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MEGATERIUM SPORES BY MANGANESE, L-ALANINE AND HEAT

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THE STIMULATION OF GERMINATION AND RESPIRATION OF *BACILLUS MEGATERIUM* SPORES BY MANGANESE, L-ALANINE AND HEAT

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Germination of spores of the genus *Bacillus* can be stimulated by heat, L-alanine, or manganese. The reversal of L-alanine stimulation by D-alanine (Hills, 1950) appears to be specific and, in the present study, affords a tool for the exploration of the mechanisms and interrelations among heat, manganese and L-alanine stimulations.

The heat stimulation of bacterial spore germination has long been known (Tarr, 1933; Evans and Curran, 1943; Levinson and Sevag, 1953). L-Alanine stimulates the germination of unheated *Bacillus anthracis* spores (Hills, 1949); of heated and unheated *B. megaterium* spores (Levinson and Sevag, 1953); and of heated spores of *B. terminalis*, *B. globigii*, *B. polymyxa*, *B. subtilis*, and *B. megaterium* (Church et al., 1954). Fixation of L-alanine by spores is not simple adsorption (Harrell and Halvorson, 1954). Levinson and Sevag (1953) have suggested that manganese could replace heat shock as a germination stimulant for *B. megaterium* spores. These authors (1954) have proposed that manganese activates spore proteolytic enzymes, resulting in the release of amino acids, including L-alanine, which stimulate germination.

EXPERIMENTAL METHODS

Harvesting of spores. Spores of *Bacillus megaterium* strain QM B1551 were grown on liver broth (Foster and Heiligman, 1949), harvested by centrifugation and washing at 4 C (Levinson and Sevag, 1953), and lyophilized after each harvest. A pool of lyophilized spores, made up in November 1954, of equal weights of the dried spores from seven harvests ranging in date from July 1953 to April 1954, was used in these experiments which ran from November 1954 through February 1955. The spores from the separate harvests making up the pool gave the same type of responses to all the agents investigated, but varied amongst themselves in the

degree of the response. For instance, the germination of heated spores incubated for 3 hr in a combination of 0.1 mM manganese, 0.2 mM L-alanine, and 25 mM glucose ranged from 79 to 95 per cent (av. 88 per cent). The percentage of germination was independent of the age of the spores. Possibly some of the spore batches contained larger numbers of viable spores than others. No vegetative cells were discernible microscopically.

Chemicals. L-Alanine and D-alanine were obtained from the Nutritional, Biochemicals Corp. and the same lots of these chemicals were used throughout. Manganese, as manganese sulfate, potassium acetate, ammonium acetate, and glucose were of CP reagent grade.

Respiration. Oxygen uptake was measured at 30 C using standard Warburg technics. The reaction systems contained 1.2 ml of spore suspension; 0.3 ml of substrate in water in the sidearm; and 0.2 ml of 10 per cent KOH in the center well. In all cases, the 1.2 ml of spore suspension contained 3.0 mg of spores in potassium and ammonium acetates (pH 6.95) adjusted so as to give a final concentration, after tipping, of 50 mM. Spore counts with a Levy-Neubauer bright line hemocytometer indicate an average of 1.35×10^8 cells per system (3.0 mg spores).

The final concentrations of L-alanine and of D-alanine were 0.2 mM except where otherwise indicated, and that of glucose was 25 mM. At an L-alanine concentration of 0.2 mM, the system contained 3×10^{-7} moles of L-alanine, or 2.2×10^{-16} moles of L-alanine per spore.

Germination. Percentage germination was determined by the staining technic developed by Powell (1950) and modified by Levinson and Sevag (1953). This technic depends on the ability of the germinated spore to take up 0.5 per cent aqueous methylene blue, and the lack of such ability on the part of the ungerminated spores.

The typically bacillary vegetative forms of *B. megaterium* were never produced within 4 hr of incubation. The observations recorded here deal rather with the transition from spore to a "pre-germinative" stage (Levinson and Sevag, 1953), and statements concerning germination or germinated spores refer to this stage.

Heat treatment. Heat activation was effected by immersion with occasional shaking of an acetate buffered spore suspension (3.0 mg spores per ml of 62.5 mM potassium and ammonium acetates, pH 6.95) in a thermostatically controlled water bath. The spores were heated at temperatures ranging from 30 to 70 C for periods ranging from 5 to 120 minutes.

