PYROPHOSPHATASE ACTIVITY OF *BACILLUS MEGATERIUM* SPORE AND VEGETATIVE CELL EXTRACTS

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We have long felt that a study aimed at correlating the manganese stimulation of germination and respiration of *Bacillus megaterium* spores (Levinson and Sevag, 1953) with Mn^{++} activation of spore enzymes of the organism would prove rewarding. In this connection, we have already shown that spore protease is activated by manganese, that spore protein can serve as a substrate for this enzyme, and that the products of hydrolysis of spore protein are stimulatory to spore germination (Levinson and Sevag, 1954*a*).

Many enzymes are contained in bacterial spores. Indeed, an enzyme of the type described here, a pyrophosphatase, has already been demonstrated in *Bacillus subtilis* spore extracts (Murrell, 1952). The object of the present report is, therefore, not to record an addition to the rapidly growing compendium of spore enzymes, but rather to demonstrate that, in *B. megaterium*, spore pyrophosphatase differs quantitatively and qualitatively from the vegetative cell enzyme. Further, we suggest a relationship between the Mn^{++} stimulation of spore germination and Mn^{++} activation of spore pyrophosphatase.

MATERIALS AND METHODS

Spores of *Bacillus megaterium* strain QM B1551, were grown according to the methods described by Levinson and Sevag (1953), and were harvested after 5 days at 30 C. A pool of lyophilized spores sufficient for all the experiments described here was prepared. Vegetative cells of *B. megaterium* were also grown on liver fraction medium (Foster and Heiligman, 1949), but they were harvested after 12 hr at 30 C. The lyophilized vegetative cells and spores were stored over anhydrous $CaSO_4$ (Drierite, W. A. Hammond Co.) at refrigerator temperature until needed.

When applicable, heat shock was accomplished by a 15 min immersion of 120 ml of a suspension containing 1.2 g of spores in a water bath at 60 C. Spores were germinated by adding 540 mg of glucose (to make 2.5×10^{-2} M) to the 120 ml of heat shocked suspension, and incubating on a reciprocal shaker (95-3 in strokes per min) for 90 min at 30 C, but were not allowed to undergo the postgerminative changes described by Hyatt and Levinson (1957). Both heat shocked and germinated spores were washed twice in water before being used for the preparation of extracts.

Enzymatically active extracts and spore coats or vegetative cell walls were prepared by grinding 300 mg of spores or vegetative cells with 8.0 ml of H_2O, 1 drop Antifoam AF Emulsion (a water dilutable dispersion of Dow Corning silicone defoamer), and 5.0 g of no. 13 Ballotini glass beads (0.08 to 0.12 mm diam) in the 10 ml glass cup of the Mickle (1948) tissue disintegrator. After 30 min vibration, no whole cells were evident. The homogenates of spores had the microscopic appearance of the spore coats described by Powell and Hunter (1956). The suspensions were washed from the Mickle cups with an additional 4.0 ml of H_2O, and the spore coats or cell walls were sedimented by centrifuging at approximately $1500 \times G$. The supernatant was saved for the preparation of extract, and the sedimented spore coats were washed 10 times with water before being frozen and dried. From 25 to 30 per cent of the original weight of spores was recovered as spore coats.

The supernatant from the spore coats or vegetative cell walls was centrifuged at 1 to 4 C at $10,000 \times G$, and the yellowish, slightly turbid watery extract so obtained (1.0 ml of extract derived from 25 mg of cells) had considerable pyrophosphatase activity. Clear extract could be obtained by using capryl alcohol (2-octanol, Eastman Kodak Co.) as an antifoam agent (Strange and Dark, 1956), but unfortunately we were unable to demonstrate pyrophosphatase activity in such extracts. The nitrogen content of extracts was determined according to the nesslerization technique of Miller and Miller (1948). Protein nitrogen was estimated by the difference

between total nitrogen and trichloracetic acid soluble (nonprotein) nitrogen. A typical set of nitrogen determinations showed for extract of resting spores, 767 μg of nitrogen per ml, of which 482 μg or 63 per cent was protein nitrogen; for extract of heated spores, 749 μg of nitrogen per ml, of which 471 μg or 63 per cent was protein nitrogen; for germinated spores, 835 μg of nitrogen per ml, of which 543 μg, or 65 per cent, was protein nitrogen; and for vegetative cells, 1757 μg of nitrogen per ml of which 1150 μg, or 65 per cent, was protein nitrogen.

