STIMULATION OF GERMINATION AND RESPIRATION OF THE SPORES OF BACILLUS MEGATHERIUM BY MANGANESE AND MONOVALENT ANIONS

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INTRODUCTION

The work reported here dealing with the spores of *Bacillus megatherium* shows that maximal rapid germination depends, at least in part, on the minerals in the medium such as manganese, which also strongly stimulates the oxygen uptake of the spores in glucose. Certain monovalent anions also play a significant role.

The phenomenon of dormancy of spores has long been a subject of interest. Burke (1919, 1923) observed long periods of dormancy both with heated and unheated spores of *Clostridium botulinum*, and Burke *et al.* (1925) demonstrated that the unheated spores of *Bacillus subtilis* and *B. megatherium* were dormant for 39 and 90 days, respectively. Evans and Curran (1943) reported, however, that preheating of the spores of *B. megatherium*, *B. subtilis*, *B. cereus*, and *B. cohaerens* decreased the time required for germination in these species. Morrison and Rettger (1930) found that there was no delay in the germination of a milk spoilage organism when the proper nutrient medium was supplied; *i.e.*, milk rather than nutrient broth. Tarr (1933) showed that the velocity of glucose oxidation by *B. subtilis* spores is greatly accelerated by preheating of the spores at 80°C. for 30 minutes. Wynne and Foster (1948a) have reported that the dormancy of *Clostridium botulinum* spores could be broken by the use of the proper medium and by the incorporation in the medium of 0.1 per cent soluble starch, which adsorbs small amounts of substances in the medium capable of repressing spore germination. The same workers (1948 b) noted a stimulatory effect on the germination of *C. botulinum* after the addition of carbon dioxide, but carbon dioxide did not accelerate the germination of several aerobic spore formers in nutrient broth.

Methods

(*a*) *Harvesting of Spores.*—Spores of *Bacillus megatherium* were obtained by growing the organism[1] (QM B1551) on liver broth (Foster and Heiligman, 1949) buffered

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[1] We are indebted to Dr. Francis E. Clark, Agricultural Experiment Station, Ames, Iowa, for confirming the identity.

617

with M/30 potassium phosphates at pH 6.8. The medium was dispensed, 25 ml. per 250 ml. Erlenmeyer flask, sterilized by autoclaving, and inoculated with 0.5 ml. of a spore suspension (containing approximately 100,000 spores per ml.) previously heated at 70°C. for 10 minutes to destroy vegetative forms. The flasks were incubated with shaking at 30°C. for 5 days, at the end of which time practically 100 per cent sporulation had occurred. Harvesting of the spores was accomplished by centrifuging the liver broth cultures in a refrigerated centrifuge. They were then washed twice with 0.005 M potassium phosphate at pH 6.8, then twice with distilled water in the refrigerated centrifuge. The final spore sediment was frozen and dried under vacuum. The dried spores were stored in a desiccator at 5–10°C. Several experiments indicated that there was no essential difference between dried and fresh spores in the experiments reported here. Analysis of several crops of spores for total nitrogen and for total phosphorus indicated a high degree of uniformity amongst crops of spores. Total nitrogen of the spores was 9.0 per cent and total phosphorus was 1.3 per cent on a dry weight basis.

(b) *Germination.*—We use essentially the technique of Powell (1950) in the measurement of the percentage of germination of spores. When 0.5 per cent aqueous methylene blue is applied to the air-dried smear of spores on a glass slide, ungerminated, heat-resistant spores stand out as unstained, highly refractile bodies, while germinated spores stain a definite blue. Percentages of germination are given as:

$$\text{Per cent germination} = \frac{\text{No. of spores staining with methylene blue}}{\text{Total No. of spores}} \times 100.$$

In all the experiments cited the percentage of germination was taken at the end of respiration experiments.

(c) *Respiration.*—

1. Reaction Systems.—Oxygen uptake was measured at 30°C. using Warburg respirometers. The reaction systems contained 1.0 ml. of spore suspension, 0.5 ml. of substrate in the side arm, and 0.2 ml. of 10 per cent KOH in the center well for a total volume of 1.7 ml. When double side arm vessels were used as in the experiment represented by Fig. 2, 1.5 ml. of spore suspension was used, 0.25 ml. of substrate was in each side arm, and 0.2 ml. of 10 per cent KOH was in the center well for a total volume of 2.2 ml. 3 mg. of dried spores were used in the experiments covered by Tables V, VI, and VII, as well as for all the figures. 4 mg. of spores were used for Table II, and 5.0 mg. were used for Tables I, III, and IV.

