CONDITIONS AFFECTING *BACILLUS MEGATERIUM* SPORE GERMINATION IN GLUCOSE OR VARIOUS NITROGENOUS COMPOUNDS

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ABSTRACT

HYATT, MILDRED T. (Quartermaster Research and Engineering Center, Natick, Mass.) AND HILLEL S. LEVINSON. Conditions affecting *Bacillus megaterium* spore germination in glucose or various nitrogenous compounds. J. Bacteriol. **83**:1231–1237. 1962.—The possibility that there is more than one metabolic pathway for triggering germination of *Bacillus megaterium* spores was investigated. Spores were germinated in seven different "physiological germinants" under varying conditions of concentration, pH, combinations of germinants, temperature before and during germination, and chemical inhibition. L-Alanine and L-valine appear to induce germination via the same metabolic pathway (same inhibitors are effective, similar germination rate and temperature requirements); and glucose and glucosamine also appear to act similarly, but by a different pathway than L-alanine and L-valine. The other germinants, L-leucine, L-proline, and KNO_3, do not correspond in all respects either to the glucose-glucosamine or to the alanine-valine pair in response to the different germination conditions. It is concluded that *B. megaterium* spore germination occurs via more than one pathway.

It has long been known that *Bacillus megaterium* spores germinate in glucose (Powell, 1951) and in L-alanine (Hills, 1950). Elsewhere (Levinson and Hyatt, 1962), we have shown that many nitrogenous compounds, including some amino acids, amines, and inorganic compounds, such as KNO_3 or KNO_2, also induce germination of heat-treated spores of *B. megaterium*.

Keynan, Murrell, and Halvorson (1961) have reported that L-alanine or calcium-dipicolinic acid (Riemann and Ordal, 1961) induce germination of *B. cereus* strain T spores by different or "multiple" pathways, both pathways, however, having a metabolic basis. The concept of "multiple pathways" for triggering germination has aroused our interest, and to test its validity for *B. megaterium* spore germination, glucose and six of the nitrogen-containing compounds shown to be germination agents (Levinson and Hyatt, 1962) were examined. In particular, we looked for differences among these seven compounds in regard to concentration requirements, pH optima, germination rates, effects of combinations of germinants, heating and incubation temperature requirements, and effects of chemical inhibitors. Similarities and differences among the tested compounds, as they affect spore germination, are discussed.

MATERIALS AND METHODS

The same pool of *B. megaterium* (QM B1551) spores and same methods described by Levinson and Hyatt (1962) were used. In general, spores, at a concentration of 1.0 mg (5×10^8 spores) per ml, were incubated at 30 C in potassium phosphate buffer (50 mM, pH 7.0), with the germination agent under investigation. Heated spores were used in all experiments except where effects of combinations of germinants were being studied and a lower base line percentage of germination was desired. With all seven germination agents, the same number of spores stained with methylene blue as appeared dark under phase microscopy (Pulvertaft and Haynes, 1951).

The seven compounds tested as germination agents were obtained from the following sources: glucose, reagent grade, from J. T. Baker Chemical Co.; KNO_3, reagent grade, from Eimer and Amend; D-glucosamine·HCl and L-leucine from Pfanstiehl Co.; L-alanine, L-valine, and L-proline from Nutritional Biochemicals Corp.

RESULTS

Concentration requirements for germination. Concentration requirements of the seven germination agents varied widely (Fig. 1). Germination

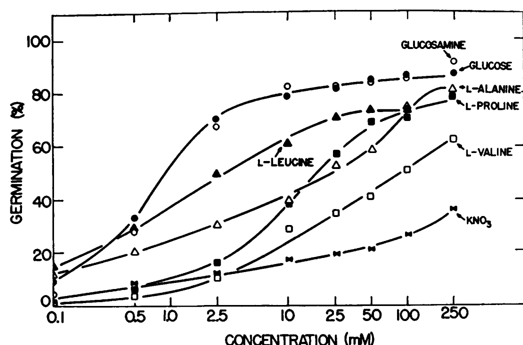

FIG. 1. *Effect of concentration of glucose or of various nitrogenous compounds on the germination of spores of Bacillus megaterium. Germination determined at 2 hr. Spores heated at 60 C for 10 min before incubation at 30 C in phosphate buffer (50 mM, pH 7.0) plus indicated germination agents.*

