seeking to determine the germinative responses of a sizable number of spore strains to a sizable number of germinative compounds, particularly to combinations of the latter. The problem was developed in two stages. The first was to obtain a primary classification of each spore strain with respect to its response to main types of germinative compounds. The second was to obtain a subsequent classification of each spore strain with respect to its response to various germinative inorganic or organic ions (21, 23, 24, 25). [Rode and Foster (21, 23, 24, 25) classified germination compounds as ionic (various salts) or nonionic (L-alanine, inosine, glucose). More suitable designations are "strong electrolytes" and "weak" (alanine, inosine) or "nonelectrolytes" (glucose), and the new terms will be used in this paper.] Here, the basal requirements, as determined in the primary classification, were furnished to the respective strains along with the other ionic supplements.

Primary classification. Glucose, L-alanine, and inosine were the principal organic substances employed. Two salts solutions were used: a mixture of sodium iodide, fluoride, nitrate, phosphate, and propionate, and a mixture of the same salts of potassium. In effect, sodium and potassium were tested in the presence of a mixture of the same anions. Along with the synthetic mixtures, a combination containing complex organic extracts was also tested.

Secondary classification. As mentioned, the object here was to determine the specific anion and cation response of the different strains, each furnished with the best germinative compounds found for it in the primary classification. Thus, the basal germination solutions would contain either the sodium or potassium ion, plus either glucose, L-alanine, inosine, or L-alanine and inosine. In these respective solutions would be tested F^-, Cl^-, Br^-, I^-, NO_3^-, CO_3^-, PO_4^-, propionate, succinate, and dipicolinate anions, and also, Mg^{++}, Ca^{++}, Sr^{++}, and Ba^{++} as cations.

RESULTS AND DISCUSSION

Primary classification. Table 1 compares 21 strains of *B. megaterium* and Table 2 compares 25 other strains in 17 different species. This mass of data could be interpreted from a variety of standpoints. Only some general conclusions will be emphasized and features of particular organisms will be pointed out. A great diversity of germination patterns is evident, taking into consideration the qualitative and quantitative responses of each organism to the large assortment of germinative combinations. About the only generalization possible is that germination was negligible in the absence of exogenous compounds and that no one combination was effective for all the different strains.

Regarding the first point, germination reported to occur in distilled water (3, 16) cannot be regarded as an intrinsic characteristic of normal bacterial spores; its apparent occurrence is probably due to adventitious germinative substances that are of exogenous origin or are adsorbed on the spore surface, or to substances of endogenous origin solubilized during heat activation. It would be difficult to eliminate this possibility.

TABLE 1. *Primary germinative compounds* for 21 strains of Bacillus megaterium*

Strain	Complex mixture	Germinative substance added														
		None			Glucose			L-Alanine			Inosine			L-Alanine and inosine		
		No salts	Na salts	K salts	No salts	Na salts	K salts	No salts	Na salts	K salts	No salts	Na salts	K salts	No salts	Na salts	K salts
QM B1551	67	2†	50	66	41	65	68	3	73	74	2	68	72	13	70	75
UC 16	35	3	1	5	10	12	3	7	9	6	5	5	8	5	8	5
KM	7	6	8	7	6	8	8	0	12	15	0	4	0	0	4	8
L	13	4	4	4	4	4	6	4	6	20	2	4	4	2	22	22
Penn	59	3	3	30	32	43	49	3	50	68	6	20	41	2	64	68
Starka	0	7	0	0	0	0	0	0	56	62	0	0	0	0	54	54
Texas	74	6	15	21	10	16	14	15	50	59	15	61	57	14	71	73
4531‡	28	2	0	0	4	4	3	3	2	37	0	2	0	0	57	49
6458	45	2	3	2	2	2	4	2	5	7	2	4	4	0	38	23
6459	50	2	3	2	2	2	0	3	3	11	0	6	4	2	66	47
7051	70	3	5	3	5	5	3	2	67	55	4	71	47	2	75	74
7052	22	0	0	2	2	0	2	2	6	8	2	4	0	2	15	10
7056	41	2	0	0	0	11	19	0	2	0	0	0	0	0	17	0
7481	33	0	3	2	2	2	2	2	4	4	0	2	3	2	28	8
7703	34	0	0	3	2	2	2	0	4	25	0	4	4	2	49	40
9885	90	5	4	2	4	2	2	4	4	24	2	4	4	0	82	53
10778	14	0	0	0	0	2	2	0	2	12	0	2	0	0	14	15
12872	64	0	8	20	13	58	65	0	57	69	0	24	44	0	65	69
13368	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13639	72	2	3	5	2	3	2	2	3	3	2	2	5	3	46	12
14581	50	2	4	2	1	26	18	0	7	3	0	2	2	2	21	8

* Final concentrations: glucose, L-alanine, and inosine, each 0.001 M; mixture of Na iodide, fluoride, nitrate, phosphate, and propionate, each 0.010 M; same for K salts mixture. The complex mixture consisted of: Difco Casamino Acids and yeast extract, each 40 μg/ml; L-alanine, inosine, and glucose, 0.0005 M each; NaCl, NaBr, $CaCl_2$, Na dipicolinate, Na propionate, 0.01 M each; tap water, 75% (v/v), all expressed as final concentrations.

