SULFUR REQUIREMENT FOR POSTGERMINATIVE DEVELOPMENT OF *BACILLUS MEGATERIUM* SPORES

MILDRED T. HYATT AND HILLEL S. LEVINSON

Pioneering Research Division, Quartermaster Research and Development Center, Natick, Massachusetts

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In *Bacillus megaterium*, the transition from bacterial spore to vegetative cell has been divided into phases of germination, swelling, emergence, elongation, and cell division. This has been demonstrated both in simple (Levinson and Hyatt, 1956) and in complex (Mandels *et al.*, 1956) media, the primary tool for quantitative measurement being the change in respiratory rate accompanying each new developmental phase.

Germination, roughly equivalent to "prevegetation" (Jensen, 1950) and to "step a" in the developmental sequence of Schmidt (1955), is defined as the spore's initial loss of heat resistance accompanied by increased metabolic activity; stainability with methylene blue; and loss of refractility. The appearance of translucent areas (Hachisuka *et al.*, 1955) may also be a criterion of germination. We describe later stages as "postgerminative development," or simply "development," which is equivalent to "outgrowth" as used by O'Brien and Campbell (1956).

The nutritional requirements for postgerminative development are not known. Spores germinate rapidly in a neutral phosphate buffered medium containing L-alanine and glucose, or $MnSO_4$ and glucose, but in the former medium they fail to develop further. $MnSO_4$ appears to act in a dual role, stimulating rapid initial germination and promoting postgerminative development.

The present study was initiated to elucidate further this dual role of $MnSO_4$. It was found that while Mn^{++} meets a requirement for rapid spore germination, $SO_4^{=}$ is necessary for development subsequent to germination. $SO_4^{=}$ is replaceable by other inorganic and organic sources of sulfur. Co^{++} and Ni^{++} are inhibitory to postgerminative development, their effects being evident at different stages.

MATERIALS AND METHODS

Lyophilized spores of *B. megaterium* strain QM B1551, grown and harvested according to the method of Levinson and Sevag (1953), were pooled in May, 1956, from a series of 18 harvests (November, 1955–April, 1956). These spores contained 0.2–0.4 per cent sulfur as determined by a modification of the method of Carius (Steyermark, 1951). Heat activated spores were used in all experiments. Heat shock was accomplished by a 5 min immersion of spores, suspended in phosphate buffer, pH 6.8, in a water bath at 50 C. Higher percentages of germination were attained by these spores than by those previously described (data of Levinson and Hyatt, 1956 shown in parentheses): in glucose alone, 65 (30); in L-alanine and glucose, 99 (82); and in $MnSO_4$ and glucose, 92 (45).

L-Alanine was obtained from Nutritional Biochemicals Corp.; L-cystine, DL-methionine, glutathione (reduced), and crystalline biotin solution from General Biochemicals, Inc.; cysteine hydrochloride from Eastman Kodak Co.; DL-homocysteine from Bios Laboratories; and spectrographically standardized (SpecPure) sulfates from Johnson, Matthey and Co., Ltd. Samples of coenzyme A and of ethylenediamine tetraacetic acid (EDTA) were kindly supplied by Dr. A. F. Brodie, Harvard Medical School, and by Alrose Chemical Co., respectively. All other chemicals were of CP reagent grade.

Percentage germination of spores, incubated in Warburg vessels under the same conditions as for respiration studies, was determined by a modification (Levinson and Sevag, 1953) of the method of Powell (1950). Germinated spores were differentiated from ungerminated spores by their stainability with methylene blue.

Oxygen consumption was measured at 30 C by conventional Warburg techniques, using 0.3 ml substrate in the sidearm, 1.5 mg of heat shocked spores in 1.2 ml of phosphate buffer, pH 6.8, in the main chamber, and 0.2 ml of 10 per cent KOH in the center well. When additional sulfur sources or chelating agents were added at different time intervals, 0.3 ml substrate and 1.5 mg of spores in 1.0 ml buffer were placed in the main

chamber, and 0.2 ml of additive tipped in from the sidearm.