Pyrophosphatase activity was determined by the estimation of the production of orthophosphate from sodium pyrophosphate ($Na_4P_2O_7 + H_2O \rightarrow 2Na_2HPO_4$), using the method of Fiske and SubbaRow (1925) for measurement of orthophosphate phosphorus. Although details varied, reaction mixtures generally consisted of 0.5 ml of barbital or acetate buffer, 5×10^{-2} M; 1.0 ml $MnSO_4$, or other metal salt, 10^{-3} M; 0.2 ml of suitably diluted extract; and after 5 min in a water bath at 37 C, 0.2 ml of $Na_4P_2O_7$, 10^{-2} M. Water was added to make the total volume 2.0 ml. These tubes and controls containing (1) no pyrophosphate nor extract; (2) extract with no pyrophosphate; or (3) pyrophosphate with no extract were incubated at 37 C for 10 min (unless otherwise indicated), chilled in an ice bath, and the reaction stopped by the addition of 0.2 ml of 100 per cent (w/v) trichloracetic acid. The precipitated protein was removed by centrifugation at 1 to 4 C, and the supernatant was tested for orthophosphate. In the system described above, total hydrolysis will yield 56.4 μg of orthophosphate phosphorus per ml, or a total of 124 μg of orthophosphate phosphorus. In most cases, the yield of orthophosphate is given in μg. Where it is desirable to compare extracts from different sources, the activities of the extracts are expressed in terms of μg phosphorus produced per mg protein nitrogen.

The hydrolysis of spore coats was tested in a manner essentially similar to that used for determination of pyrophosphatase activity. Generally, the reaction systems consisted of 1.0 ml of a barbital buffer (pH 6.8) suspension of 20 mg of spore coats; 2.0 ml of 10^{-3} M metal sulfate; 0.5 ml of extract; 0.4 ml of 0.1 per cent merthiolate (Eli Lilly and Co.) to retard bacterial growth; and water to make the total volume 4.0 ml. Controls lacking spore coats, or extract, or both were also included. The mixtures were incubated 18 hr in a water bath at 30 C, and after addition of 0.4 ml of 100 per cent (w/v) trichloracetic acid and centrifugation at approximately 10,000 × G, the supernatant solution was tested for orthophosphate phosphorus.

RESULTS

Metal requirement. Extracts of unheated spores show no pyrophosphatase activity at a neutral pH in the absence of Mn^{++}, but in the presence of Mn^{++} (as the sulfate or chloride) considerable activity is apparent. No other metal is as effective as Mn^{++} in activation of the pyrophosphatase (table 1). Optimal conditions of Mn^{++} concentration and temperature were 5×10^{-4} M (figure 1) and 37 C (figure 2) respectively. Extracts of heated spores and of germinated spores have a similar requirement for Mn^{++}.

By contrast, the pyrophosphatase activity of vegetative cell extracts does not increase with addition of Mn^{++}. It was thought that the lack of a Mn^{++} requirement for the pyrophosphatase activity of extracts of vegetative cells (table 1) might be due to the presence of an adequate quantity of Mn^{++} in the extract. However,

TABLE 1

The metal ion requirement for neutral pyrophosphatase activity of spore and vegetative cell extracts

Metal Ion Added†	Relative Pyrophosphatase Activity*			
	Unheated spores	Heated spores	Germinated spores	Vegetative cells
None	2.4	1.8	0	10.5
Co^{++}	5.8	5.4	0	20.6
Mg^{++}	2.3	0	0	13.8
Mn^{++}	55.9	100.0	30.4	11.8
Zn^{++}	9.8	16.1	7.9	13.1
Ni^{++}	2.4	5.4	0	9.5
Ca^{++}	0.8	3.8	0	
Fe^{++}	0	0		
Sr^{++}	2.3	0	2.6	
Cu^{++}	2.3	1.1	0	
Cd^{++}	0	2.0	0	
Ba^{++}	0	1.1	0	

* Relative activity is based on orthophosphate phosphorus produced per mg extract protein nitrogen. Barbital buffer, 1.25×10^{-2} M, pH 6.8 to 7.0. Incubated 10 min at 37 C.