in three of the compounds (glucose, glucosamine, and L-leucine) reached near maximal levels at concentrations as low as 10 or 25 mM; but germination with the other agents continued to increase with concentrations as high as 250 mM. Furthermore, for 50% germination, 1.0 mM glucose or glucosamine or 2.5 mM L-leucine was required; for 50% germination in the other compounds, higher orders of concentration were necessary (25 mM L-alanine or L-proline; 100 mM L-valine); and in KNO_3, only 35% of the spores germinated in the highest concentration used.

Effect of pH on germination. Heated spores were incubated for 1 hr in phosphate buffer, at pH levels from 5 to 8, in the presence of the seven germinants at 25 mM (Fig. 2). Germination in glucose, L-valine, and KNO_3 occurred over a wide pH range. With glucose and L-valine, germination reached somewhat higher levels on the acid side, and with KNO_3 there was slightly more germination at neutrality. Germination in L-proline and in glucosamine appeared to be favored at alkaline pH levels. Of the seven compounds tested, only L-leucine and L-alanine had definite pH optima (at pH 6). Similar pH responses were obtained when these compounds were used at 2.5 mM.

Germination rate. Heated spores, incubated in the presence of either 25 mM glucose or glucosamine, reached their full germination potential (no further increase in percentage of germination with continued incubation; Hyatt and Levinson, 1961) within 30 min (Fig. 3). In the five other compounds, germination was more gradual, although the full germination potential was attained, in all cases, within 2 hr. The slower rate of germination in the latter five compounds is not a function of concentration; similar rate relationships were obtained when these germination agents were used at concentrations between 2.5 and 100 mM. Nor is the slower germination rate a function of pH, since comparable results were obtained when these compounds were used at the pH level most favorable for germination in each individual agent.

Effects of combinations of germination agents. Unheated spores, and various germinants in concentrations sufficient to support about 10% germination when used singly, were incubated in combination with 10 mM glucose or L-alanine (Table 1). With glucose, the largest stimulation of germination was found with the addition of L-valine and L-leucine, and smaller increases with L-alanine, KNO_3, and L-proline. The apparent glucosamine stimulation of germination in glucose may be due to the presence of the chloride ion (Levinson and Sevag, 1953). With L-alanine, the highest percentage of stimulation resulted from the addition of KNO_3, glucose, glucosamine, or

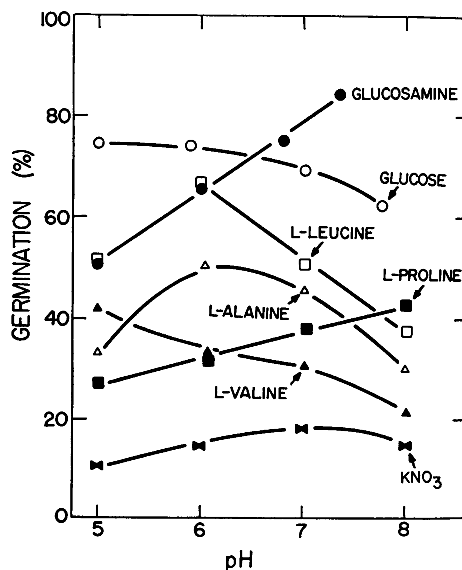

FIG. 2. *Effect of pH on germination of spores of Bacillus megaterium in glucose or in various nitrogenous compounds. Germination determined at 1 hr. Spores heated at 60 C for 10 min before incubation at 30 C in phosphate buffer (50 mM) at the indicated pH levels, plus germination agents (25 mM).*