All reaction systems contained glucose, 25 mM, and phosphate buffer (KH_2PO_4, 50 mM; ammonium acetate, as a nitrogen source, 50 mM) adjusted to pH 6.8 with KOH. L-Alanine, when used, was included at a concentration of 2.0 mM. Metallic salts were used at 0.2 mM, as were sulfur sources, except for $K_2S_2O_3$ and cystine, where 0.1 mM gives an equivalent sulfur concentration (6.4 ppm). EDTA, 0.4 mM, was made up in phosphate buffer and adjusted to pH 6.8.

All concentrations are final, after tipping.

RESULTS

The spores used in these experiments show the same pattern of respiratory activity as those used previously (Levinson and Hyatt, 1956). The following data show the times at which respiratory and morphological changes occur, and establish a basis for comparison with results obtained in later experiments.

The maximum germination of spores incubated in glucose alone is only 65 per cent, and the low respiratory rate (figure 1) does not increase after attainment of maximum germination. The germinated spores swell slightly, but do not develop further. After 50 min of incubation, spores in $MnSO_4$ and glucose reach their maximum (92 per cent) germination (figure 1, *B*). The germinated spores swell from 50 to 150 min,[1] at about which time the spore coat cracks and emergence occurs (figure 1, *C*). The cells elongate, and at 230 min (figure 1, *D*) cell division commences. The respiratory rate (figure 1) changes with the initiation of each phase of morphological development. L-Alanine and glucose support very rapid germination (maximum, 99 per cent), but the germinated spores do not emerge. The rate of oxygen consumption (figure 1) does not increase after the initial rapid rate is reached. However, when spores are incubated in a mixture of L-alanine, glucose, and $MnSO_4$, postgerminative development does occur with concomitant changes in the rate of respiration (figure 1). $MnSO_4$ appears to serve a dual role, in stimulating rapid initial germination and in being required for postgerminative development.

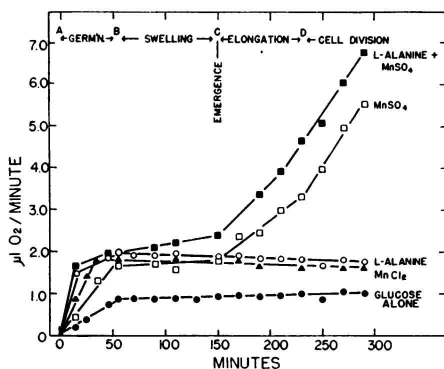

Figure 1. Effect of $MnSO_4$, $MnCl_2$, and L-alanine on the rate of oxygen consumption of *Bacillus megaterium* spores. All reaction systems contained glucose and phosphate buffer, pH 6.8. Developmental phases *A*, *B*, *C*, and *D* refer only to data obtained with $MnSO_4$ present (upper two curves).

Preliminary experiments. The removal, with EDTA, of manganous ions from suspensions of spores germinating in $MnSO_4$ and glucose provided a useful tool in clarification of the dual role of $MnSO_4$. When EDTA is added simultaneously with $MnSO_4$ and glucose, the level of spore germination is reduced to that in glucose alone. However, the germinated spores swell, emerge, elongate, and divide. The depressed germination is reflected in lower respiratory rates accompanying germination and post-germinative development. When EDTA is added after the completion of germination, emergence, or elongation, there is no change in the typical pattern of morphological development or in the respiratory rate.

These results indicate that $SO_4^=$ may be required for postgerminative development. The necessity for $SO_4^=$ is further suggested by the fact that, even after 6 hr, spores germinated with $MnCl_2$ do not develop beyond a slight swelling. There is no increase in respiratory rate beyond the initial rise (figure 1) which accompanies the rapid germination (95 per cent).

Sulfur requirement for postgerminative development. (1) $SO_4^=$ and other sulfur sources:—We have substantiated the necessity for $SO_4^=$ in postgerminative development. The addition, to glucose, of as simple a source of $SO_4^=$ as H_2SO_4 (0.2 mM) renders this medium capable of sup-

[1] Exact time of these morphological and respiratory changes varies slightly from experiment to experiment. Occasionally, emergence occurs as early as 130 min and cell division begins as early as 210 min.