† All metals added as chlorides to give a final concentration of 5×10^{-4} M.

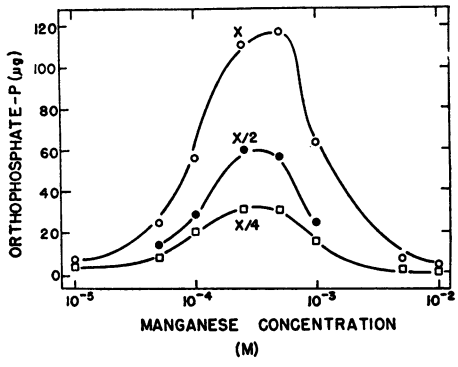

Figure 1. The effect of Mn^{++} concentration on the hydrolysis of sodium pyrophosphate by extract of *Bacillus megaterium* spores. X = full strength extract; X/2 and X/4 = half and quarter strength extracts. All reaction mixtures contained Mn^{++} as $MnSO_4$; sodium pyrophosphate, 10^{-3} M; and were incubated at pH 6.8 to 7.0 (barbital buffer) for 10 min at 37 C.

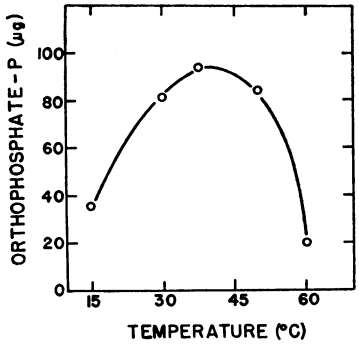

Figure 2. The effect of temperature of incubation on pyrophosphatase activity of *Bacillus megaterium* spore extract. Reaction mixtures contained $MnSO_4$, 5×10^{-4} M; sodium pyrophosphate, 10^{-3} M; and were incubated at pH 6.8 to 7.0 (barbital buffer) for 10 min.

dialysis of the vegetative cell extract against distilled water for 24 hr did not result in a Mn^{++} requirement. The possibility remains that the Mn^{++} of the vegetative cell extract is in a bound condition and not removable by dialysis.

We have not extended these observations to the spores of other bacterial species, but the pyrophosphatases of resting and germinated spores of the fungus, *Myrothecium verrucaria* strain QM 460, hydrolyze almost twice as much pyrophosphate in the presence of Mn^{++} as in its absence. Thus, the fungal pyrophosphatase is stimulated by Mn^{++}, but is not completely dependent upon it, as is the bacterial spore pyrophosphatase.

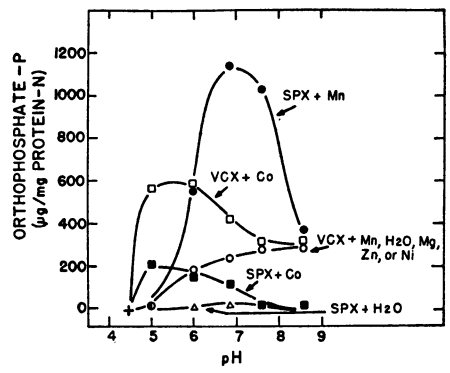

Figure 3. The pH-activity curves of pyrophosphatases of spore and vegetative cell extracts of *Bacillus megaterium*. SPX = spore extract; VCX = vegetative cell extract. All metals as the spectrographically pure sulfate, at 5×10^{-4} M final concentration. Acetate buffer for pH levels of 6.0 or less, barbital buffer for pH levels greater than 6.8. All reaction mixtures contained sodium pyrophosphate, 10^{-3} M, and were incubated at 37 C for 10 min.

Optimum pH levels. Further differences between the spore and vegetative cell pyrophosphatases become apparent when the reactions are run at pH levels other than neutrality (figure 3). The spore extract contains not only the Mn^{++} stimulated pyrophosphatase most active at neutral pH, but a Co^{++} activated acid pyrophosphatase evident as a minor component. In vegetative cell extract, on the other hand, the Mn^{++} activated enzyme is no longer apparent, whereas the Co^{++} activated acid pyrophosphatase is increased.

