NITROGENOUS COMPOUNDS IN GERMINATION AND POSTGERMINATIVE DEVELOPMENT OF *BACILLUS MEGATERIUM* SPORES

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Received for publication December 18, 1961

ABSTRACT

LEVINSON, HILLEL S. (Quartermaster Research and Engineering Center, Natick, Mass.) AND MILDRED T. HYATT. Nitrogenous compounds in germination and postgerminative development of *Bacillus megaterium* spores. J. Bacteriol. **83**:1224–1230. 1962.—Of the 48 inorganic and organic nitrogenous compounds tested, only L-alanine, glucosamine, and *N*-acetylglucosamine promote germination of unheated spores of *Bacillus megaterium*. Heated spores also germinate in nitrate, nitrite, L-cysteine, DL-isoleucine, L-leucine, DL-methionine, DL-norleucine, L-proline and L-valine.

A source of nitrogen is required for postgerminative development, but nitrogenous compounds which effect germination do not necessarily support postgerminative development. Nitrogenous compounds which support postgerminative development include $(NH_4)_2SO_4$, nitrates, D-alanine, L-alanine, L-arginine, L-asparagine, L-aspartic and L-glutamic acids, glutamine, L-proline, adenine, adenosine, and guanosine. Oxygen consumption rates during postgerminative development are different with different nitrogen sources, and these rates, in general, reflect the extent of postgerminative development. Utilization of amino and ammonium nitrogen during postgerminative development was followed, and the concentration requirements were determined (ca 10 mM for at least one cell division of 5×10^8 spores). Inhibitor studies on postgerminative development are included.

Spores of *Bacillus megaterium* germinate in simple media containing glucose, L-alanine, or KNO_3 (Hills, 1950; Powell, 1951; Hyatt and Levinson, 1961). It is, therefore, evident that neither carbon nor nitrogen sources are specifically required for germination. However, postgerminative development (Levinson and Hyatt, 1956) of the germinated spore into a vegetative cell requires sources of phosphorus, sulfur, and nitrogen in addition to a metabolizable carbon source, such as glucose (Hyatt and Levinson, 1957, 1959, 1961).

Levinson and Sevag (1953) reported that certain amino acids such as aspartic and glutamic acids fail to support spore germination in *B. megaterium*. Woese, Morowitz, and Hutchison (1958) found that, in *B. subtilis*, a series of L-alanine analogues either initiated germination, inhibited L-alanine-induced germination, or were inactive. Hachisuka et al. (1955) have demonstrated that, in *B. subtilis*, the utilization of a nitrogen-containing compound for germination does not necessarily imply its adequacy in supporting postgerminative development.

The present report describes a more detailed and extended investigation of the effects of many nitrogenous compounds, both inorganic and organic, on *B. megaterium* spore germination and postgerminative development and on the differences in nitrogen requirements for these stages in the transition from dormant spore to vegetative cell.

MATERIALS AND METHODS

Spores of *B. megaterium* (QM B1551), harvested from liver broth by centrifugation and washing at 4 C, were dried from the frozen state. A pool of lyophilized spores, sufficient for all the experiments described in this report, was prepared in April 1960 and stored over anhydrous $CaSO_4$ in a desiccator at 4 C. These spores contain approximately 11% total nitrogen (Nesslerization, according to the method of Miller and Miller, 1948). Water suspensions of spores were heated for 10 min at 60 C when treatment was required.

For studies of the promotion of germination

and postgerminative development, 3.0 ml of spore suspension and nitrogenous compounds were shaken in 50-ml Erlenmeyer flasks in a water bath at 30 C. Spores, at a concentration of 1.0 mg (5×10^8 spores) per ml, were incubated with the nitrogen source under investigation in potassium phosphate buffer (50 mM, pH 7.0). When a medium suitable for postgerminative development was required, glucose (25 mM) and K_2SO_4 (1 mM) were included. Nitrogenous compounds were generally used at 25 mM. Concentrations are based on nitrogenous compounds with one nitrogen atom per molecule. For example, L-alanine and KNO_3, each with one nitrogen atom, were used at 25 mM; $(NH_4)_2SO_4$ and asparagine, each with two atoms of nitrogen were used at 12.5 mM. Because of solubility limitations, certain purine and pyrimidine derivatives were used at lower concentrations. All concentrations given are final, and are expressed as millimolar (mM).