2. Substrates.—The concentration of adenosine in these systems was 0.00075 M; glucose 0.028 M; and monosodium glutamate 0.006 M. Concentrations other than these are indicated with the results of the individual experiments.

3. Salt Mixtures.—In the experiments, the results of which are represented in Tables II and III, the inorganic salt mixture consisted of sodium chloride, 0.085 M; magnesium sulfate, 0.0008 M; ammonium dihydrogen phosphate, 0.0087 M; and potassium dihydrogen phosphate, 0.24 M (adjusted to pH 6.8).

The concentration of salts used in experiments represented by Tables I and IV was for sodium chloride, ammonium dihydrogen phosphate, and potassium dihydrogen phosphate (adjusted to pH 6.8), 0.05 M, and for magnesium sulfate, 0.005 M.

The concentration of the salts used in experiments represented by Figs. 1, 2, and 3 was for ammonium acetate and potassium acetate (pH 6.6), 0.05 M.

(d) *Heat Activation.*—When heat activation of spores was used for an experiment, it was accomplished by heating a suspension of spores in a water bath at 60°C. for 10 minutes.

(e) *Glucose.*—Glucose concentrations were measured at the beginning and at the end of Warburg experiments by the dinitrosalicylic acid method of Sumner and Somers (1944).

(f) *Turbidity.*—The turbidity of suspensions for Fig. 1 was determined with a Klett-Summerson photoelectric colorimeter using a 470 to 530 mμ filter.

TABLE I

The Effect of Manganese Treatment on Germination and Oxygen Uptake

Spore treatment	MnCl$_2$ concentration	O$_2$ uptake	Germination
	M	$\mu l.$	*per cent*
1. Heated in MnCl$_2$	5×10^{-2}	524	93
2. Heated in water	—	230	65
3. 10 min. in MnCl$_2$, no heating	5×10^{-2}	454	92
4. MnCl$_2$ added after heating in water	5×10^{-2}	580	100
5. Heated in water. MnCl$_2$ in re-action system	9.1×10^{-4}	435	95
	1.8×10^{-4}	327	74
	1.8×10^{-5}	255	66
6. No heat. No MnCl$_2$	—	19	10

Spore suspensions were washed once with 4 volumes of water after treatments 1 through 5. Medium for respiration and germination consisted of salts, glucose, glutamate, and adenosine. For conditions, see Methods (c). Reaction period was 120 minutes.

RESULTS

Factors Influencing Germination and Oxygen Consumption

(a) *Heat.*—An experiment confirming the heat effect for *B. megatherium* spores may be cited (Table I, treatments 2 and 6). The striking effect of the preheating of spores is also demonstrable when they are heated in phosphate buffer. However, a high concentration of the potassium phosphate in the heating medium exercises a depressing effect (Table II). As is shown in Table III, an outstanding exception to the depression of germination and respiration obtained by heating spores in salts is the heating of spores in manganous chloride. Here, the total oxygen consumption in 140 minutes is almost twofold the respiration of water-heated spores, and the germination is increased.

(b) *Manganese.*—The question of how and when the manganese chloride exercises its stimulatory effect on spore germination was investigated. The data

TABLE II

The Effect of Phosphate Concentration in the Heating Medium on Oxygen Consumption

Spores heated in	O₂ uptake*
	$\mu l.$
Water	345
Salts‡ without potassium phosphates	189
Salts with 0.04 M potassium phosphates	162
Salts with 0.24 M potassium phosphates	94

* Medium for respiration consisted of salts (with 0.24 M potassium phosphates), glucose, glutamate, and adenosine. For conditions, see Methods (c). Reaction period was 160 minutes.

‡ See Methods, section c3.