The vegetative cell pyrophosphatase, tested either with or without Mn^{++}, does not appear to be identical to the Mn^{++} activated spore enzyme. This vegetative cell enzyme has no clear pH optimum within the range tested, and its maximum activity occurs at higher pH than that of the Mn^{++} activated spore pyrophosphatase.

Mn^{++}, extract, and substrate relationship. Pyrophosphatase activity is a linear function of the spore extract concentration (figures 1 and 4). The ratio, Mn^{++} concentration:substrate concentration is an important one. The velocity of the reaction depends on the concentration of Mn^{++}, with a maximum velocity being attained at a Mn^{++} concentration of 5×10^{-4} M, and a pyro-

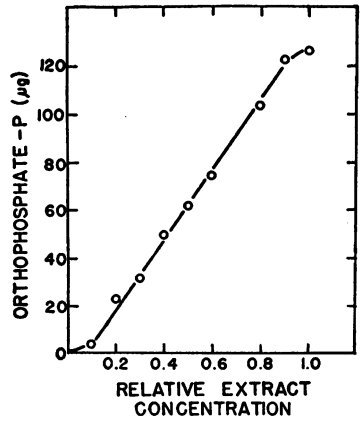

Figure 4. The effect of *Bacillus megaterium* spore extract concentration on pyrophosphatase activity. All reaction mixtures contained 10^{-3} M sodium pyrophosphate; 5×10^{-4} M $MnSO_4$; barbital buffer, pH 6.8 to 7.0; and were incubated at 37 C for 10 min.

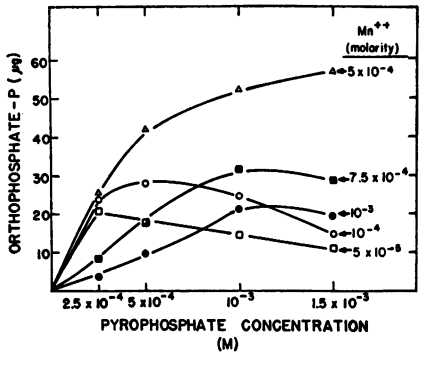

Figure 5. The effect of substrate and of Mn^{++} concentration on pyrophosphatase activity of *Bacillus megaterium* spore extract. Mn^{++} was present as the spectrographically pure sulfate. Incubation at pH 6.8 to 7.0 (barbital buffer) for 10 min at 37 C.

phosphate concentration of approximately 1.5×10^{-3} M (figure 5).

The data of figure 5 (with 5×10^{-4} M Mn^{++}), plotted as the reciprocal of the velocity as a function of the reciprocal of the substrate concentration (Lineweaver and Burk, 1934), give a Michaelis-Menten (1913) constant for this crude pyrophosphatase preparation of approximately 3×10^{-4} M.

Relative activity of extracts at pH 7.0. In the presence of 5×10^{-4} M Mn^{++}, at a pH near neutrality, extracts of heated spores hydrolyze pyrophosphate more than twice as rapidly as extracts of unheated spores (figure 6). Thus, heat shock, which can serve as a stimulant of spore germination, also results in increased pyrophosphatase activity, and the pyrophosphatase of intact spores is resistant to heating at 60 C.

The transition from spore to vegetative cell is accompanied by a diminution of neutral pyrophosphatase activity (figure 6). The rate of hydrolysis of pyrophosphate by extract of germinated spores is approximately one-fourth that by extract of heated spores, and approximately one-half that by extract of unheated spores. Since only 75 to 80 per cent of the spores in our suspension of "germinated spores" had actually germinated, it is possible that the pyrophosphatase activity of germinated spore extracts actually represents the activity of that fraction of the suspension which had not germinated. The trend of reduction in neutral pyrophosphatase as the spores germinate is continued to vegetative cells. Extracts of these cells have less activity at neutral pH than do spores and, as has been noted, their pyrophosphatase is independent of added Mn^{++}.