Oxygen consumption at 30 C was measured by conventional Warburg techniques. Both microscopic and respirometric techniques were used in the measurement of germination and of the subsequent stages of morphological development. Nitrogen utilization was estimated by assay of ninhydrin-reactive material (amino or ammonium nitrogen; Moore and Stein, 1948) remaining in the supernatant fluids from the reaction mixtures in Warburg flasks.

In studies of the inhibition of postgerminative development, spores were incubated for 30 min in phosphate buffer, containing glucose and sulfate, to give approximately 80% germination and then incubated for 15 min with a suitable concentration of inhibitor, before the addition of the nitrogen source.

RESULTS

Germination of spores of B. megaterium. 1) Effect of various nitrogenous compounds:—Of the 48 inorganic and organic nitrogenous compounds which were examined, only L-alanine, glucosamine, and *N*-acetylglucosamine supported appreciable germination of unheated spores (Table 1). With heated spores, nitrate, nitrite, L-cysteine, DL-isoleucine, L-leucine, DL-methionine, DL-norleucine, L-proline, and L-valine were also effective in inducing germination. None of the purine or pyrimidine derivatives was active as a germination agent, either for heated or for unheated spores.

2) Stimulation of glucose-induced germination by nitrogenous compounds:—Although numerous nitrogen-containing compounds were ineffective in supporting the germination of unheated *B. megaterium* spores (Table 1), some of these compounds were capable of stimulating the germination of unheated spores in glucose (see also Levinson and Hyatt, 1955). For example, 30% of unheated spores germinated when incubated with 25 mM glucose; 4% germinated in L-valine; but, when unheated spores were incubated with both glucose and L-valine, there was 71% germination, an increase of 110%. Other nitrogenous compounds which, by themselves, failed to support germination of unheated spores (NH_4NO_3, L-cysteine, DL-isoleucine, L-leucine, DL-methionine, and DL-norleucine) also increased glucose-induced germination by approximately 100%. However, L-proline, glucosamine, and *N*-acetylglucosamine failed to exert this effect. Further data relevant to combinations of germinants are provided elsewhere (Hyatt and Levinson, 1962).

Postgerminative development of spores of B. megaterium. 1) Effect of various nitrogenous compounds:—Promotion of spore germination by a nitrogenous compound does not necessarily imply utilization of the compound in postgerminative development (Table 1). Indeed, the 48 compounds which we tested may be arbitrarily classified according to their ability to support germination and postgerminative development of heated spores. These classes included: (i) those compounds which support germination, but not subsequent cell division (nitrite, L-cysteine, glucosamine, DL-isoleucine, L-leucine, DL-methionine, *N*-acetylglucosamine, DL-norleucine, and L-valine—DL-isoleucine and L-leucine, however, did support a considerable amount of elongation); (ii) those compounds which supported both appreciable germination and subsequent cell division of heated spores (nitrates, L-alanine, and L-proline); (iii) those compounds which did not support germination, but which can be used for postgerminative development ($(NH_4)_2SO_4$, D-alanine, L-arginine, L-asparagine, L-aspartic acid, adenine, adenosine, and guanosine; and L-glutamic acid and glutamine, which supported only a small amount of germination, but which were, nevertheless, excellent nitrogen sources for postgerminative development) and; (iv) those

TABLE 1. *Germination and postgerminative development of Bacillus megaterium spores in the presence of nitrogenous compounds*