† Results are expressed as per cent reduction in optical density.

‡ Beginning here, numbers are American Type Culture Collection designations.

The so-called "spontaneous germination" may be expected to be contingent on the density of the spore population (*see also* 7, 14).

With regard to the second point of the generalization, even the "complex mixture" that was very potent for most of the organisms tested was totally or negligibly active for three strains of *B. megaterium* and 10 of the others. On still other strains, the action of this "all-purpose" germination solution was feeble. It is clear from Table 1 that the germination response of any one strain is not characteristic of other strains of the same species, and that generalizations on the basis of results with one or even several strains are imprudent. Nor does germinative response conform to established lines of classification. Similar conclusions had been reached by Wolf and Thorley (27, 31) in their investigation of 65 strains of four species of *Bacillus*.

Fractional germination. The conspicuous extent to which intermediate degrees of reduction in OD occurred (Tables 1 to 4) warrants explanation. Germination is an "all-or-none" phenomenon in any one spore, judged by the various parameters which can be employed (14, 19). Once commenced, the process is quickly completed in a matter of seconds or minutes (22, 28). Kinetic data and the sequential use of different germinative compounds often reveal that spores in a given population which have remained ungerminated after 1 hr (Tables 1 to 4) have different requirements, or at least respond differently, than the promptly germinating members of that population. Heterogeneity of spore populations (*see* 1) with respect to germinative responses, "fractional germination" (23), probably is common. This type of variation would be of obvious advantage for the species in nature.

Another manifestation of fractional germination is concentration response. A suboptimal level of a germinative compound(s) will induce germination of some individuals in a spore population and not others. Higher concentrations may effect germination of more or even all of

TABLE 2. *Primary germinative compounds* for species of Bacillus*

Species	Complex mixture	Germinative substance added														
		None			Glucose			L-Alanine			Inosine			L-Alanine and inosine		
		No salts	Na salts	K salts	No salts	Na salts	K salts	No salts	Na salts	K salts	No salts	Na salts	K salts	No salts	Na salts	K salts
B. atterimus	7	13†	15	7	0	13	11	6	16	25	4	7	11	7	14	12
B. cereus T	67	0	10	13	5	2	0	3	8	1	0	56	3	2	67	68
B. cereus var. *mycoides* A6	59	0	2	9	0	7	4	0	2	5	5	7	10	8	59	62
B. cereus UC8	59	2	3	5	0	2	2	3	65	49	14	64	65	25	63	66
B. subtilis var. *globigii* (Ft. Detrick)	11	13	13	8	11	18	14	10	16	25	15	15	10	14	17	20
B. subtilis var. *globigii* K (UT)	7	17	9	9	5	19	18	8	20	23	8	12	9	6	23	20
B. subtilis UC23	57	3	6	7	0	3	8	17	65	65	0	11	12	10	65	65
B. subtilis UC29	40	8	14	7	7	9	10	0	44	62	8	50	43	2	73	74
B. subtilis UT	8	7	6	9	5	17	12	5	15	15	5	6	11	3	10	6
B. larvae UC15	60	4	8	7	0	8	6	2	7	6	6	7	9	20	68	64
B. polymyxa UC20	42	2	12	10	10	13	12	5	38	27	12	39	40	10	39	40
B. stearothermophilus	29	2	5	12	0	11	10	1	45	39	1	6	5	5	45	41
Bacillus 350	0	0	0	0	0	0	0	0	0	0	4	0	0	4	0	0
Bacillus 1A28	42	3	2	4	2	7	2	27	44	51	2	2	4	27	45	42
Bacillus 1A34	52	2	3	3	3	8	6	37	56	60	3	0	2	35	57	60
B. badius 14574‡	10	0	0	2	2	2	4	0	15	15	0	2	4	0	14	8
B cereus 7064	49	3	4	6	0	2	2	0	7	10	0	2	4	4	61	43
B circulans 4513	7	0	0	0	0	12	0	0	0	0	0	0	0	0	10	16
B. circulans 7049	9	0	0	3	0	0	0	0	21	27	0	9	0	0	33	38
B. coagulans 10545	6	2	0	2	4	0	0	0	0	0	0	0	0	0	2	2
B. firmus 14575	9	3	0	0	0	0	0	0	0	0	0	0	0	0	0	9
B. laterosporus 9141	2	0	2	4	2	2	2	0	4	4	0	2	4	0	2	2
B. licheniformis 9259	59	2	4	5	4	4	4	4	9	6	4	7	6	17	69	72
B. polymyxa 842	22	0	16	25	0	8	23	8	14	20	4	19	11	0	25	21
B. subtilis 6051	56	8	10	10	2	5	11	31	66	65	7	4	9	33	65	69

* Same as in Table 1.

† Results are expressed as per cent reduction in optical density.

‡ Beginning here, numbers are American Type Culture Collection designations.

these spores. This reflects population heterogeneity. Tables 1 to 4 are replete with examples of both qualitative and quantitative "fractional germination."