TABLE III

The Effect of Heating of Spores in Various Salts on Germination and Oxygen Uptake

Spores heated in	Concentration	O₂ uptake	Germination	Glucose utilized
	M	$\mu l.$	per cent	per cent
1. Distilled water	—	351	83	71
2. Magnesium chloride	0.05	237	67	46
3. Sodium chloride	0.05	219	52	41
4. Calcium chloride	0.05	191	43	31
5. Manganous chloride	0.05	637	100	90
6. Potassium phosphate*	0.05	165	59	46
7. Potassium phosphate*	0.1	141	56	38
8. Potassium phosphate*	0.3	129	39	36
9. Potassium chloride	0.025	269	67	56
10. Potassium chloride	0.05	216	62	53
11. Potassium chloride	0.3	163	38	35
12. Sodium phosphate*	0.05	212	37	35
13. Ammonium phosphate*	0.05	170	25	34
14. Potassium phthalate*	0.05	132	28	35

* Adjusted to pH 6.8–7.0.

Spore suspension was washed once with 4 volumes of water after heating. Medium consisted of salts, glucose, glutamate, and adenosine. For conditions, see Methods (c). Reaction period was 140 minutes.

presented in Table I reveal that while heating of the spores with manganese results in a higher oxygen uptake than merely heating in water, it is not essential for the mangenese to be present in the heating medium. This has been observed repeatedly.

The oxygen consumption and germination of the spores heated in water are

increased by the addition of glutamate and adenosine to the salts-glucose medium (Table IV). But this effect of glutamate and adenosine is equaled by adding manganese to water-heated spores respiring only in inorganic salts-
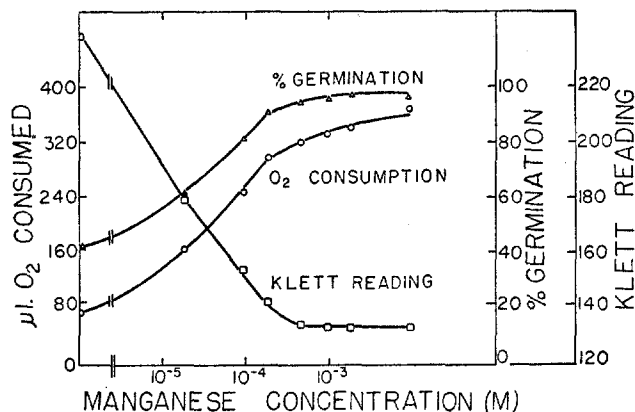

Fig. 1. The effect of manganese concentration on germination, oxygen uptake, and turbidity of spore suspensions. Indicated Klett readings represent turbidities. Spores were not heated. Manganese was as $MnSO_4$. Medium was composed of salts and glucose (0.025 M). For conditions, see Methods (c). Reaction period was 140 minutes.

TABLE IV

The Effect of Manganese, Heat Treatment, Glutamic Acid, and Adenosine on Germination and Oxygen Uptake

Spore treatment	Medium*	O_2 uptake	Germination
		μl.	*per cent*
1. Heated in water.....................	S-G-Gl-Ad	232	83
2. Heated in water.....................	S-G	38	13
3. Suspended in $MnCl_2$, 0.05 M, no heat....	S-G	228	75
4. No heat. No $MnCl_2$.................	S-G	0	0
5. No heat. No $MnCl_2$.................	S-G-Gl-Ad	19	10

* Medium composition is indicated as S = salts; G = glucose; Gl = glutamate; Ad = adenosine. Spore suspension was washed once with 4 volumes of water after treatments 1 to 3. For conditions, see Methods (c). Reaction period was 120 minutes.

glucose. The effect of manganese is increased as the manganese concentration increases (Fig. 1). The effect of manganese is greatest at concentrations ranging from 0 to 4.5 × 10^{-4} M, and levels off when the concentration is about 9.1 × 10^{-4} M or higher. A striking relationship in oxygen uptake, per cent germination, and turbidity is noted.

An experiment was conducted to determine whether manganese or glucose

is stimulatory *per se* to the germination of the spores (Fig. 2). Unheated spores were incubated with manganese (at 9.1×10^{-4} M) for 60 minutes before the addition of glucose; and another aliquot of spores was incubated with glucose for the same length of time before the addition of manganese. There was no difference in the respiration of the two sets of spores.