RESULTS

Unheated Spores

L-Alanine. L-Alanine stimulates oxygen consumption (figure 1) and germination in the presence of glucose. In the following experiments, a concentration of 0.2 mM L-alanine was used. This concentration is sufficient, under the experimental conditions, to bring about a 9-fold increase in total oxygen consumption over a period of 3 hours. Our spores show no uptake of oxygen when incubated in L-alanine without glucose. Complete oxidation of 1.5 ml of 0.2 mM L-alanine would result in the uptake of between 30 and 40 μ L of oxygen.

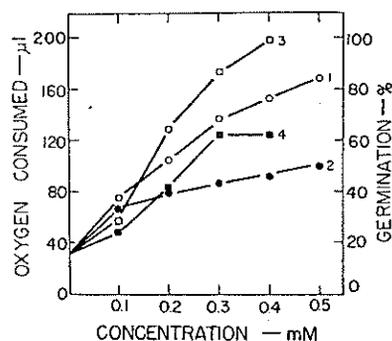


Figure 1. The effect of concentrations of L-alanine and of manganese on germination and oxygen consumption. Each vessel contained 25 mM glucose and 3.0 mg of unheated spores. Curves 1 and 2, L-alanine; curves 3 and 4, manganese. Curves 1 and 3, oxygen consumption data; curves 2 and 4, germination data (both after 3 hours at 30 C).

The rate of consumption of oxygen by the spores rapidly approaches a maximum (figure 2). Generally, with L-alanine, one-half the maximal Q_{O_2} (μ L oxygen consumed per mg dried spores per hour) is reached in from 40 to 60 min, and this is independent of L-alanine concentration in the range from 0.1 to 0.5 mM, where the actual maximal Q_{O_2} ranges from 13 to 33.

Rapid germination accompanies this rapid attainment of the maximum Q_{O_2} . Forty-four per cent of the spores germinate in the first half hour of incubation, and very few more in the ensuing $2\frac{1}{2}$ hours of the experiment.

Manganese. The respiration and germination of spores in manganese and in L-alanine present different pictures (figure 1). The curves showing cumulative oxygen consumption for 3 hours with L-alanine and with manganese (curves 1 and 3, figure 1) have the same general relationship to each other as do the curves for germination (curves 2 and 4, figure 1), bringing out the close relationship between germination and oxygen consumption. However, unless one is fully cognizant of the constituents of a germination medium, predictions as to oxygen consumption drawn from germination data may be invalid. Thus (figure 1), the percentage of germination in 0.2 mM manganese is approximately equal to that in 0.2 mM L-alanine, but the oxygen consumption in manganese is approximately 25 per cent higher than that in L-alanine.

A more impressive comparison with the observations in the L-alanine experiments may

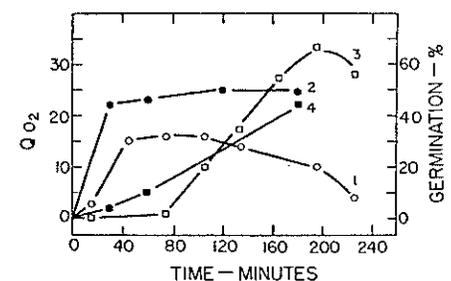


Figure 2. Comparison of the germination and respiration responses of spores to L-alanine and to manganese. Curves 1 and 2, L-alanine + glucose; curves 3 and 4, manganese + glucose. Curves 1 and 3, Q_{O_2} data; curves 2 and 4, germination data. Each vessel contained 3.0 mg unheated spores. Incubation was at 30 C.

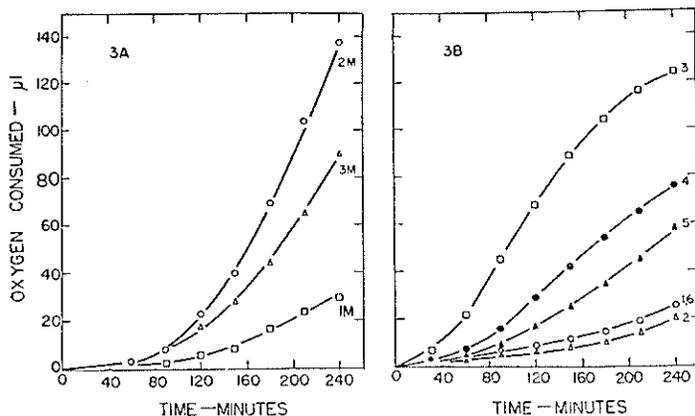


Figure 3. The effect of D-alanine on the oxygen consumption of spores in manganese (3A) and in L-alanine (3B). Curve 1M, glucose; curve 2M, manganese + glucose; curve 3M, manganese + D-alanine + glucose; curve 1, glucose; curve 2, D-alanine (0.2 mM) + glucose; curve 3, L-alanine (0.2 mM) + glucose; curves 4 through 6, L-alanine (0.2 mM) + glucose + D-alanine at concentrations of 0.008, 0.02, and 0.2 mM, respectively. Each vessel contained 3.0 mg unheated spores. Incubation was at 30 C.