Nitrogen source (25 mM)	Germination[a]		Postgerminative development[b]			
			Unheated spores		Heated spores	
	Unheated spores	Heated spores	Elong.	Div.	Elong.	Div.
	%	%				
Inorganic compounds						
$(NH_4)_2SO_4$	0	1	++++	++++	++++	++++
NH_4NO_3	2	41	++++	++++	++++	++++
KNO_3	1	22	+++	+++	+++	+++
KNO_2	<1	25	+	−	+	−
NaN_3	0	<1	−	−	−	−
$NH_2OH \cdot HCl$[c]	0	11	−	−	−	−
Organic compounds						
Amino acids and amines						
β-Alanine	0	3	+	−	+	−
D-Alanine	0	<1	+++	+++	+++	+++
L-Alanine	21	52	+++	+++	+++	+++
α-NH_2-isobutyric acid	0	3	−	−	−	−
DL-α-NH_2-*n*-butyric acid	0	5	−	−	−	−
L-Arginine·HCl[c]	0	4	++++	++++	++++	++++
L-Asparagine	0	<1	++	++	++++	++++
L-Aspartic acid	0	<1	++	++	++++	++++
L-Cysteine·HCl[c]	<1	38	±	±	±	±
L-α,ε-Diaminopimelic acid	0	0	±	−	±	−
D-Glucosamine·HCl[c]	30	87	±	−	−	−
N-acetylglucosamine	30	84	±	−	±	−
L-Glutamic acid	<1	10	++++	++++	++++	++++
Glutamine	0	11	++++	++++	++++	++++
Glycine	0	<1	−	−	−	−
L-Histidine·HCl[c]	0	4	±	−	±	−
DL-Isoleucine	3	40	++	−	+++	−
L-Leucine	9	70	+++	−	+++	−
L-Lysine·HCl[c]	0	5	−	−	−	−
DL-Methionine	2	25	−	−	±	−
DL-Norleucine	2	32	±	−	±	−
L-Phenylalanine	0	12	++	−	++++	+
DL-β-Phenylalanine	0	11	++	+	++++	+
L-Proline	5	58	+++	+++	+++	+++
DL-Serine	0	<1	−	−	−	−
DL-Threonine	0	<1	−	−	−	−
DL-Tryptophan	<1	8	−	−	±	−
L-Valine	4	42	+	−	+	−
Purine and pyrimidine derivatives						
Adenine[d]	0	<1	++	++	++	++
Adenosine	0	<1	++	++	++	++
Guanosine[d]	0	0	+++	+++	+	+
Hypoxanthine[d]	0	0	++	++	+	−
Inosine	0	0	±	−	±	−
Isocytosine	0	0	−	−	−	−
Thymine	0	0	−	−	−	−
Uracil	0	1	±	−	±	−
5-Aminouracil	0	3	±	−	±	−
Uridine	0	1	++	−	±	−
Xanthine[d]	0	<1	++	++	++	−
Xanthosine[d]	0	<1	±	−	±	−
Other compounds						
Dipicolinic acid	0	0	−	−	−	−
Urea	<1	<1	−	−	−	−

[a] Germination determined after 2 hr incubation in phosphate buffer (50 mM, pH 7.0) plus nitrogen source. No germination in phosphate buffer alone.

[b] Postgerminative development determined after 6 hr incubation in a medium containing phosphate buffer (50 mM); glucose (25 mM); K_2SO_4 (1 mM); and nitrogen source; ++++ indicates ca. 100% elongation (elong.) or cell division (div.); +++ = 75%; ++ = 50%; + = 25%; ± = an occasional cell elongated or divided; − = no elongation or cell division.

[c] KCl (25 mM) effects 10% germination of heated spores, and this figure should be subtracted when compounds are used as the hydrochloride.

[d] Nitrogen sources used at 2.5 mM owing to solubility limitations.

compounds which supported neither germination nor postgerminative development (glycine, DL-serine, DL-threonine, etc.). It should be noted that, in the case of certain purine and pyrimidine derivatives, the low concentration (2.5 mM) may have limited postgerminative development.

2) Oxygen consumption in the presence of various nitrogenous compounds:—The morphological stages of spore germination and subsequent growth coincided with changes in respiratory activity, the respiratory rate curves being characterized by linear increases in rate corresponding to the phases of germination, swelling, emergence, elongation, and cell division (Levinson and Hyatt, 1956).

Heated spores, incubated in a medium suitable for postgerminative development, had different oxygen-uptake rate plots (Fig. 1) with different sources of nitrogen, and, in general, these curves reflected the extent of postgerminative development on the various nitrogen-containing compounds. L-Alanine and $(NH_4)_2SO_4$ (as well as L-aspartic and L-glutamic acids, L-asparagine, glutamine, and L-arginine) promoted the most rapid and most complete development, and development of germinated spores in D-alanine was only slightly delayed. Other differences in oxygen consumption rates with the different nitrogenous compounds were worth noting. With L-proline, there were lower rates during elongation and cell division. In KNO_3 (and with adenosine), the oxygen-uptake rate changes accompanying elongation were delayed until 150 min (vs. 50 to 70 min), and rate changes associated with cell division did not start until 270 min (vs. 110 to 130 min). It was interesting that oxygen consumption rate changes accompanying elongation with L-leucine and with L-phenylalanine (not shown) occurred, but, in the absence of a significant amount of cell division (Table 1), the rate of oxygen uptake leveled off. Oxygen-consumption rates in nitrogenous compounds, such as glucosamine, L-valine, and DL-serine, which did not support postgerminative development, failed to increase after the spores germinated (data not shown).