The observed stimulation of germination and of oxygen consumption by manganese made it desirable to investigate whether other divalent cations had
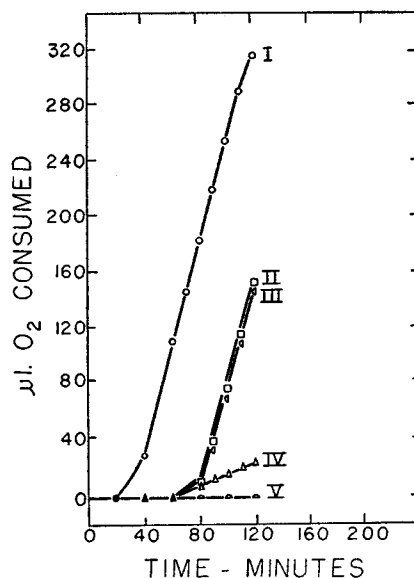

FIG. 2. The effect of time of addition of manganese and glucose on oxygen uptake. Spores were not heated. Medium consisted of salts, glucose (0.025 M), and manganese as the sulfate (9.1×10^{-4} M). For conditions, see Methods (*c*). Curve I, manganese and glucose tipped at 0 minutes, salts at 60 minutes. Curve II, glucose tipped at 0 minutes, manganese at 60 minutes. Curve III, manganese tipped at 0 minutes, glucose at 60 minutes. Curve IV, glucose tipped at 0 minutes, salts at 60 minutes. Curve V, manganese tipped at 0 minutes, salts at 60 minutes. On the zero abscissa, solid triangles represent curves II to V; solid circle represents curves I to V.

the same effect. Cations, as the sulfates, were added with the substrate to acetate-buffered glucose. Water-heated spores were used. Clearly (Fig. 3) cobalt and zinc have some stimulatory effect on germination, although not as great as that observed with manganese. Iron and copper exert an inhibitory effect.

The question of whether the observed noteworthy effect of manganese was demonstrable with spores of organisms other than *Bacillus megatherium* was investigated. Manganese did not exercise a similar stimulation of the germination and oxygen uptake of the spores of *B. subtilis* and *B. cereus*.

(c) *Monovalent Anions.*—Most of the experiments up to this point have used a complete inorganic salt medium containing sodium chloride, ammonium dihydrogen phosphate, and potassium phosphates (pH 6.8) at a concentration of 0.05 M, and magnesium sulfate at a concentration of 0.005 M. The respiration of spores heated in 0.05 M manganous chloride was measured in a substrate consisting of glucose and salts prepared so as to be deficient in one ion of the complete inorganic salt solution (Table V). Chloride omission represses ger-
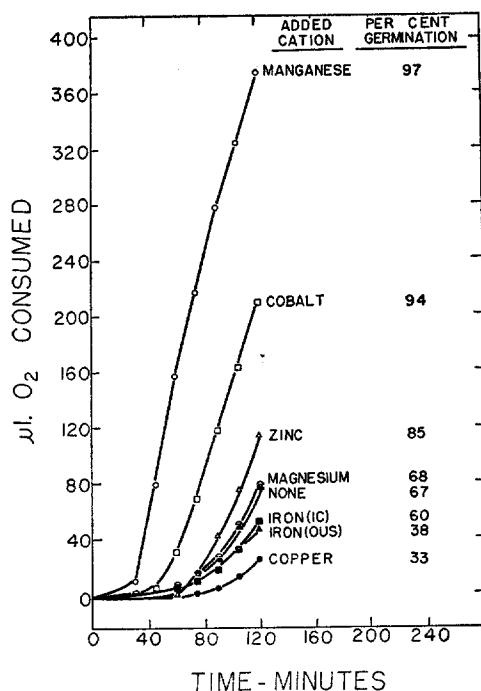

FIG. 3. The effect of added cations on germination and oxygen uptake. Spores were heated in water. Medium was composed of salts, glucose, and added cation as the sulfate in a concentration of 0.00077 M. Added cations are divalent except where indicated. For conditions, see Methods (c).

mination and respiration strikingly. The effect of phosphate omission might be due to the increased acidity, the pH changing from 6.0 to 4.2 during the experimental period of 1.5 hours in this unbuffered solution, while none of the other omissions showed any significant change from the initial pH of 6.8–6.9.