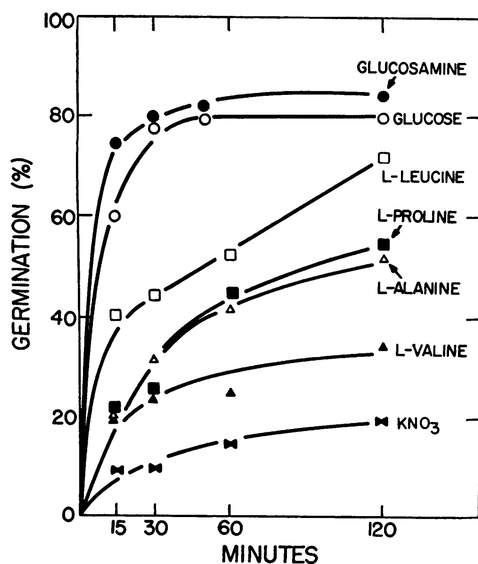

FIG. 3. *Germination of spores of Bacillus megaterium in glucose or in various nitrogenous compounds. Germination plotted as a function of time. Spores heated at 60 C for 10 min before incubation at 30 C in phosphate buffer (50 mM, pH 7.0) plus indicated germination agents (25 mM).*

L-proline (L-leucine having a slighter effect). L-Valine had only a slight stimulatory effect. Comparable results were obtained with heated spores.

Germination and oxygen consumption. The oxygen consumption data (Table 2) of spores germinating in glucose and other compounds suggested that the increased oxygen consumption, which we previously found to accompany germination in glucose (Levinson and Sevag, 1953), was not a necessary concomitant to germination. It seemed more likely that the observed oxygen consumption was a result of the metabolism of the germination agents by the germinated spores. These agents were not all metabolized by the same mechanism, and therefore the level of oxygen consumption did not necessarily reflect the percentage of germination. For example, spores in glucose (2.5 mM) and in L-proline (100 mM) germinated at almost equal rates, but the rates of oxygen consumption in the two compounds were significantly different. Concentrations of glucose, glucosamine, L-alanine, L-leucine, and L-proline could be adjusted to give essentially equal percentages of germination in 2 hr (Table 2), yet the total oxygen consumption in these compounds differed considerably.

These data also suggested that oxidative deamination was not necessary for the utilization of the amino acids in triggering germination, since germination may precede detectable oxygen consumption. Furthermore, various keto acids, the presumed products of oxidative deamination of amino acids, were tested, at pH 5.5, 7.0, and 8.0, as germination agents, both in the presence and in the absence of ammonia [as $(NH_4)_2SO_4$]. Only negligible germination (0 to 9%) of heated spores, incubated for 2 hr with pyruvate (from L-alanine), α-ketoisocaproate (from L-leucine), α-ketoisovalerate (from L-valine), or α-ketovalerate (from L-proline), was obtained.

Germination and temperature of "heat shock." Water suspensions of spores were "heat shocked" for 10 min at various temperatures before incubation in the presence of the various compounds (25 mM at 30 C; Fig. 4). Heating at 40 C, prior to incubation, had little effect on germination; the critical temperature for stimulation of germination appeared to be near 50 C. With the exception of L-alanine-induced germination, where optimal germination occurred after "heat shock" at 70 C, heat treatment at 80 C resulted in the greatest stimulation. Heating at 90 C decreased the percentage of germination, but

TABLE 1. *Effect of addition of various compounds on germination of unheated Bacillus megaterium spores in glucose or in L-alanine*

Additive		Germination (2 hr)				
Compound	Concn	—	+ Glucose (10 mM)		+ L-Alanine (10 mM)	
	mM	%	%	*% increase**	%	*% increase**
None	—	—	24	—	13	—
Glucose	1.0	7	—	—	52	160
Glucosamine ·HCl[1]	2.5	11	40	14	56	133
L-Alanine	1.0	10	53	56	—	—
L-Valine	25	5	68	134	21	16
L-Leucine	10	9	67	103	36	64
L-Proline	25	9	46	39	46	109
KNO_3	25	1	37	48	52	270
[1]KCl	2.5	0	35	46	15	15

* Represents increase over sum of germination in additive plus glucose or plus L-alanine.