It was not necessary for a nitrogen source to be present from the beginning of incubation, before the spores had germinated, for postgerminative development to occur. When a nitrogen source was added, at 30 or 70 min, to spores incubating in phosphate buffer, glucose,

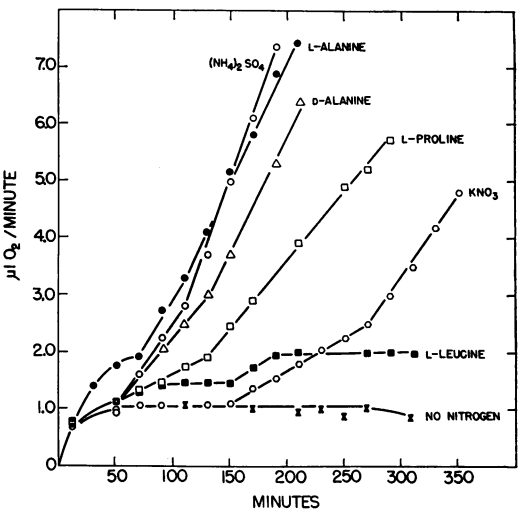

FIG. 1. *Oxygen consumption rate of Bacillus megaterium spores incubated with various nitrogenous compounds. Reaction systems contained heated spores; phosphate buffer (50 mM, pH 7.0); glucose (25 mM); K_2SO_4 (1 mM); and nitrogen source (25 mM). L-Aspartic and L-glutamic acids, L-asparagine, glutamine, and L-arginine (data not plotted) give rate curves similar to L-alanine and $(NH_4)_2SO_4$. Rate curve with adenosine (not shown) approximates that of KNO_3.*

and K_2SO_4, there was no delay in the initiation of respiratory changes associated with postgerminative development. Emergence, and a concomitant increase in oxygen-uptake rate, occurred at the same time as when nitrogen was present from the beginning. When nitrogen was added at 110, 130, 150, 190, or 230 min, elongation started immediately, indicating that the presence of a nitrogen source was critical for initiation of postgerminative changes.

3) Concentration requirements and utilization of nitrogen:—Heated spores were incubated in a medium suitable for postgerminative development, containing from 0 to 25 mM $(NH_4)_2SO_4$ or L-alanine. Oxygen consumption in all concentrations of nitrogen ensued rapidly with the initiation of germination (Fig. 2). In the absence of a source of nitrogen, the oxygen-uptake rate did not increase further after about 50 min and declined gradually during the remaining 3 to 4 hr of the experiment. In the presence of nitrogen, even at so low a concentration as 2.5 mM, swelling, emergence, and elongation occurred, and these were accompanied by increasing rates of oxygen