Germination by salts only. Strains capable of substantial germination in the absence of exogenous weak electrolytes or nonelectrolyte organic compounds are few (Tables 1 and 2). *B. megaterium* QM B1551, previously known in this respect (7, 12, 23), is outstanding and, if full germination of the suspension is the yardstick, is unique among the 46 strains tested. The infrequency of this type is also indicated by the fact that, of 40 random soil spore isolates, only one was of this type (26). A few other strains exhibited a partial response to Na or K salts, or both (Tables 1 and 2). *B. polymyxa* 842 germinated as well in salts solution as it did in any other solution, but the suspension did not germinate fully.

Glucose. Certain carbohydrates, including glucose, are germinative (8), but substantially full germination was obtained here in only three strains, all *B. megaterium* (Tables 1 and 2). Any special function ascribed to glucose in germination must be assessed against the fact that the sugar was dispensable; there were two instances of response to glucose substantially greater than that obtained by salts only, and, in both, alanine or inosine was a satisfactory substitute. None of the strains failing to respond to alanine or inosine responded to glucose (Tables 1 and 2). On the basis of the low incidence of the glucose response among the strains studied here, as well as replaceability of the sugar (7, 23), whether spores of any strain of *B. megaterium* really "need glucose specifically for germination" (17) is worth verifying. The relationship between glucose and ions is discussed later.

Inosine. This riboside (interchangeable with

TABLE 3. *Ionic germination of strains of Bacillus megaterium*

Strain	Spore class[a]	Ionic supplements															
		None	F	Cl	Br	I	NO_3	CO_3	PO_4	Propionate	Succinate	$MgCl_2$	$CaCl_2$	$SrCl_2$	$BaCl_2$	CaDPA[b]	MgDPA[b]
QM B1551	K	0[c]	19	20	52	40	54	11	11	51	8	18	45	36	43	62	14
UC 16	Na; Al + In	3	4	5	2	5	8	4	7	10	6	9	9	10	10	5	11
KM	Na; Al + In	0	20	11	4	11	8	27	23	19	20	13	4	0	7	8	6
L	K; Al + In	0	5	2	2	2	6	6	2	19	2	2	2	4	2	2	22
Penn	K; Al	6	7	11	31	34	42	2	5	7	4	10	45	48	46	20	7
Stárka	Na; Al	1	15	2	0	0	0	14	47	49	52	27	5	2	0	0	2
Texas	K; Al + In	11	19	15	28	39	18	66	65	75	69	28	37	30	26	52	64
4531[d]	K; Al	5	13	5	4	6	4	7	15	32	28	23	20	21	21	10	43
6458	Na; Al + In	0	5	0	3	5	4	5	9	34	9	22	31	21	25	2	52
6459	Na; Al + In	3	13	4	7	31	19	17	23	64	15	4	19	8	33	4	48
7051	Na; Al + In	0	73	4	6	16	12	47	75	75	71	67	22	23	23	5	52
7052	Na; Al + In	2	9	3	1	7	4	5	5	16	5	10	7	5	5	7	33
7056	K; Gl	2	17	6	13	14	14	6	14	15	14	11	16	10	9	10	13
7481	Na; Al + In	2	5	0	2	19	9	6	5	33	7	4	5	2	7	4	48
7703	K; Al	2	2	2	4	11	4	8	4	20	4	5	4	2	6	4	4
9885	Na; Al + In	2	34	2	3	3	5	0	5	5	2	2	17	18	16	0	65
10778	K; Al	2	2	2	4	11	4	8	4	20	4	5	4	2	6	4	4
12872	K; Gl	11	34	36	50	47	49	19	36	67	35	2	9	7	12	19	3
13368	Na; Al + In	0	2	2	0	2	2	2	2	4	4	4	4	2	2	2	4
13639	Na; Al + In	5	15	5	7	20	11	7	7	56	9	5	7	7	7	3	56
14581	Na; Gl	0	15	2	2	6	7	27	21	22	17	14	9	13	11	12	2

[a] Based on data in Table 1. The first nine anions were present as either Na or K salts (column 2)' according to which was most germinative in Table 1. Also present were the best organic compounds according to Table 1. Abbreviations: Al, L-alanine; In, inosine; Gl, glucose. All ionic supplements, 40 mM, excepting bivalent chlorides, 20 mM. Al, In, Gl, 1 mM each.

[b] DPA, 40 mM, was titrated to *p*H 7 with $Ca(OH)_2$ or $Mg(OH)_2$. No other inorganic ions were added. Al, In, or Gl was added as designated in spore class column. QM B1551 contained only dipicolinate.

[c] Results are expressed as per cent reduction in optical density.