be made on the basis of Q_{O_2} measurements (figure 2). There is a lag of between 70 and 80 min before spores in manganese and glucose start to consume oxygen at an appreciable rate. With L-alanine, oxygen consumption starts after only a short lag and the maximum acceleration is greater than that with manganese. However, the spores in manganese reach a considerably higher rate of oxygen consumption than is attained in L-alanine.

Whereas spores respiring in L-alanine and in glucose germinated practically as much as they were going to in the first half hour of incubation, the spores in manganese and glucose reached their maximal germination more gradually (curves 3 and 4, figure 2). Q_{O_2} values as high as 70 may be reached with manganese (10 mM), but no tested concentration of L-alanine will give values higher than 35.

D-Alanine. D-Alanine inhibits the L-alanine

TABLE 1

Effect of D-alanine on germination of heated and unheated spores in manganese and in D-alanine

Germination Medium	Per Cent Germination					
	Unheated spores incubated			Heated spores incubated		
	1 hr	2 hr	3 hr	1 hr	2 hr	3 hr
Glucose +						
1. —	6	13	15	9	12	22
2. D-alanine	5	10	16	4	10	19
3. L-alanine	45	49	49	60	68	72
4. L-alanine + D-alanine	6	15	17	10	21	35
5. Mn	10	23	44	45	78	79
6. Mn + D-alanine	5	8	26	8	28	37
7. Mn + L-alanine	61	73	73	81	85	84
8. Mn + L-alanine + D-alanine	10	36	36	23	61	62

Heat treatment was at 60 C for 10 min. Final concentration of glucose was 25 mM; L-alanine (L-al), 0.2 mM; D-alanine (D-al), 0.2 mM; manganese (Mn) as manganous sulfate, 0.1 mM. Each vessel contained 3.0 mg spores. Germination data recorded after shaking on Warburg respirometer for indicated time periods at 30 C.

TABLE 2
The influence of manganese and of D-alanine on oxygen consumption by spores of *Bacillus megaterium*

	Concentration in System		Oxygen Consumption Excess μL
	Manganese mM	D-alanine mM	
1	0.0	0.0	0
2	0.0	0.2	0
3	0.01	0.0	17
4	0.05	0.0	45
5	0.1	0.0	94
6	0.01	0.2	19
7	0.05	0.2	35
8	0.1	0.2	46
9	0.1	0.008	91
10	0.1	0.02	69

Final glucose concentration in all cases was 25 mM. The oxygen consumption excess is the difference between the oxygen uptake in four hours with manganese and/or D-alanine at 30 C and that with glucose alone (21 μL) in the same time. Each vessel contained 3.0 mg unheated spores.

stimulation of spore respiration (figure 3B) and germination (lines 3 and 4, table 1). Germination data, recorded at the end of the 4 hours of the experiment represented by figure 3B, showed that D-alanine had no effect on the germination of spores in glucose. However, germination of spores in L-alanine (about 45 per cent) was reduced to the level of spores germinating in glucose alone by the addition of an equimolar quantity of D-alanine.

If D-alanine is a specific inhibitor of L-alanine in germination, then a D-alanine inhibition of manganese stimulation might conceivably be attributed to inhibition of the stimulation resulting from the L-alanine produced by manganese. The results of an experiment relating to this hypothesis are shown in table 2. When 0.2 mM D-alanine was added to 0.1 mM manganese, the 4-hr oxygen consumption was reduced approximately 51 per cent. When 0.2 mM D-alanine is added to spores in 0.05 mM manganese and glucose, the reduction is only about 22 per cent, and D-alanine is ineffective when added to spores in 0.01 mM manganese and glucose.

The inhibitory effect of D-alanine on manganese stimulation is further illustrated in figure 3A for

respiration data and by table 1 (lines 5 and 6) for germination data.