TABLE 1

*Effect of various sulfur sources on postgerminative development of spores, as reflected in relative rates of oxygen consumption**

Sulfur Source	Relative O_2 Uptake Rate	
	55 min	290 min
None	1.0	1.0
H_2SO_4	1.0	3.1
K_2SO_4	1.1	3.6
K_2SO_3	1.1	4.1
$K_2S_2O_3$	1.3	4.9
DL-Methionine	1.4	3.4
DL-Homocysteine	1.1	3.0
Cysteine	1.4	4.2
L-Cystine	1.5	3.9
Glutathione	0.9	3.0
Thiamin	1.0	1.3
Biotin	1.0	1.5
Coenzyme A	1.7	1.8

* Phosphate buffer, pH 6.8, and glucose were used in all experiments. The sulfur concentration was 6.4 ppm, except in the case of biotin, where for reasons of solubility, only 0.66 ppm were used.

porting typical development with corresponding changes in respiratory activity (table 1). Not only $SO_4^=$, but many other inorganic and organic sulfur sources meet this requirement for spore development subsequent to germination (table 1). Of the sulfur compounds tested, only thiamin, biotin, and coenzyme A do not promote postgerminative development, perhaps for reasons of permeability or configuration. Selenate does not substitute for $SO_4^=$ as a requirement for this development.

K_2SO_4 was selected as a standard in further investigations of the effect of the addition of sulfur. An excess of K^+ was already present in the buffer. K_2SO_4 (0.2 mM) does not stimulate germination as $MnSO_4$ would, but it does permit spores germinating in glucose to emerge (150 min), elongate, and divide (230 min). These morphological changes are not as clear-cut as in $MnSO_4$, since germination is spread out over a longer period (up to 90 min, when the maximum of 62 per cent have germinated), and the subsequent changes do not occur synchronously. The respiratory pattern of spores in K_2SO_4 and glucose (figure 2) is essentially the same as that observed with $MnSO_4$ incubated spores (figure 1), but the levels of oxygen uptake rates with K_2SO_4 are lower than those with $MnSO_4$ where there is more germination. No postgerminative development of spores in L-alanine and glucose occurs after the very rapid attainment of 99 per cent germination. The addition of K_2SO_4 to this medium from the beginning of incubation allows the spores to complete their development into dividing vegetative cells. The morphological development is reflected in the rates of oxygen consumption (figure 2) which are elevated because of the higher percentage of germination with L-alanine.

(2) Time of addition of sulfur:—K_2SO_4 was added at various time intervals to spores preincubating in glucose (figure 3). When K_2SO_4 is added at 50, 90, or 130 min, there is no delay in the initiation of postgerminative morphological and respiratory changes. Emergence occurs at 150 min, just as it does when K_2SO_4 is present at zero time. When K_2SO_4 is added at 170 min, emergence and elongation start immediately, and division begins at 250 min. If K_2SO_4 is not added until 210 min, elongation also commences immediately, but the duration of this phase is doubled, after which normal cell division takes place. This increase in elongation time is reminiscent of the delay in initiation of cell division when spores are shaken for 2 hr in glucose before the addition of L-alanine and $MnSO_4$ (Levinson and Hyatt, 1956).

(3) Concentration of sulfur:—The effect of K_2SO_4 concentrations on respiratory rate is shown in figure 4. Concentrations greater than 0.1 mM (3.2 ppm of sulfur) have no appreciable additional effect on postgerminative development or on respiration. Nor do higher $SO_4^=$ concentrations alter the duration of any morphological

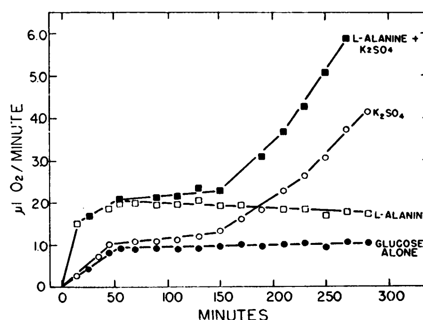

Figure 2. Effect of K_2SO_4 on the rate of oxygen consumption of *Bacillus megaterium* spores in glucose, with and without L-alanine. All reaction systems buffered with phosphate, pH 6.8.