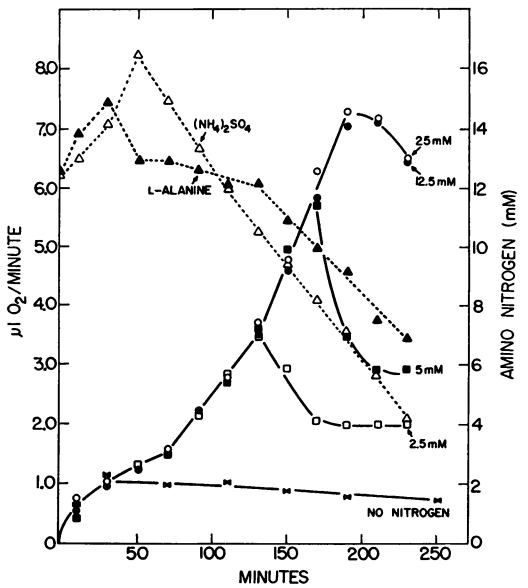

FIG. 2. *Oxygen uptake rate and utilization of amino or of ammonium nitrogen during germination and postgerminative development of Bacillus megaterium spores. Reaction systems contained heated spores; phosphate buffer (50 mM, pH 7.0); glucose (25 mM); K_2SO_4 (1 mM); and varying concentrations of* L-*alanine or $(NH_4)_2SO_4$. These nitrogenous compounds used at original concentration of 12.5 mM for determination of utilization of amino nitrogen (dotted lines). Oxygen uptake data (solid lines) are given for $(NH_4)_2SO_4$;* L-*alanine (not plotted) is virtually identical, except for slightly higher rate during first 70 min (see Fig. 1).*

consumption. An L-alanine or $(NH_4)_2SO_4$ concentration of 2.5 mM was, however, inadequate to support an appreciable amount of cell division or a continued high rate of oxygen consumption, and at 130 min there was a decline in the oxygen-uptake rate (Fig. 2). With higher concentrations of nitrogen, there was a change in the slope of the oxygen-uptake rate curve at 130 min, marking the beginning of cell division. In 5 mM nitrogen, about 50% of the germinated spores divided, and the rate of oxygen consumption fell at 170 min, when the nitrogen supply was essentially exhausted. With 12.5 and 25 mM concentrations of the nitrogenous compounds, the germinated spores underwent at least one cell division before the rate of oxygen consumption ceased to increase at 190 min.

The utilization of the amino nitrogen of L-alanine or of the ammonium nitrogen of $(NH_4)_2SO_4$ (at an initial concentration of 12.5 mM) was determined at intervals during the incubation of heated spores undergoing germination and postgerminative development as described above (Fig. 2). Ninhydrin-positive material, probably a sugar amine (Strange and Powell, 1954), was excreted from the spores during the period of germination. The major disappearance of nitrogen from the medium took place after completion of germination, and, by the completion of one cell division, about 50% of the 12.5 mM originally present ninhydrin-reactive material disappeared from the medium. It was not surprising, therefore, that 2.5 or 5 mM nitrogen was insufficient to allow spores to complete their development. On the other hand, when more than 12.5 mM nitrogen was originally present, the decline in oxygen-uptake rate after approximately 200 min of incubation was not due to exhaustion of nitrogen. We feel that this decline was more likely due to depletion of glucose, or perhaps to accumulation of toxic products. With compounds such as L-valine, DL-methionine, and DL-serine, which did not support postgerminative development, there was essentially no disappearance of amino nitrogen through 6 hr of incubation.

4) Chemical inhibitors:—None of the various chemical inhibitors (except perhaps DL-serine) had any differential effect on the postgerminative development of spores incubated with different nitrogen sources. The differential inhibition of germination in various compounds is discussed in another paper (Hyatt and Levinson 1962).

Spores were incubated in a medium suitable for postgerminative development, and contained either L-alanine, L-proline, $(NH_4)_2SO_4$, L-aspartic acid, or KNO_3 as nitrogen source. In no case was postgerminative development inhibited by 10 mM D-alanine, NaF, or glycine; 25 mM dipicolinic acid; 1 mM 2,3-dimercaptopropanol (BAL), or isonicotinic acid hydrazide. (With the last two compounds, postgerminative development in KNO_3 is delayed.) Postgerminative development in all of these nitrogen sources was inhibited by 10 mM NaN_3, 2,4-dinitrophenol, potassium iodoacetate, KCN, $NaAsO_2$, and ethylenediaminetetraacetic acid; and by 1 mM 8-hydroxyquinoline, $HgCl_2$, atebrin, and *p*-chloromercuribenzoate. However, DL-serine at 25 mM inhibited postgerminative development with all of the tested nitrogen compounds except L-alanine. Hachisuka, Sugai, and Asano (1958), however, reported

DL-serine inhibition of growth of germinated *B. subtilis* spores incubated in a synthetic medium, including a combination of L-glutamic acid, L-alanine, and L-asparagine.