Manganese and L-alanine. Manganese enhances the typically early response of spores to L-alanine (figure 4). The response to manganese and L-alanine in combination (curve 6, figure 4) is not merely an additive effect of the responses to these two agents separately.

Heated Spores

The higher the temperature of heating (from 0 to 70 C) and the longer the exposure to heat (from 5 to 60 min), the higher are the oxygen consumption and germination on glucose (table 3). A plot, not shown here, of 3-hr oxygen consumption in glucose as a function of time of heating, reveals an essentially linear relationship for exposures of from 5 to 60 min. D-Alanine suppresses the oxygen uptake and the germination (figure 5, tables 1 and 3) of heated spores in glucose, and at 60 C this suppression is more

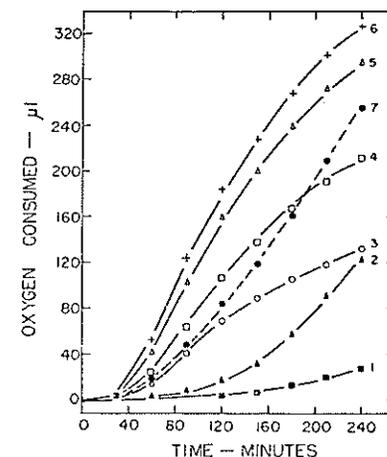


Figure 4. The effect on oxygen consumption of the addition of manganese to spores in L-alanine and glucose. All vessels contained 25 mM glucose and 3.0 mg unheated spores. L-Alanine was at 0.2 mM when used. Curve 1, glucose; curve 2, manganese (0.1 mM); curve 3, L-alanine; curve 4, L-alanine + manganese (0.01 mM); curve 5, L-alanine + manganese (0.05 mM); curve 6, L-alanine + manganese (0.1 mM) by addition of curves 2 and 3. Incubation was at 30 C.

TABLE 3

Effect of duration and temperature of heating and of D-alanine on oxygen consumption and on germination

Heating Conditions		Concentration of D-Alanine	Oxygen Uptake		Germination	
Temperature	Time		3 hr	4 hr	3 hr	4 hr
C	min	mM	μ L	μ L	%	%
0	30	0.0	14	25	12	13
	30	0.2	12	—	11	—
	30	1.0	12	—	11	—
30	30	0.0	13	27	11	15
40	30	0.0	24	47	14	19
50	30	0.0	35	68	30	40
	60	0.0	98	159	47	—
	60	0.2	76	134	33	—
	60	1.0	75	129	35	—
	120	0.0	147	214	55	—
	120	0.2	112	186	44	—
60	5	0.0	32	53	12	20
	10	0.0	46	76	22	27
	15	0.0	60	102	27	32
	30	0.0	100	167	41	49
	30	0.2	51	—	23	—
	30	1.0	47	—	18	—
	45	0.0	142	220	50	54
60	0.0	206	289	60	65	
70	30	0.0	157	254	66	67

Incubation of spores subsequent to heat treatment was in Warburg vessels at 30 C. Each vessel contained 3.0 mg spores and glucose in a final concentration of 25 mM.

apparent when spores have been heated for 30 min than for 10 min.

Heating of spores reduces the lag in oxygen consumption both with manganese and with L-alanine. However, the relation between oxygen consumption in manganese and in L-alanine remains approximately the same as with unheated spores (curves 2 and 3, figure 4, with curves 3 and 4, figure 5). Heated spores in L-alanine consume oxygen similarly to unheated spores in a combination of manganese and L-alanine (curve 6, figure 4, with curve 3, figure 5).

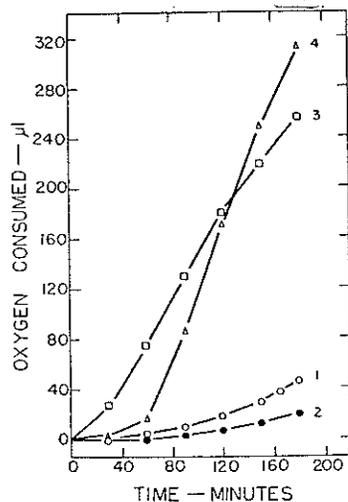


Figure 5. The effect of manganese, L-alanine, and D-alanine on the oxygen consumption of heated spores. Each vessel contained 3.0 mg spores heated at 60 C for 10 minutes. Curve 1, glucose; curve 2, glucose + D-alanine; curve 3, L-alanine + glucose; curve 4, manganese + glucose. Incubation was at 30 C.