Specificity of the pyrophosphatase. The extracts are quite specific in their hydrolysis of inorganic

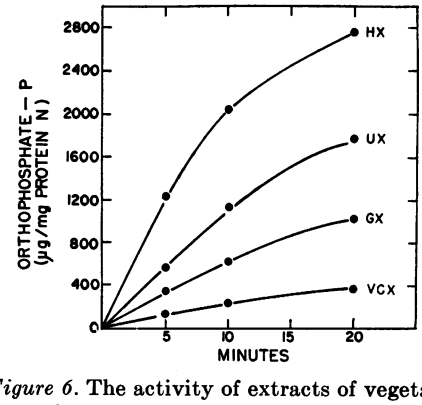

Figure 6. The activity of extracts of vegetative cells, and of extracts of unheated, heated, and germinated spores of *Bacillus megaterium*. UX = extract of unheated spores; HX = extract of heated spores; GX = extract of germinated spores; and VCX = extract of vegetative cells. All reaction mixtures incubated at 37 C, at pH 6.8–7.0 (barbital buffer), and in the presence of 5×10^{-4} M $MnSO_4$, and 10^{-3} M sodium pyrophosphate.

pyrophosphate. While sodium and potassium pyrophosphates were equally susceptible to the enzymatic action, adenosine triphosphate, glucose-1-phosphate, sodium glycerophosphate, sodium trimetaphosphate, and sodium tripolyphosphate were not hydrolyzed at acid, neutral and alkaline pH, in the absence of added metals, or in the presence of 5×10^{-4} M Mg^{++}, Co^{++}, Zn^{++}, or Ni^{++}.

Twenty hr incubation of extracts of spores or vegetative cells with 20 mg of spore coats or vegetative cell walls as substrate, either in the presence or in the absence of Co^{++} or Mn^{++}, gave no detectable release of orthophosphate although 20 mg of spore coats contained a total of 696.0 μg of phosphorus, of which 5.3 μg and 46.0 μg appeared as orthophosphate after 7 min and 180 min hydrolysis in N/1 HCl at 100 C.

Heat inactivation. The heat lability of the extracted Mn^{++} activated neutral pyrophosphatase is in marked contrast to the heat stability of the intact spore, as well as to the stability of the enzyme within the intact spore. About 50 per cent of the original pyrophosphatase activity of extract of unheated spores is lost on exposure to a temperature of about 40 C for 10 min. An equivalent percentage of the activity of extract of heated spores is lost on exposure to 32.5–35 C. In spite of the greater heat sensitivity of extract of heated spores, Mn^{++} protects both extracts similarly against heat inactivation (figure 7*A*, *B*). Ca^{++}, an ion which commonly has a stabilizing effect on enzymes, has no protective effect in this case ($CaCl_2$, 5×10^{-4} M).

The pyrophosphatase of germinated spores is less heat sensitive than that of resting unheated spores or of heated spores. Together with this diminution in heat sensitivity, the pyrophosphatase of germinated spores is no longer quite as dependent on the protective effect of Mn^{++} (figure 7 *C*) as are the pyrophosphatases of resting spores and of heated spores. This trend continues, and the unprotected neutral pyrophosphatase of vegetative cells (figure 7*D*) is just as stable as is the enzyme in the presence of Mn^{++}.

These results bring up the question as to whether the observed dependence of the spore enzyme on Mn^{++} for its activity may not be due entirely to protection of the enzyme by Mn^{++} during incubation. In order to test the validity

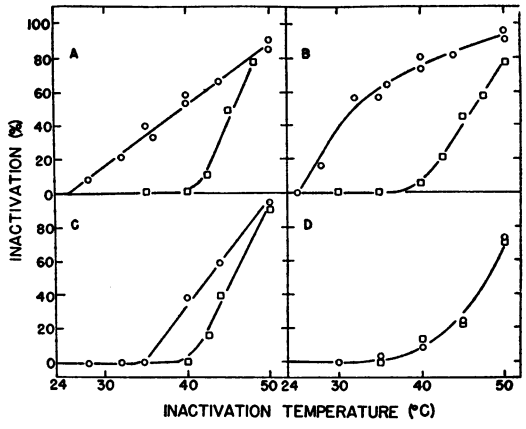

Figure 7. The heat inactivation of extracts of vegetative cells, and of extracts of unheated, heated, and germinated spores of *Bacillus megaterium*. *A*, extract of unheated spores; *B*, extract of heated spores; *C*, extract of germinated spores; *D*, extract of vegetative cells. Circles represent inactivation at the indicated temperature in the absence of Mn^{++}; squares, inactivation for 10 min in the presence of 5×10^{-4} M Mn^{++}. All reaction mixtures were incubated 10 min at 37 C, pH 6.8 to 7.0 (barbital buffer), and contained 10^{-3} M sodium pyrophosphate, 5×10^{-4} M $MnSO_4$, and "inactivated" extract.

of such a theory, spore extract was tested for pyrophosphatase activity at 20 C, a temperature at which there is no inactivation in 10 min in the absence of Mn^{++}. Even at this temperature, Mn^{++} was necessary for enzyme activity.