DISCUSSION

Much of the earlier work on the effect of nitrogenous compounds on spore germination and growth is difficult to interpret since there was, by present day criteria, insufficient separation of the processes of germination and postgerminative development. In the present paper, where we considered germination as the initial step in the transition from spore to vegetative cell (determined by stainability), we noted that utilization of a nitrogenous compound for germination does not carry with it the implication that the compound is also useful in supporting the further development of the germinated spore into a dividing vegetative cell. The data of Table 1 show clearly that the 48 nitrogenous compounds which we examined may be arranged into several groups based on the ability or inability to support germination or cell division. We realize, of course, that support of postgerminative development by nitrogenous compounds involves utilization of nitrogen for synthesis of cellular material, but germination may involve a "trigger" or activation mechanism not necessarily using the nitrogenous germination agent as a source of nitrogen. Indeed, Rode and Foster (1960) have postulated that many "physiological germinants" may react nonspecifically with surface components of the spore, destroying the integrity of a water-impermeable layer, and leading to a display of the reactions commonly associated with germination.

B. megaterium spores germinate, even in the absence of a nitrogen source (e.g., 30% germination of unheated spores in 25 mM glucose). However, in the absence of glucose from the germination medium, only three of the tested nitrogen compounds (L-alanine, glucosamine, and *N*-acetylglucosamine) supported germination of unheated spores. Heating, at 60 C for 10 min prior to incubation with the nitrogenous compounds, enables *B. megaterium* spores to germinate in a wider variety of nitrogen-containing compounds, both inorganic and organic, and increases the percentage of germination in those compounds which support germination of unheated spores. Whether, as we have postulated (Hyatt and Levinson, 1961), heating of spores brings about the production, internally, of substance(s) capable of reacting together with added compounds to initiate germination, or whether heating alters the permeability of spores to make them more susceptible to the "triggering" action of a greater variety of compounds, remains unresolved. There appears to be no outstanding structural relationship among the compounds supporting germination, nor does there seem to be any clear structural resemblance among those compounds which do not support germination. It is interesting, too, that many levorotatory amino acids are ineffective, and many racemic mixtures of amino acids (the L-forms of which were not tested) did support germination.

Some differences between the nitrogen requirements for germination of *B. subtilis* spores, incubated with glucose (Woese, Morowitz, and Hutchison, 1958; Hachisuka et al., 1955), and those which we have described for *B. megaterium* spores, may be attributed to species differences or to the presence of glucose. Indeed, Hachisuka et al. (1955) found L-valine to be the only nitrogenous compound tested which supported an appreciable amount of germination (39%) in the absence of glucose. Results of other workers, too, suggest that various species and strains differ in their responses to physiological germinants. For example, Kosaki (1958) found that inorganic nitrogenous compounds did not promote germination of *B. cereus* strain T (*B. terminalis*) spores; and Thorley and Wolf (1961) demonstrated that there are even differences in germination agents among strains of the same species, some of their *B. megaterium* strains, in contrast to our own, germinating in adenosine and in inosine. Differences in the sporulation medium may also profoundly influence the ability of spores to germinate in various agents (Levinson, 1961).

O'Connor and Halvorson (1961) reported that, of the 40 L-alanine analogues tested, only those substrates of L-alanine dehydrogenase, which are precursors of pyruvate, namely, L-alanine and L-α-NH_2-*n*-butyric acid, were effective germination agents for *B. cereus* strain T. Although L-cysteine was an effective germination agent, it is apparently metabolized by another pathway. We feel, too, that germination in different nitrogenous compounds, or in glucose, may involve different mechanisms; this will be discussed further (Hyatt and Levinson, 1962).

Although Knaysi (1945) indicated that spores of *B. mycoides*, grown on a meat infusion medium containing tryptone and glucose, had a sufficient reserve of nitrogenous material to enable the spores to grow, even in the absence of added nitrogen, other workers have found that added nitrogen is necessary for growth after germination. Although our *B. megaterium* spores contain ca. 11% total nitrogen, this cannot be utilized by the germinated spore for postgerminative development. Hachisuka et al. (1955) found that *B. subtilis* spores (grown on meat extract agar) required added nitrogen for growth after germination; and we agree with their basic finding that the same nitrogenous compounds need not necessarily support both germination and growth. Differences between the compounds, which they list as effective for growth and those which we report, may reflect species differences, or the fact that we observed cell division at 6 hr (when there may have been only one division), while Hachisuka et al. used the extensive cell division necessary to form a visible pellicle (48 hr) as their criterion for growth.

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