TABLE 2. *Germination and oxygen consumption of spores* of Bacillus megaterium*

Germination agent	Concn	Germination (%)			O_2 consumption (μl)		
		Time (min)			Time (min)		
		30	60	120	30	60	120
	mM						
Glucose	2.5	69	70	70	12	40	89
Glucosamine ·HCl	2.5	50	55	67	3	17	46
L-Alanine	100	50	64	69	0	11	36
L-Valine	100	30	40	50	0	7	19
L-Leucine	25	45	53	73	0	7	21
L-Proline	100	62	65	72	0	14	58
KNO_3	250	25	32	35	0	0	7

* Heated spores (60 C, 10 min) incubated in phosphate buffer (50 mM, pH 7.0) plus germination agent.

germination in glucosamine remained at a fairly high level (67%) even after this heat treatment.

Germination and temperature of incubation. Both initial rate of germination (during the first 30 min) and total extent of germination (in 120 min) depended on the temperature of incubation as well as on the particular germination agent used (Fig. 5). However, initial rate and extent of germination were not affected by incubation temperature in the same way. At 13.5 C, germination during the first 30 min of incubation was slow in all of the compounds tested. After 2 hr of incubation (13.5 C), however, spores in glucose or in glucosamine have attained 65% of their maximal germination, but the extent of germination in the other compounds remains low (26% or less of the maximum). At 20 C, spores incubated with L-alanine, L-proline, L-valine, or KNO_3 initially germinated more slowly than in L-leucine. However, by the end of 2 hr, although germination of spores in L-alanine, L-valine, or L-proline increased to the point where they approached the percentage germinating in L-leucine, the extent of germination in KNO_3 remained notably lower (only 21% of the maximum). The initial rates of germination in L-alanine, L-valine, L-proline, L-leucine, and KNO_3 were higher at 30 than at 40 C. The extent of germination in 120 min continued to be lower at 40 than at 30 C in the case of L-alanine and L-valine; with L-leucine, L-proline, and KNO_3, total germination at 120 min was higher at 40 than at 30 C. In the remaining two germinants, glucose and glucosamine, neither the rate of germination nor total germination changed appreciably when the incubation temperature was increased from 30 to 40 C. Incubation at 50 C was inhibitory to germination in all the compounds tested, but perhaps less so in glucosamine than in the other germinants. However, if, after 5 hr incubation at 50 C, the temperature was dropped to 30 C, germination occurred

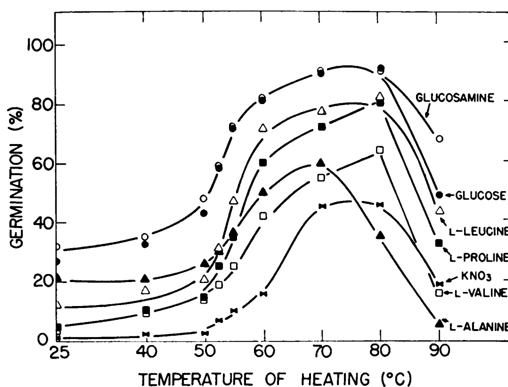

FIG. 4. *Effect of temperature of "heat shock" on the germination of spores of Bacillus megaterium in glucose or in various nitrogenous compounds. Germination determined at 2 hr. Spores heated for 10 min at the indicated temperatures before incubation at 30 C in phosphate buffer (50 mM, pH 7.0) plus germination agents (25 mM).*

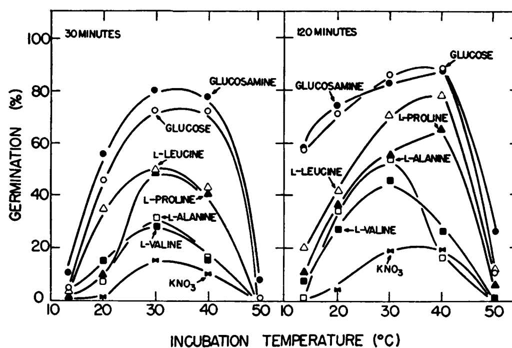

FIG. 5. *Effect of temperature of incubation on the initial rate and on the extent of germination of spores of Bacillus megaterium in glucose or in various nitrogenous compounds. Initial germination rate determined at 30 min, and total extent of germination at 120 min. Spores heated at 60 C for 10 min before incubation at the indicated temperatures in phosphate buffer (50 mM, pH 7.0) plus germination agents (25 mM).*