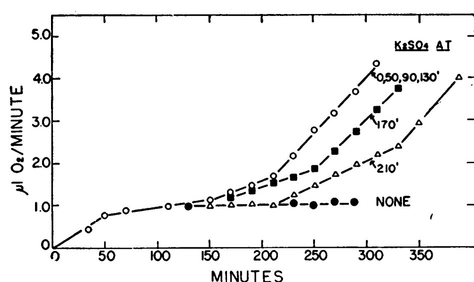

Figure 3. Effect of the addition of K_2SO_4 on the rate of oxygen consumption of *Bacillus megaterium* spores incubated with glucose. All reaction systems contained phosphate buffer, pH 6.8. K_2SO_4 added at the indicated times.

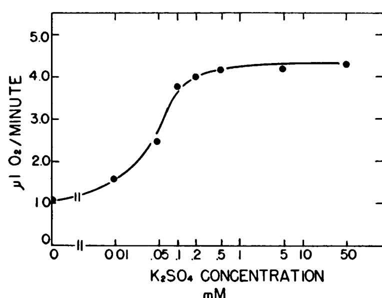

Figure 4. Effect of K_2SO_4 concentration on the rate of oxygen consumption of *Bacillus megaterium* spores, at 310 min. All reaction systems contained glucose and phosphate buffer, pH 6.8.

phase. A few cells emerge and elongate with 0.01 mM K_2SO_4, but without significant accompanying respiratory rate changes. In 0.05 mM K_2SO_4, approximately 50 per cent of the germinated spores divide, but respiratory rate changes are atypical. Cysteine is no more effective than K_2SO_4 at equivalent concentrations.

Effects of cations on germination and development. Spores were incubated in phosphate buffer with glucose and SpecPure metal sulfates. Only Mn^{++} exhibited any marked stimulation of germination (table 2). Postgerminative development occurred in the presence of Mn^{++}, Mg^{++}, Fe^{++}, Cu^{++}, and Zn^{++}. Changes in oxygen consumption rate accompany this morphological development, with the respiratory rates (table 2) of the spores germinated in Mn^{++} being higher because of the greater number of germinated spores.

Co^{++} and Ni^{++} do not inhibit germination, although Ni^{++} depresses the respiratory rate during the first 4 hr of incubation below that in glucose alone. However, in the presence of Co^{++} or of Ni^{++}, postgerminative development is delayed until approximately 290 min, when there is a slight increase in respiratory rate and a few cells emerge and divide. Normal postgerminative development and typical respiratory changes occur when the Co^{++} and Ni^{++} are complexed with EDTA.

Co^{++} or Ni^{++} was added at different stages of postgerminative development to spores incubating in glucose and K_2SO_4. With Co^{++} added at zero time or at 50 min, there is no increase in the postgerminative respiratory rate until 290 min (figure 5). However, when Co^{++} is added at 130 min, when emergence is about to occur, the

TABLE 2

*Effect of various cations on germination, and on postgerminative development of spores, as reflected in relative rates of oxygen consumption**

Cation	Germination	Relative O_2 Uptake Rate	
	360 min	55 min	290 min
	%		
None	65	1.0	1.0
Mn^{++}	92	1.8	4.9
Mg^{++}	66	1.0	3.7
Zn^{++}	69	0.9	3.6
Cu^{++}	74	1.2	4.3
Fe^{++}	67	1.1	4.1
Co^{++}	72	0.9	1.0
Ni^{++}	72	0.7	0.8

* All reaction mixtures contained phosphate buffer, pH 6.8, and glucose. Cations added as sulfates (0.2 mM).

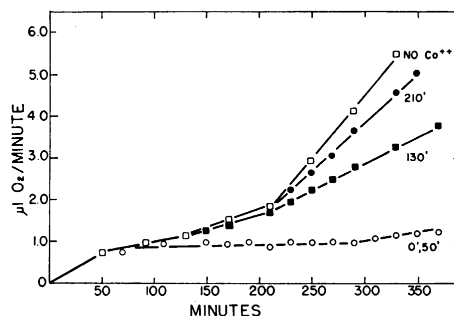

Figure 5. Effect of the addition of Co^{++} on the rate of oxygen consumption of *Bacillus megaterium* spores. Co^{++}, as $CoSO_4$, added at the indicated times to spores in phosphate buffer, pH 6.8, with K_2SO_4 and glucose.