DISCUSSION

Manganese activates a proteolytic enzyme of the spores of *B. megaterium* and stimulates spore germination (Levinson and Sevag, 1953, 1954). This protease is capable not only of hydrolyzing gelatin and egg albumin but also of breaking down spore protein. One of the products of the protein hydrolysis, L-alanine, is known to be stimulatory to spore germination. The effect of L-alanine is catalytic and can be only partially due to oxidation of this substance. It was postulated that the stimulation of germination by manganese resulted from its activation of proteolytic enzymes. The germination and respiration data presented here do not negate this hypothesis, but indicate that manganese has additional roles. Spores incubated with L-alanine and glucose germinate almost immediately. Within the first half hour of incubation, practically all the spores which will germinate have done so. The rate of oxygen consumption, too, rapidly approaches the maximum that will be attained. On the other hand, spores incubated

with manganese and glucose have a much longer lag before the maximal Q_{O_2} is attained, and the percentage of germination increases gradually. These phenomena have been found to be characteristic for germination and respiration in L-alanine and in manganese. If the theory involving proteolysis comprised the complete explanation, one might conclude that the long lag exhibited by spores incubated with manganese is due to the time necessary for the accumulation of hydrolytic products.

If manganese acted only to stimulate production of L-alanine, incorporation of higher concentrations of the latter should give germination and maximal Q_{O_2} values at least equal to those attained in manganese. We have not been able, with any concentration of L-alanine, to attain the Q_{O_2} and germination percentages that obtain with high concentrations of manganese.

If L-alanine were the only substance, or even the main substance, having a role in the stimulation of spore germination, and the function of manganese were merely to activate the production of L-alanine, it would not be expected that the addition of manganese to spores respiring in L-alanine would be stimulatory. But the addition of manganese in this case is stimulatory. The effect of manganese and L-alanine in combination is not merely additive, but is rather an exaggerated L-alanine type of response. This includes both the typical early rise in Q_{O_2} and the early germination.

Our experiments with D-alanine also suggest that manganese may have a dual role. Whereas the stimulatory effect of 0.2 mM L-alanine on germination is completely nullified by the addition of 0.2 mM D-alanine, this concentration of D-alanine reduces the germination of spores incubated in manganese by only 41 per cent. Respiration data, too, indicate that D-alanine cannot completely reverse the stimulation obtained with manganese as it does that with L-alanine. The lower the concentration of manganese, the lower is the capacity of D-alanine to reverse the manganese stimulation. Possibly in low concentration manganese acts without the release of L-alanine. As its concentration increases, manganese may function in the release of L-alanine as well as in raising the metabolic level of the spores. The stimulation resulting from the use of higher concentrations of manganese

is then partially reversed by D-alanine. It is conceivable that D-alanine reverses that portion of manganese stimulation which is involved in L-alanine release, while the general metabolic stimulation is unaffected.

The partial reversal of heat stimulation by D-alanine may indicate that, as with manganese stimulation, heat treatment acts in part to produce L-alanine (reversible by D-alanine) and in part as a more general metabolic stimulant.

Heat treatment, like manganese, raises the metabolic level of the spores without greatly altering the basic type of response. Both thermal shock and manganese enhance the respiratory activity of spores in glucose. Heated spores incubated in L-alanine and glucose respire and germinate in a manner similar to unheated spores in the presence of manganese, L-alanine and glucose. The germination of spores in glucose for 3 hours is increased similarly by heat (from 12 per cent without heat to 41 per cent by pre-heating 30 min at 60 C) and by manganese (to 44 per cent).

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SUMMARY

Both L-alanine (0.2 mM) and manganese (0.1 mM) stimulate the respiration and germination of spores of *Bacillus megaterium*, each in a characteristic manner. L-Alanine effects rapid attainment of maximal germination and rate of oxygen consumption; with manganese, maximal germination and respiration are attained after a longer lag, although ultimately higher levels are reached.

D-Alanine completely reverses the stimulation with L-alanine, but only partially reverses stimulation by manganese or by heat.

We no longer subscribe to the theory that manganese acts in the stimulation of spore germination solely through activation of a spore protein hydrolyzing enzyme yielding stimulatory substances such as L-alanine. We now propose that the major effect of manganese is to raise the over-all metabolic level of the spores by an unknown mechanism. Stimulation of L-alanine formation may still be involved.

The similarities between heat and manganese stimulation of spore germination and respiration are discussed.

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