In summarizing some of the effects thus far described (Table VI), the following facts become evident. Manganese (or cobalt) increases the germination and oxygen uptake of *B. megatherium* spores. This effect is much enhanced in the presence of chloride. The curve in Fig. 4 represents the titration of the effect of chloride as the specific anion. The effect of chloride is not so evident

when adenosine is present as when it is absent in the substrate for manganese-treated spores. This "substitution" of adenosine for chloride is not due to chloride present in the adenosine preparation used. In fact, adenosine in four

TABLE V

The Effect of Ion Deficiency on Germination and Oxygen Uptake

Ion omitted	O_2 uptake	Germination
	μl.	*per cent*
None	164	92
NH_4^+	87	52
Na^+	160	93
Mg^{++}	128	79
K^+	156	79
$PO_4^{\equiv}$	145	77
Cl^-	5	23
SO_4^-	147	78

Spore suspension was heated in 0.05 M of manganous chloride, then washed once with 3 volumes of water. Reaction systems consisted of glucose to which was added inorganic salt solution containing 0.005 M concentration of Mg^{++} and SO_4^-; and 0.05 M concentration of other ions. For conditions see Methods (c). Oxygen uptake was determined for 100 minutes, per cent germination at the end of 150 minutes.

TABLE VI

The Effect of Manganese and Chloride on Oxygen Uptake

Spore treatment	NaCl	O_2 uptake in:						
		G	Gl	Ad	G-Gl	G-Ad	Gl-Ad	G-Gl-Ad
		μl.	*μl.*	*μl.*	*μl.*	*μl.*	*μl.*	*μl.*
Heated in water	+	13	0	0	67	28	0	331
	−	14	0	0	47	6	0	88
Heated in 0.05 M manganous sulfate	+	400	0	0	573	550	57	619
	−	26	0	0	121	377	13	574

Spore suspension was washed once with 3 volumes of water after heating. Sodium chloride concentration was 0.05 M. Medium consisted of 0.05 M concentration of ammonium and potassium phosphates (pH 6.8) and 0.001 M concentration of magnesium sulfate. G = glucose; Gl = monosodium glutamate (0.005 M); and Ad = adenosine. For conditions, see Methods (c). Reaction period was 160 minutes.

times the concentration used in the experiment gave no chloride precipitate with N/10 silver nitrate. Investigation of the specificity of the action of chloride (Fig. 5) shows that except for fluoride and formate, all the monovalent anions tested produced a stimulation of germination when compared with a substratum lacking monovalent anions. We call the effect of the monovalent anions

the "anion effect." With less concentrated potassium phosphate buffer (0.0005 M rather than 0.05 M), the "anion effect" is not so apparent (Table VII). The "anion effect" is, however, independent of the concentration of sulfate and ammonium ions. Adenosine gave essentially a percentage of germination as great as that found with the added anions. In parallel experiments, the presence of 0.00075 M of adenine, adenylic acid, adenosinetriphosphate, guanylic acid, uracil, or guanine failed to exercise any effect comparable to adenosine.
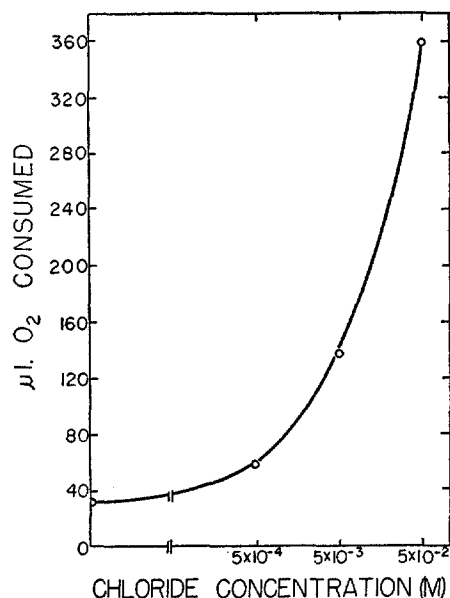

FIG. 4. The effect of the concentration of chloride in the medium on oxygen uptake. Spore suspension was washed once with 3 volumes of water after heating in 0.05 M of manganous sulfate. Medium consisted of 0.05 M of potassium phosphates (pH 6.8) and ammonium dihydrogen phosphates; 0.001 M of magnesium sulfate; glucose; and chloride as the sodium salt. For conditions, see Methods (c). Reaction period was 150 minutes.