[d] From here on, numbers are American Type Culture Collection designations.

adenosine but not xanthosine or guanosine; 5, 6, 16, 27) was germinative for five strains of *B. megaterium* (Table 1) and a few other species (Table 2). Like glucose, inosine, whenever it was the sole organic compound active, was with a single exception replaceable by glucose or L-alanine (*see also* 15). The exception was one of the three strains of *B. cereus* tested. Whereas germination of *B. cereus* strain T can be induced with L-alanine (10, 13, 25), concentrations much higher than those we used are required. The data in Tables 1 and 2 show that several organisms are much more sensitive to L-alanine than is *B. cereus* T. Knowledge of the relative potencies of the various germinative compounds, in conjunction with extrinsic factors determining their capacity to express their germinative activity, e.g., *p*H, temperature, and selected ions, expedites a direct access to primary germination mechanisms.

L-Alanine. This amino acid is but one of several with germinative activity for bacterial spores (4, 6, 11, 29). Whether L-alanine has a unique or even special significance among the amino acids is not yet clear. As Murrell (14) put it, "L-Alanine has most commonly shown activity, but it has also been tested more frequently." Nevertheless, in keeping with tradition, we employed L-alanine as a model amino acid in this work.

As others have found (e.g., 4, 30, 31), L-alanine in the absence of the riboside did not induce germination of all spore strains. Only a minority of the strains responded (Tables 1 and 2). For half of these, however, the alanine proved not to be essential; inosine and glucose were very satisfactory substitutes. Regardless, as Tables 1 and 2 reveal, a strong electrolyte environment was prerequisite for maximal alanine activity; in most cases, it was required for any germinative activity of alanine at all.

L-Alanine and inosine. This mixture was previously found effective in concentrations in which each compound singly was relatively ineffective (21, 23, 24). In this work, it was clearly the synthetic germination mixture *par excellence.* It

TABLE 4. *Ionic germination of species of Bacillus**

Species	Spore class	Ionic supplements															
		None	F	Cl	Br	I	NO_3	CO_3	PO_4	Propionate	Succinate	$MgCl_2$	$CaCl_2$	$SrCl_2$	$BaCl_2$	CaDPA	MgDPA
B. atterimus	K; Al	6	10	9	13	11	13	11	15	14	18	10	6	12	15	10	7
B. cereus T	Na; Al + In	6	12	8	4	9	8	19	55	65	59	11	7	10	11	18	33
B. cereus var. *mycoides*	Na; Al + In	3	18	4	9	3	13	10	57	66	55	12	9	19	15	60	54
B. cereus UC8	Na; Al + In	7	59	29	27	17	19	52	68	67	67	23	31	32	25	62	53
B. subtilis var. *globigii* (Ft. Detrick)	K; Al	8	14	16	19	17	16	15	14	23	16	13	16	16	11	14	13
B. subtilis var. *globigii* K (UT)	K; Al	19	18	9	9	16	15	13	20	26	20	12	12	9	10	13	13
B. subtilis UC23	K; Al	23	46	24	32	49	34	49	58	63	59	33	33	30	31	58	60
B. subtilis UC28	K; Al	3	8	18	18	11	18	43	43	66	34	26	32	29	29	16	40
B. subtilis UT	K; Al	6	8	9	7	12	20	13	18	14	18	17	28	21	19	30	20
B. larvae UC15	Na; Al + In	22	51	33	28	15	10	57	67	66	64	28	48	41	32	50	46
B. polymyxa UC20	Na; Al + In	10	34	21	17	13	13	34	43	41	44	14	25	25	18	39	35
B. stearothermophilus	Na; Al	2	7	2	5	5	4	27	7	45	10	7	5	3	3	5	7
Bacillus 350	Na; Al−In	2	4	0	2	6	0	0	6	4	0	6	8	2	9	4	4
Bacillus 1A58	K; Al	27	35	35	38	27	25	25	46	28	47	23	33	34	31	48	51
Bacillus 1A34	K; Al	35	52	49	50	57	48	46	59	56	62	42	46	45	44	54	55
B. badius 14574	Na; Al + In	2	18	4	2	6	8	27	10	35	26	8	11	14	11	8	9
B. cereus 7064	Na; Al + In	6	12	8	4	9	8	19	55	65	59	11	7	10	11	18	33
B. circulans 4513	Na; Al + In	3	0	5	7	0	4	15	8	16	8	17	0	4	0	0	6
B. circulans 7409	K; Al + In	6	21	20	17	9	10	13	19	34	17	12	11	13	11	7	9
B. coagulans 10545	Na; Al + In	0	8	2	0	0	4	8	4	4	2	2	4	4	2	4	4
B. firmus 14575	Na; Al + In	3	12	6	9	12	6	8	3	6	7	4	8	9	7	6	0
B. laterosporus 9141	Na; Al + In	0	2	2	0	0	2	4	2	0	0	0	0	0	0	2	0
B. licheniformis 9259	Na; Al + In	14	62	46	29	12	20	62	64	69	66	70	25	29	23	49	54
B. polymyxa 842	Na; Al + In	0	21	7	6	5	3	23	23	22	19	15	9	0	4	11	8
B. subtilis 6051	Na; Al	30	47	40	43	38	46	50	60	62	56	49	40	42	35	40	47

* Footnotes same as in Table 3.

worked with many strains on which glucose, alanine, or inosine individually failed. In other cases, it elicited a marked augmentation over that with the individual compounds. In the entire survey, only three strains responded less well to alanine-inosine than to the "complex mixture."