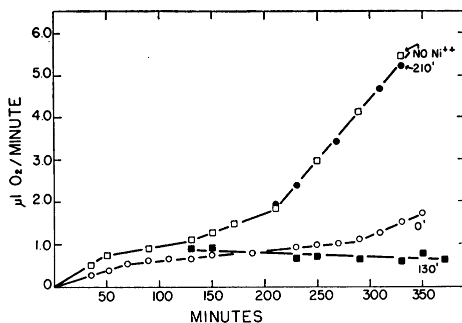

Figure 6. Effect of the addition of Ni^{++} on the rate of oxygen consumption of *Bacillus megaterium* spores. Ni^{++}, as $NiSO_4$, added at the indicated times to spores in phosphate buffer, pH 6.8, with K_2SO_4 and glucose.

respiratory rate curve is parallel to the normal K_2SO_4 curve during the period of elongation, but is somewhat depressed during cell division (figure 5). If Co^{++} is added at 210 min, when the elongated cells are about to divide, the ensuing respiratory rate (figure 5) approximates that in K_2SO_4 alone. The morphological observations corroborate the respiratory data. The respiratory rate of spores to which Ni^{++} is added at zero time also increases slightly at about 290 min (figure 6). When added at 50 or 130 min, Ni^{++} depresses the respiratory rate, and there is no emergence. When the cells have already elongated and are about to divide (210 min), the addition of Ni^{++} permits the normal respiratory pattern (figure 6) and cell division to continue. Neither Co^{++} nor Ni^{++} inhibits germination. However, Co^{++} inhibits stages of postgerminative development prior to emergence, and Ni^{++} inhibits all stages of postgerminative development prior to cell division.

DISCUSSION

The transition of a resting bacterial spore into a dividing vegetative cell is apparently a complex phenomenon, as evidenced by the changes in respiratory activity concomitant with morphological development. Previous demonstration of a defined medium (Levinson and Hyatt, 1956) in which this transition can occur provided a basis for possible delimitation of the nutritional requirements for each of the stages of postgerminative development. The data reported here lend experimental support to the postulate that the nutritional requirements, and possibly the metabolic pathways, undergo definite qualitative changes as the germinated spore swells, emerges, elongates, and divides.

In a phosphate buffered medium containing glucose, L-alanine greatly stimulates spore germination, but does not promote postgerminative development of the spores (Levinson and Hyatt, 1956; Fitz-James, 1955). While the stimulation of germination by a substance not supporting vegetative growth had been reported previously (Pulvertaft and Haynes, 1951), it had had no adequate explanation. We hypothesized that the spores were injured in some way on exposure to L-alanine. Contrary to the situation with L-alanine, $MnSO_4$ not only stimulates germination, but also meets a requirement for the development of the germinated spores into dividing vegetative cells. While Mn^{++} does meet a requirement for rapid spore germination, we have shown that it is the $SO_4^=$ of $MnSO_4$ which is essential for development of the germinated spore. Indeed, our theory concerning the injury of spores by L-alanine was invalidated when we found that the addition of $SO_4^=$ as K_2SO_4 would permit L-alanine germinated spores to go through all the subsequent morphological stages to cell division.

A wide variety of sulfur compounds, including sulfates, sulfites thiosulfates, and amino acids containing sulfur, supports the postgerminative development of *B. megaterium* spores. While the spores themselves contain about 0.3 per cent sulfur, this sulfur apparently cannot be utilized by the germinated spore. An additional sulfur source must be provided for postgerminative development. Selenium cannot replace sulfur in this development, as it can in promoting cell division in certain of the yeastlike fungi (Nickerson *et al.*, 1956). It is interesting that Shepherd (1956) uses spore germination as a quantitative measure of the response of *Aspergillus nidulans* to various sulfur compounds. Spore germination in fungi is defined as extrusion of a germ tube, a stage which, in *B. megaterium*, we consider to be postgerminative.