(d) *Amino Acids.*—While our studies regarding the effect of amino acids on germination are fragmentary, we can report that glutamic, *l*-aspartic, *dl*-aspartic acids, *dl*-alanine, and β-alanine do not stimulate germination of heated or unheated *B. megatherium* spores when used at a concentration of 0.025 M in a system containing potassium acetate and ammonium acetate whether manganese is present or absent. On the other hand, 77 per cent of heated spores incubated with *l*-alanine and manganese germinated in 120 minutes, and 12 per cent germinated when no manganese was present. Of unheated spores, 4 per cent germinated with manganese, while only 1 per cent germinated when
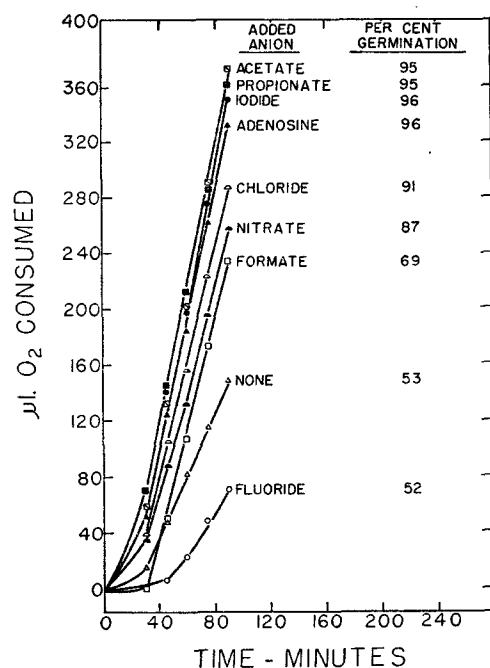
FIG. 5. The effect of some monovalent anions on germination and oxygen uptake. Spore suspension was washed once with 3 volumes of water after heating in 0.05 M of manganous sulfate. Medium consisted of 0.025 M of ammonium and potassium phosphates (pH 6.8); glucose; and 0.05 M of the sodium salts of the monovalent anion (or adenosine instead of the anion). For conditions, see Methods (c).

TABLE VII

The Effect of Composition and Concentration of Salts on the "Anion Effect" in Germination, Oxygen Uptake, and Glucose Utilization

Composition of salts			O₂ uptake	Germination	Glucose utilized
KH₂-K₂HPO₄ (pH 6.8)	(NH₄)₂SO₄	NaCl			
M	M	M	μl.	per cent	per cen
0.05	0.05	0.05	262	94	54
0.05	0.05	0.0	54	27	26
0.0005	0.05	0.05	263	95	56
0.0005	0.05	0.0	214	92	51
0.05	0.0005	0.05	172	58	44
0.05	0.0005	0.0	30	3	12
0.0005	0.0005	0.05	123	41	37
0.0005	0.0005	0.0	161	54	35

Spore suspension was washed once with 3 volumes of water after heating in 0.05 M of manganous sulfate. Medium consisted of glucose; potassium hydrogen phosphates and ammonium sulfate in the concentrations indicated; and 0.001 M concentration of magnesium sulfate. For conditions, see Methods (c). Reaction period was 120 minutes.

626

manganese was absent. Contrary to the situation with glucose, no oxygen consumption accompanied germination when *l*-alanine was the substrate. In this connection, Hills (1949, 1950) has reported that *l*-alanine was essential for rapid germination of a laboratory strain of *B. subtilis*, and the effect of *l*-alanine was strongly inhibited by *d*-alanine at a molar ratio of 1:30.

DISCUSSION

Spores of *Bacillus megatherium* incubated in water, in inorganic salt solution, or in phosphate buffer did not germinate, failed to consume oxygen, and suspensions failed to become less turbid in 2 to 4 hours. Germination of the spores in glucose solutions as followed by the methylene blue staining technique is closely related to the oxygen consumption. Our method for the determination of the percentage of germination is admittedly subject to error, but the close relationship between germination and oxygen uptake of spores in glucose is striking. There was also demonstrated a close correlation between germination and turbidity changes (Fig. 1), a correlation previously pointed out by Powell (1951). When glucose is used in the substrate, the per cent of glucose utilized also becomes a measure of the degree to which the spores have germinated. In all the experiments it was found to be important that the measurements be made after a relatively short incubation period, since on extended incubation cell division will occur, and there is then no way of determining the number of original spores which germinated.