The pioneering surveys of Wolf and Thorley (27, 31) were done before the importance of exogenous ions in germination was realized (23, 24, 25), but their experiments were done in phosphate buffer, so a strong electrolyte environment was present. However, their tests were done with alanine and inosine separately. Our findings highlight the remarkable interplay between ions, alanine, and inosine, and especially the striking superiority of the mixture. In fact, many organisms germinated weakly or insignificantly ("fractional germination") when one of the organic pair was omitted. The effects induced by the mixture are obtainable with concentrations that are considerably less than required to secure the same results with the components individually.

Salts. A glance at Tables 1 to 4 reveals that salts are vital for the activity of all of the organic germinative compounds. The strong electrolyte requirement (22–25) seems to apply to virtually all spores. Even in the one possible exception, *B. megaterium* QM B1551, the effects of sugars were remarkably enhanced by salts (7, 8, 23). In this work, the several strains that responded to glucose were clearly aided by the salts (Table 1). Hyatt and Levinson (8) stated that deionized glucose was no less germinative than unpurified glucose. Deionized mannose was, however, less germinative. The extent of enhancement, by addition of appropriate ions, of the germination believed by those authors to be intrinsic to glucose was not considered. In our experience, it has always been possible to demonstrate a strong augmentation of the germination caused by reagent glucose or mannose by the addition of ionic substances. Any intrinsic germination-inducing properties of glucose for a few organisms would seem to be of less general importance than the overriding fact that the appropriate ions themselves can substitute fully for glucose

(Table 1). A vital role for strong electrolytes is also evidenced by their essentiality for alanine-inosine germination. Indeed, ions are the only universally required agents of physiological germination.

As regards Na versus K salts, there was little to choose from. K salts were superior for the Penn and 12872 strains of *B. megaterium* (Table 1). But the most striking finding (Table 2) was the confirmation of the special importance of Na^+ for *B. cereus* T, previously reported by Rode and Foster (25). Such results suggest that due regard must be given to the buffer or other salts used, if generalizations are to be made. As seen later, spore responses to diverse ions are as varied as the responses to weakly ionized or nonionic substances.

"Reluctant" spores. This expression may be infelicitous, but it is intended to imply only that negligible germination took place in some suspensions in 1 hr. Semantics notwithstanding, strains in this category are readily discernible in Tables 1 and 2; they include some apparently inert strains and others with feeble germination tendencies. Additional study of this group is described later in this paper. A similarly reluctant *B. subtilis* was encountered by Wolf and Thorley (31).

Germination inertia may well be genotypic. Such strains would then be at a selective disadvantage in serial culture. Fast genotypes would eventually predominate, in theory. Consequently, the incidence of such types is best determined on natural populations. Of the random isolates of *Bacillus* from soils, 35% were of this type (26).

Ionic responses. Having classified the spore strains primarily according to response to weakly ionic and nonionic agents, as well as to Na^+ and K^+, a secondary classification having to do with other individual ions was now possible. Each spore strain received the respective germinative agents best for it; to this "class" solution were added ionic species individually, and the germination was measured. The results with 46 strains (Tables 3 and 4) confirm and extend our previous conclusions regarding ionic germination made with only three strains (21, 23–25). Every one of the strains responded to the strong electrolyte environment. Although several were able to germinate substantially when no ions were added, even they were strikingly affected by the addition of strong electrolytes. Tables 3 and 4 show that, whereas not all the ions tested were effective for any one strain, neither was there an absolute requirement for a particular ion species.

Phosphate is traditionally employed as a "buffer" in germinative studies. This undoubtedly obscured a different function, namely, as a germinative ion. The buffer influence of phosphate could be incidental and unnecessary in germination; its use, however, has fortuitously assisted germination research. Several examples in Tables 3 and 4 are, nevertheless, reminders that phosphate ion is inadequate for the germination of some strains. In such cases, several other ions, but not all, were effective. Obviously, expression of the maximal germinative potential of any particular strain requires a "calibration" against a spectrum of ions.

As a general conclusion, more strains germinated maximally in the presence of organic ions than inorganic ions. Propionate, succinate, and dipicolinate (DPA) ions were essentially equivalent germinatively. Under our conditions, the DPA was fully effective for numerous strains, but not all. As in earlier work reported from this laboratory (21, 24), we find no indication that calcium dipicolinate (CaDPA) per se (18) is germinative. Other ions are at least essential. Our earlier papers also did not confirm that other pyridine dicarboxylic acids could not substitute for DPA as germination compounds; both pyridine dicarboxylic acid and monocarboxylic acids were active in our system. Moreover, as seen here, in the proper environment other organic acids not regarded as chelators also substitute for DPA. Similarly, Ca^{++} in conjunction with DPA, while effective in many strains, was overshadowed by magnesium DPA. This is particularly evident for strains of *B. megaterium*, for which CaDPA was inactive. The nonspecificity of CaDPA (24) has also been confirmed recently by Jaye and Ordal (9). For a strain of *B. megaterium* in which pure CaDPA is nongerminative, the presence of Na^+ and Cl^- renders it germinative (23). The efficient substitution and even superiority of various anions and cations for metal salts of DPA in our systems, and the importance of the presence of other substances, is construed as a nonspecific ion effect of DPA on germination rather than a specific chelation effect. Since CaDPA solubilization and release is a consequence of germination, we deduce a posteriori that spore DPA does not induce germination (18).