TABLE 3. *Effect of inhibitors on germination of Bacillus megaterium spores**

Inhibitor	Concn	Percentage inhibition of germination in						
		Glucose	Glucosamine	L-Leucine	L-Proline	L-Valine	L-Alanine	KNO_3
	mM							
D-Alanine	10	—‡	—	6	—	71	100	—
Atebrin	1	—	—	—	—	—	—	—
p-Chloromercuribenzoate	1	—	—	—	—	—	—	—
2,3-Dimercaptopropanol	1	—	—	—	—	73	94	—
2,4-Dinitrophenol†	10	—	—	45	—	39	64	—
Dipicolinic acid	25	45	42	45	51	—	—	89
Dipicolinic acid	10	17	17	—	—	—	—	—
Ethylenediaminetetraacetic acid	10	—	—	—	—	—	—	—
Glycine	10	—	—	6	—	71	52	—
8-Hydroxyquinoline	5	25	23	75	48	95	99	25
8-Hydroxyquinoline	1	—	—	29	—	55	63	—
Isonicotinic acid hydrazide	1	—	—	—	—	—	—	—
Mercuric chloride	10	100	100	100	100	100	100	100
Mercuric chloride†	1	100	69	—	20	—	23	100
Potassium cyanide	10	—	—	—	—	—	—	—
Potassium iodoacetate†	10	21	24	25	18	—	—	100
DL-Serine	25	—	—	—	—	59	70	—
DL-Serine	10	—	—	—	—	57	47	—
DL-Serine	1	—	—	—	—	31	11	—
Sodium arsenite	10	—	—	—	—	—	—	—
Sodium azide†	10	28	69	53	38	65	63	89
Sodium fluoride†	100	Rate slower in all germination agents						

* Heated spores (60 C, 10 min) preincubated with inhibitors for 15 min in phosphate buffer (50 mM) before addition of germination agents (25 mM).

† No inhibition with 1/10 the concentration of these compounds.

‡ — = no inhibition.

promptly in all the tested compounds, indicating that prolonged exposure of spores at this temperature did not irreversibly inactivate any system necessary for germination.

Chemical inhibition of germination. Differential inhibition by various specific enzyme inhibitors and chelating agents of the utilization of various compounds in promoting spore germination was demonstrated (Table 3). Germination in L-valine and L-alanine (not in the other compounds) was inhibited by D-alanine, 2,3-dimercaptopropanol (BAL), glycine, and DL-serine. Germination in these compounds, and also in L-leucine, was inhibited by 2,4-dinitrophenol and by 1 mM 8-hydroxyquinoline. Germination was not inhibited in L-valine or in L-alanine by 25 mM dipicolinic acid or by 10 mM potassium iodoacetate, but it was inhibited in the other germinants. Germination in glucose or in KNO_3 was completely inhibited by 1 mM $HgCl_2$. Sodium azide inhibited germination in all compounds to some extent. Many compounds, such as atebrin, *p*-chloromercuribenzoate, and sodium arsenite, which have been reported to inhibit germination in other species of *Bacillus*, were ineffective in inhibition of *B. megaterium* spore germination.

DISCUSSION

Murrell (1961) recently reviewed a number of postulates purporting to explain the "triggering" of bacterial spore germination by various agents. With the exception of the "mechanical germination" and the "surfactant germination" of Rode and Foster (1960 *a,b*), all of these postulated mechanisms involve some metabolic pathway. These investigators suggested that permeability changes resulting from the disruption of the spore's cortical layer are the basis for spore germination. They further suggested that even so-called "physiological germinants" may act in the

same way, i.e., nonenzymatically by destroying the integrity of a water-impermeable layer. Keynan, Murrell, and Halvorson (1961) have, however, presented evidence that metabolic pathways are involved in at least two germination systems (calcium-dipicolinic acid and L-alanine), and that these pathways differ with the germination agent used.

The experiments which we report here were designed to determine whether any of the seven "physiological germinants," described elsewhere (Levinson and Hyatt, 1962), act similarly in their promotion of the germination of heated *B. megaterium* spores. If we could show that two or more of the germinants (and none of the other compounds) respond in the same way to alteration of such conditions of germination as chemical inhibitors, pH, or temperature, then these particular germination agents might be assumed to be acting in the same way in triggering germination. We find that there are, indeed, significant differences and similarities among the germination characteristics of spores in the presence of the various agents.