Germination may then involve two phases: (*a*) "pregermination," or the process undergone by the spore when it becomes stainable and has started to consume oxygen, but before it has elongated and become typically bacillary in shape, and (*b*) "germination," which we conceive of as having occurred when the bacillus has emerged from the spore case.

A similar relationship has been noted by Powell (1950) and by Pulvertaft and Haynes (1951). The latter using phase microscopy, showed essentially what we term a "pregerminative" phase in *B. cereus* and in *B. subtilis* following almost immediately on the addition of adenosine. This was followed by the emergence of the bacillus, or "germination." No motile vegetative cells were observed by Tarr (1933) in spore preparations of *B. subtilis* which had oxidized glucose for 2 hours. Tarr interpreted this as showing that the oxygen uptake was due to spores, and not to vegetative cells. We can agree, at least partly, with this interpretation. However, in view of the present data, we should say that his spores were in a "pregerminative phase."

Keilin and Hartree (1947) have described the stimulation of *B. subtilis* spore germination by yeast extract. The stimulation of germination of *B. anthracis* spores by yeast extract and the replacement of yeast extract by adenosine were reported by Hills (1949). Powell (1951) has reported that in a strain of *B. megatherium*, glucose was specifically essential for germination of

heated spores. We have not been able to confirm this essentiality of glucose with our strain of *B. megatherium*. Indeed, spores heated in water and incubated in an inorganic salt-glucose medium showed only a small amount of germination (Table IV). However, the addition of adenosine and glutamate to the inorganic salts-glucose substrate increases the germination considerably.

Our results show that if the spores, instead of being heated in water, are heated in manganous chloride or manganous sulfate, germination and oxygen consumption in inorganic salts-glucose are markedly increased (Table IV). Moreover, as long as there is sufficient manganese in the system, heat activation appears to be no longer necessary for optimal germination. On the other hand, iron and copper inhibit germination at concentrations at which manganese is stimulatory.

It is not known whether this effect of manganese is due to an activation of an enzyme system. Hochster and Quastel (1952) reported that MnO_2 can act as an oxidant of a variety of biologically important substances. The relation of this observation to ours cannot at present be indicated. Experiments are in progress to determine whether any specific enzymes in the spores are activated by manganese as a cofactor. In view of the non-specificity of the manganese activation (since cobalt and zinc are also effective, though to a minor degree), it may be possible that manganese activates enzymes essential for germination, and concomitantly respiration, in essentially the same way as does heat activation; that is, in relieving a respiratory block (Goddard, 1939). Another possibility is that manganese so alters the permeability of the spore that glucose may enter and be metabolized. If this interpretation were correct, preincubation of the spores in manganese should accelerate germination following the addition of glucose. As is seen in Fig. 2, preincubation does not show this effect. Neither glucose nor manganese acts alone to "sensitize" the spores for rapid germination and respiration. It is interesting to speculate on a possible cyclical nature of the germination process, with manganese occupying a mediating role both in the formation of spores (sporulation) and in the transformation of spores into vegetative cells (germination). Charney, Fisher, and Hegarty (1951) have shown that manganese stimulates the sporulation of several species of *Bacillus*, and that sporulation is dependent on the presence of manganese in concentrations greater than those required for vegetative growth.

The nature of the ions of the inorganic salt solution is of great importance. In an inorganic salt solution containing 0.05 M phosphate, omission of chloride results in almost complete suppression of germination and oxygen uptake (Table VII). This "anion effect" is far from specific, being obtainable, in more or less degree, with any one of a number of monovalent anions (Fig. 5) or with adenosine. The effect is nullified as the phosphate concentration is lowered (Table VII), indicating that there may be some sort of ionic balance necessary for optimal germination. It does not seem likely that adenosine and chloride

are acting in the same way. We hope that completion of current work will give us a fuller knowledge of the bases for these effects.

SUMMARY

1. The addition of manganese in a concentration as low as 1.8×10^{-5} M stimulates both respiration and germination markedly and eliminates the need for heat activation of spores respiring in inorganic salts-glucose.

2. Rapid germination and respiration of *B. megatherium* spores require a proper balance of ions in the nutrient medium. The repressive effect of a high phosphate concentration can be eliminated or balanced by chloride, or by other monovalent anions. Adenosine can relieve the necessity for chloride in such a system.

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