Excepting chloride, halide ions are usually regarded as toxic; but they are efficiently germinative for nearly half of all the spore strains tested (Tables 3 and 4). The superiority of Br^- and I^- for *B. megaterium* QM B1551 (23) does not hold for all strains in which halides induce germination. For example, F^- was much superior in some strains (e.g., *B. megaterium* 7051 and *B. licheniformis* 9259) and as good as the other halides in many other strains.

In all strains, the germinative activity of halides

was matched by nitrate, carbonate, or phosphate ions. On the other hand, more strains responded to the latter group than to the halides.

The inorganic chlorides as a group were active in about half of the strains. No indication was obtained of a specific requirement; in varying degrees, the four inorganic chlorides were interchangeable. A few superiorities within the group were noteworthy. For example, $MgCl_2$ was decidedly superior for *B. megaterium* 7051 and *B. licheniformis* 9259 but decidedly inferior for *B. megaterium* QM B1551 and Penn.

In summary of all the foregoing, there is only one feature common to optimal physiological germination of all spores, namely, a requirement for strong electrolytes. Carbohydrates, amino acids, and ribosides individually effect germination in some strains, but relatively few. These substances are not specifically required and can be replaced by other substances. An amino acid-riboside mixture (L-alanine and inosine) is far superior to either alone. This applies to numbers of strains affected, rate of germination, extent of germination, and molar potency. With few exceptions, strong electrolytes are essential for the expression of the germinative effects of weak electrolytes and nonionic compounds. In some cases, strong electrolytes alone suffice. Even in the exceptions, ions greatly augment germination. These results strengthen the view that the cardinal event in germination is ion-dependent and may be ionic itself—"ionic germination" (23, 24).

Anomalous germination behavior. B. megaterium Stárka germinated readily in alanine-salts, but, in the multicomponent germination mixture which contained both, it did not germinate at all. Obviously, one or more components of the mixture was strongly inhibitory.

B. megaterium 9885 behaved in an extraordinary manner. Its germination was extremely rapid, and the OD reduction considerably greater than in any other strain. It was highest in the complex mixture (Table 1), suggesting an involvement of amino acids. Some individual amino acids were strongly stimulatory, viz., leucine, isoleucine, phenylalanine, proline, valine, and glutamine (Table 5). There is no correlation apparent between structure and germinative activity.

Figure 1 illustrates the profound effect of L-leucine on the germination of strain 9885. The reduction in OD without leucine was slow and quite incomplete (compare the basal germination mixture). In the presence of leucine, however, the reduction exceeded 90%. This remarkable degree of clearing of the suspension suggested that germination was accompanied by spontaneous lysis of the germinated spores. Microscopic examination confirmed the occurrence, practically simultaneously, of germination and lysis. The loss of refractility is seen as a darkening process originating at the periphery of the spore and proceeding inward. Concomitantly

TABLE 5. *Amino acids and the germination of Bacillus megaterium 9885**

Amino acid, 100 μg/ml	OD reduction
	%
None	48
Glycine	25
L-Serine	42
L-Tryptophan	52
L-Lysine	54
L-Arginine	55
DL-Asparagine	55
DL-Ornithine	59
DL-Homocysteine	61
DL-Glutamine	80
L-Valine	82
L-Proline	86
DL-Phenylalanine	87
L-Isoleucine	88
DL-Leucine	92

* Spores preheated 60 min at 60 C; germination, 1 hr at 40 C. Basal germination mixture: L-alanine, inosine, and glucose, 0.001 M each; NaCl, NaBr, $CaCl_2$, Na propionate, and NaDPA, 0.01 M each.

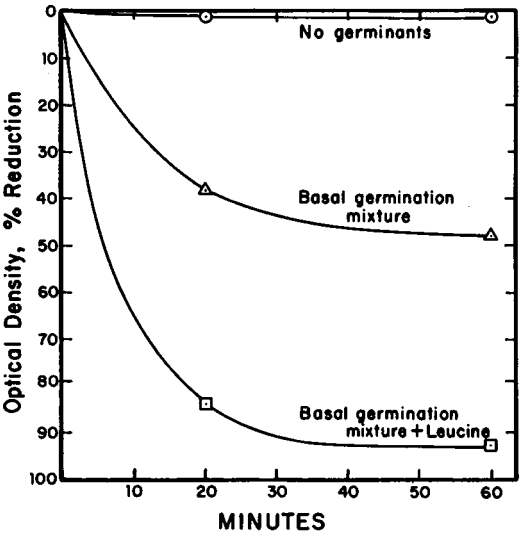

FIG. 1. *Germination of Bacillus megaterium 9885. Germination solution: L-alanine, glucose, inosine, each 0.001 M; NaCl, NaBr, $CaCl_2$, Na propionate, NaDPA, each 0.01 M. L-Leucine, 100 μg/ml. Spores preheated 60 min at 60 C. Temperature, 40 C.*

with darkening, the spores swell. Shortly after darkening is complete, the spores shed the coats. The shedding is sudden, but the coats remain attached through a small region, at least temporarily, to the germinated, dark cell. After 1 hr or longer, free coats or ghostlike remains are seen without intact cells. The entire picture is typical of a situation in which mass lysis has occurred. This lethal germination phenomenon has been studied extensively and will be described in a separate publication (Holdom and Foster, *unpublished data*).