The metabolic pathways in the utilization of sulfur compounds in postgerminative development of spores are unknown. The very low concentration of sulfur required to exert a maximum effect, and the immediate response of germinated spores to the addition of sulfur seem to indicate its utilization in the synthesis of substances active in catalysis (enzymes, coenzymes, etc.). In the growth of *Escherichia coli*, sulfate is used for the synthesis of cysteine and of methionine (Bolton

et al., 1952). Scherr and Weaver (1953), in their review of the dimorphism phenomenon in yeasts, implicate sulfhydryl groups in the process of cell division. In promotion of postgerminative spore development, however, cysteine does not appear to act by virtue of its sulfhydryl group, since it is no more active than is $SO_4^{=}$.

Co^{++} and Ni^{++} have no effect on germination, but their differential inhibition of postgerminative development is extremely interesting and appears to provide a useful tool for further delimitation of the nutritional requirements and of the metabolic mechanisms characteristic of each developmental stage. Co^{++}, added to spores incubating with K_2SO_4 and glucose, is inhibitory only at stages prior to emergence, i.e., swelling, while Ni^{++} is inhibitory at stages prior to cell division, i.e., swelling, emergence, and elongation. The differential inhibition by Co^{++} and Ni^{++} of the formation and utilization of enzyme precursors in an adaptive enzyme system of *Pseudomonas aeruginosa* (Bernheim, 1954) may be relevant here. The toxicity of Co^{++} and Ni^{++} to bacteria and fungi has been attributed to interference with the normal role of magnesium in cellular metabolism (Abelson and Aldous, 1950; Webb, 1951; Shankar and Bard, 1955). Since magnesium is present in the spore (Curran *et al.*, 1943), Co^{++} and Ni^{++} may be interfering with a role of this metal in postgerminative development. It is also possible that Co^{++} acts to inhibit sulfhydryl enzymes (Levy *et al.*, 1950). The change in respiratory rate after long incubation of spores with Co^{++} and Ni^{++} may be due to binding of these cations by substances secreted into the medium by germinating spores.

We propose that, in addition to a sulfur source, added phosphate at neutral pH may be required for the completion of the transition from germinated spore to vegetative cell. Phosphate is not necessary for germination itself. Spores will germinate in acetate (Levinson and Hyatt, 1956), barbital, or tris buffered systems (unpublished data), but no postgerminative development occurs. Germinated spores do not develop in phosphate buffered media at pH 6.0 (Levinson and Hyatt, 1956). Our data are inconclusive as to the necessity for phosphate, as acetate, tris and barbital buffers, either do not maintain a pH near neutrality or are in themselves inhibitory to postgerminative changes. Inorganic phosphate does disappear from a medium in which spores are developing (unpublished data). Hachisuka *et al.* (1956) suggest that there is a difference in the phosphate requirement for glucose oxidation in resting spores, germinating spores, and vegetative cells of *Bacillus subtilis*. Glucose and a nitrogen source (ammonium acetate) have been included in all experiments, so we can reach no conclusion regarding nutritional requirements for these substances in germination and at the various stages of postgerminative development.

The amino acid requirements for vegetative cell growth in certain thermophilic bacteria and in *Bacillus cereus* var. *terminalis* are different from the requirements for germination and outgrowth (O'Brien and Campbell, 1956). We have distinguished between the requirements for germination of *B. megaterium* spores and those for postgerminative development (outgrowth). Our evidence suggests that a different metabolic pathway requiring a compound of sulfur content and, possibly, added phosphate may be operative in postgerminative development.

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SUMMARY

A source of sulfur is required for the postgerminative development of *Bacillus megaterium*, but not for spore germination. Sulfates and other compounds containing sulfur meet this requirement.

Co^{++} and Ni^{++} do not affect germination, but have differential inhibitory effects on postgerminative development. Co^{++} inhibits at stages of development prior to emergence, i.e., swelling; and Ni^{++} is inhibitory at stages prior to cell division, i.e., swelling, emergence, and elongation. Mn^{++} stimulates germination, but has no effect on subsequent development.

The separation of nutritional requirements for germination from those for postgerminative development, and the differential inhibition by Co^{++} and Ni^{++} of the stages of postgerminative development, may provide a useful tool in further studies of the metabolic pathways characteristic of each developmental phase.

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