L-Alanine-L-valine. Metabolically, the most significant data deal with the chemical inhibition of germination (Table 3). Germination in both L-alanine and in L-valine, but not in the other compounds, is inhibited by glycine, D-alanine, 2,3-dimercaptopropanol, and DL-serine. Germination in L-leucine, as well as in L-alanine and in L-valine, is inhibited by 2,4-dinitrophenol and 8-hydroxyquinoline (1 mM). In the presence of dipicolinic acid or potassium iodoacetate, germination in five of the germinants is inhibited, but that in L-valine and in L-alanine remains unaffected. These data, then, suggest that the same initial site of action may be involved when germination is triggered by L-alanine or L-valine. Aside from the chemical inhibition studies, other data also appear to reflect similarity in the action of these two amino acids, although there are exceptions which do not permit exclusion of the possibility that other germinants may also act like L-alanine or L-valine. Germination of spores in L-alanine is not increased by the addition of L-valine, and both L-alanine and L-valine stimulate glucose-induced germination, although L-valine is somewhat more effective than L-alanine in this respect; incubation at 40 C inhibits germination in these amino acids, but not in any of the other germinants; the same order of magnitude of concentration is necessary to achieve 50% germination with these two amino acids, and this differs from the concentration requirement of any of the other germinants (except L-proline); the initial germination rate (per cent germination per minute, in the first 15 min of incubation) in these compounds is equal (1.3% per min), and this differs from the rate in all of the other germinants (except L-proline).

Glucose-glucosamine. In general, the data suggest that glucose and glucosamine share a "pathway" for germination differing from that utilizing L-alanine and L-valine. Both glucose and glucosamine require the same concentration to achieve 50% germination; the initial germination rate in these compounds is essentially equal (glucose, 4% per min; glucosamine, 5% per min), and this rate differs substantially from that in the other germinants; "heat shock" at 90 C reduces the maximal germination attained in these compounds less than in the other germination agents. There is significantly more germination in glucose and in glucosamine at 13.5 C than there is in the other compounds; the addition of glucosamine does not stimulate germination in glucose; and both compounds stimulate L-alanine-induced germination.

Although the evidence for the similarity of action of L-alanine and L-valine and the differences in the action of this pair of germinants from that of glucose and glucosamine is not clear-cut, notably in regard to pH optima and to the effect of "heat shock" at 80 C, it does appear certain that germination of *B. megaterium* spores can be induced through at least two pathways. Of the other physiological germinants, L-leucine, L-proline, and KNO_3 can not be said to correspond completely in their action to either of the above pairs of germinants.

It is interesting to speculate that, not only may there be several pathways for utilization of various compounds in triggering the germination of *B. megaterium* spores, but that the pathway for utilization of any one germinant may vary with the species of spore-former. O'Connor and Halvorson (1959) reported that an inhibitor of pyruvate oxidation (10^{-2} M sodium arsenite) inhibits L-alanine-induced germination in *B. cereus* strain T (*B. terminalis*), and concluded that pyruvate oxidation is necessary for germination. Furthermore, Falcone, Salvatore, and Covelli

(1959) found that germination of *B. subtilis* spores in L-alanine is inhibited by atebrin (an inhibitor of oxidative deamination) and by *p*-chloromercuribenzoate (an inhibitor of oxidative decarboxylation of pyruvate), and they therefore concluded that both of these enzymes are necessary for L-alanine-induced germination of *B. subtilis* spores. Our present data, on the other hand, indicate no inhibition of L-alanine-induced germination of *B. megaterium* spores, by atebrin, by *p*-chloromercuribenzoate, or by arsenite. Oxidative deamination of L-alanine and pyruvate oxidation may not, then, necessarily be involved in *B. megaterium* spore germination. This is further suggested by our oxygen consumption data. No oxygen consumption is detectable when spores germinate in L-alanine and in some of the other amino acids, even when a considerable percentage of the spores have germinated. Furthermore, the products of oxidative deamination (ammonia and keto acids) do not support an appreciable amount of germination.

ACKNOWLEDGMENT

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