"Reluctant" strains. This designates organisms that would not germinate under conditions adequate for the great majority of other strains. The term may be a misnomer; as shown in the following, in most instances it is merely a matter of ascertaining special requirements for such organisms, whereupon they respond promptly. In any event, the term is a useful denotation.

Temperature and the presence of leucine were the critical factors for all but one of our "reluctant" strains. With respect to the first of those factors, whereas 40 C is suitable for most organisms, that relatively low temperature was inhibitory to a few; they did germinate satisfactorily at room temperature (approximately 25 C). This was discovered when microscopic examination revealed loss of refractility in a substantial fraction of the spore population in a suspension which exhibited a negligible change in OD at the usual 40 C incubation temperature. Removal of a sample and preparation of a wet mount for microscopy was sufficient exposure to room temperature for germination. The existence of spores inhibited by physiological temperatures (37 to 40 C), but germinating at lower temperatures, was first described by Thorley and Wolf (27). They likewise showed that 40 C and even higher was suitable for most spore strains. Results with one such temperature-sensitive strain are presented in Table 6. *B. laterosporus* 9141 also exhibited similar temperature symptoms. At 25 C nearly complete germination occurred in 1 hr, but at 40 C it was negligible even after 2 hr (Table 6). From 2 to 5 hr were required at the higher temperature, clear evidence of inhibition even though germination eventually asserted itself. This experiment also shows that the abnormal temperature behavior of physiological germination did not apply to chemical germination with the surfactant, *n*-dodecylamine. Characteristically, the higher temperature accelerated germination by this universally effective germinative compound (20, 22). Several other "reluctant" strains also germinated promptly in *n*-dodecylamine.

The second factor critical for the so-called "reluctant" strains turned out to be L-leucine. Unnecessary for more than 40 of the organisms studied in this work, this amino acid had a striking effect when added to the alanine-containing mixture of germinative compounds that otherwise was adequate for all the others. For the first four organisms listed in Table 7, the basal germinative solution exhibited only the slightest effect; with leucine added, germination of three was normal; that of *Bacillus* 350 was incomplete. For comparative purposes, strain 9885 was included; its response to leucine (described earlier) appeared to be different from that of the other strains, which did not exhibit lysis. Eighteen other amino acids were tested individually as leucine substitutes. For strains 13639, 10778, and 350, no other amino acid was satisfactory. For strain 14581, however, several other amino acids were as effective as leucine.

TABLE 6. *Germination of temperature-sensitive Bacillus megaterium 13368*

Germinative compound	Per cent reduction in optical density					
	Hr at 25 C			Hr at 40 C		
	1	2	5	1	2	5
None	0	2	0	0	0	0
Physiological*	42	58	62	2	6	57
N-Dodecylamine†	20	35	43	45	47	47

* L-Alanine, inosine, glucose, each 0.001 M; Ca propionate, 0.01 M.
† At 0.0004 M.

TABLE 7. *Germinative activity of L-leucine for some "reluctant" spores*

Strain	Per cent reduction in optical density in 1 hr	
	Basal germination solution*	Basal germination solution + L-leucine†
Bacillus megaterium 13639	7	55
B. megaterium 14581	4	54
B. megaterium 10778	3	42
Bacillus 350	2	24
B. megaterium 9885	58	80

* Glucose, L-alanine, inosine, each 0.001 M; NaCl, $CaCl_2$, NaBr, Na propionate, each 0.01 M.
† At 0.001 M.

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LITERATURE CITED

1. CHURCH, B. D., H. HALVORSON, D. S. RAMSEY, AND R. S. HARTMAN. 1956. Population heterogeneity in the resistance of aerobic spores to ethylene oxide. J. Bacteriol. **72**:242–247.
2. CURRAN, H. R., AND F. R. EVANS. 1945. Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. J. Bacteriol. **49**:335–346.
3. CURRAN, H. R., AND F. R. EVANS. 1946. The viability of heat-activatable spores in distilled water or glucose solutions as influenced by prestorage or poststorage heating. J. Bacteriol. **51**:567–568.
4. HACHISUKA, Y., N. ASANO, N. KATO, M. OKAJIMA, M. KITAORI, AND T. KUNO. 1955. Studies on spore germination. I. Effect of nitrogen source on germination. J. Bacteriol. **69**:399–412.
5. HILLS, G. M. 1949. Chemical factors in the germination of spore-bearing aerobes. The effect of yeast extract on the germination of *Bacillus anthracis* and its replacement by adenosine. Biochem. J. **45**:353–362.
6. HILLS, G. M. 1949. Chemical factors in the germination of spore-bearing aerobes. The effect of amino acids on the germination of *Bacillus anthracis*, with some observations on the relation of optical form to biological activity. Biochem. J. **45**:363–370.
7. HYATT, M., AND H. S. LEVINSON. 1961. Interaction of heat, glucose, L-alanine, and potassium nitrate in spore germination of *Bacillus megaterium*. J. Bacteriol. **81**:204–211.
8. HYATT, M. T., AND H. S. LEVINSON. 1964. Effect of sugars and other carbon compounds on germination and postgerminative development of *Bacillus megaterium* spores. J. Bacteriol. **88**: 1403–1415.
9. JAYE, M., AND Z. J. ORDAL. 1965. Germination of spores of *Bacillus megaterium* with divalent metal-dipicolinate chelates. J. Bacteriol. **89**: 1617–1618.
10. KEYNAN, A., W. G. MURRELL, AND H. O. HALVORSON. 1961. Dipicolinate acid content, heat-activation, and the concept of dormancy in the bacterial spore. Nature **192**:1211–1212.
11. LEVINSON, H. S., AND M. T. HYATT. 1962. Nitrogenous compounds in germination and postgermination development of *Bacillus megaterium* spores. J. Bacteriol. **83**:1224–1230.
12. LEVINSON, H. S., AND M. SEVAG. 1953. Stimulation of germination and respiration of the spores of *Bacillus megaterium* by manganese and monovalent anions. J. Gen. Physiol. **36**: 617–629.
13. MCCORMICK, N. G. 1965. Kinetics of spore germination. J. Bacteriol. **89**:1180–1185.
14. MURRELL, W. G. 1961. Spore formation and germination as a microbial reaction to the environment. p. 100–150. *In* G. G. Meynell and H. Gooder [ed.], Microbial reaction to environment. Cambridge Univ. Press, New York.
15. POWELL, J. F. 1957. Biochemical changes occurring during spore germination in *Bacillus* species. J. Appl. Bacteriol. **20**:349–358.
16. POWELL, J. F., AND J. R. HUNTER. 1955. Spore germination in the genus *Bacillus*: the modification of germination requirements as a result of preheating. J. Gen. Microbiol. **13**:59–67.
17. POWELL, J. F., AND R. E. STRANGE. 1953. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. **54**:205–209.
18. RIEMANN, H., AND Z. J. ORDAL. 1961. Germination of bacterial spores with calcium and dipicolinic acid. Science **133**:1703–1704.
19. RODE, L. J., AND J. W. FOSTER. 1960. Mechanical germination of bacterial spores. Proc. Natl. Acad. Sci. U.S. **46**:118–128.
20. RODE, L. J., AND J. W. FOSTER. 1960. The action of surfactants on bacterial spores. Arch. Mikrobiol. **36**:67–94.
21. RODE, L. J., AND J. W. FOSTER. 1961. Physiological and chemical germination of spores of *Bacillus megaterium*. Z. Allgem. Mikrobiol. **1**:307–322.
22. RODE, L. J., AND J. W. FOSTER. 1961. Germination of bacterial spores with alkyl primary amines. J. Bacteriol. **81**:768–779.
23. RODE, L. J., AND J. W. FOSTER. 1962. Ionic germination of spores of *Bacillus megaterium* QM B1551. Arch. Mikrobiol. **43**:183–200.
24. RODE, L. J., AND J. W. FOSTER. 1962. Ionic and nonionic compounds in the germination of spores of *Bacillus megaterium*. Texas Arch. Mikrobiol. **43**:201–212.
25. RODE, L. J., AND J. W. FOSTER. 1962. Ions and the germination of spores of *Bacillus cereus* T. Nature **194**:1300–1301.
26. RODE, L. J., AND J. W. FOSTER. 1965. Gaseous hydrocarbons and the germination of bacterial spores. Proc. Natl. Acad. Sci. U.S. **53**:31–38.
27. THORLEY, C. M., AND J. WOLF. 1961. Some germination factors of mesophilic spore formers, p. 1–13. *In* H. O. Halvorson [ed.], *Spores II*. Burgess Publishing Co., Minneapolis.
28. VARY, J. C., AND H. O. HALVORSON. 1965. Kinetics of germination of *Bacillus* spores. J. Bacteriol. **89**:1340–1347.
29. WOESE, C. R., H. J. MOROWITZ, AND C. A. HUTCHINSON III. 1958. Analysis of action of L-alanine analogues in spore germination. J. Bacteriol. **76**:578–588.
30. WOLF, J., AND S. A. Z. MAHMOUD. 1957. The effects of L- and D-alanine on the germination of some *Bacillus* spores. J. Appl. Bacteriol. **20**: 373–383.
31. WOLF, J., AND C. M. THORLEY. 1957. The effects of various germination agents on the spores of some strains of *B. subtilis*. J. Appl. Bacteriol. **20**:384–389.