Hydrolysis of pyrophosphate by intact cells. No orthophosphate could be detected after 90 min incubation of 1.0 mg and 12.5 mg of spores with 10^{-3} M sodium pyrophosphate, either in the presence or in the absence of Mn^{++}. It made no difference whether the spores were heated, unheated, or germinated.

In contrast to the situation with spores, 2.5 mg vegetative cells of *B. megaterium* hydrolyzed 15 per cent of the pyrophosphate substrate in 15 min at pH 6.0; 40 per cent at pH 7.0; and almost 70 per cent at pH 8.0. This activity was evident in the absence of added metals, or in the presence of 5×10^{-4} M Mg^{++}, Mn^{++}, Zn^{++}, or Ni^{++}. In the presence of Co^{++}, 50 per cent of the substrate was hydrolyzed at pH 6.0; 80 per cent at pH 7.0; and 100 per cent at pH 8.0. This is in marked contrast to the pH relationship of the Co^{++}

activated pyrophosphatase extracted from vegetative cells (figure 3).

DISCUSSION

Pyrophosphatases are ubiquitous enzymes, having been found in bacteria, fungi, yeasts, higher plants, and in many animal organs (Schmidt, 1951). The Mn^{++} activated pyrophosphatase of *B. megaterium* spores appears to be distinguished from many other pyrophosphtases, both on the basis of the metal requirement, and the optimal pH for activity. For example, pyrophosphatase of crushed spores of *B. subtilis* has a pH optimum well in the alkaline range, and is stimulated by Ca^{++}, Mg^{++}, and Mn^{++} in decreasing order (Murrell, 1952). Our Mn^{++} activated enzyme is also distinct from the Co^{++} activated, alkaline pyrophosphatase, and from the Mg^{++} activated acid pyrophosphatase (Oginsky and Rumbaugh, 1955) of *Streptococcus faecalis*. In some aspects, of course, our enzyme is similar to other pyrophosphatases. It is specific for inorganic pyrophosphate, and it has a pH optimum similar to that of the Mg^{++} activated yeast pyrophosphatase of Bailey and Webb (1944). It is conceivable that Mn^{++} relieves the pyrophosphate inhibition of *iso*citric dehydrogenase (Adler *et al.*, 1939) by activation of a pyrophosphatase with consequent destruction of the inhibitory pyrophosphate.

There appears to be a definite optimal Mn^{++} concentration. However, the ratio between Mn^{++} and enzyme concentrations does not seem to have great significance. Inactivation of enzyme at high concentrations of activating cation occurs in many systems (Adler *et al.*, 1939; Malmström, 1953). An explanation for a distinct cation concentration optimum (Hagen and Jackson, 1953) may not be applicable here since the Mn^{++}:pyrophosphate concentration ratio seems to have major significance in determining the activity of the enzyme. Brown (1955) has estimated that, for the pyrophosphatase of *Bacillus stearothermophilus*, a Mg^{++}:$Na_4P_2O_7$ ratio of 2:1 is optimal. Our data do not permit the establishment of any definite optimum ratio for activity, yet we can see (figure 5) that with increase in concentration of Mn^{++} from 5×10^{-5} M to 5×10^{-4} M, the total orthophosphate phosphorus production is increased. At Mn^{++} concentrations above 5×10^{-4} M, the maximum orthophosphate phosphorus produced decreases with increasing Mn^{++} concentration, and the maximum attained for any Mn^{++} concentration tends to occur at increasing concentrations of pyrophosphate. Perhaps at high ratios of Mn^{++}:substrate, the Mn^{++} is precipitating as well as activating the substrate, thus removing some of the insoluble $Mn_2P_2O_7$ from the field of reaction.

Our interest in the spore pyrophosphatase does not lie in a strict biochemical characterization of the enzyme. We are more concerned with differences in the enzyme as it occurs in spores and in vegetative cells, as well as in the possibility of a relationship between the enzyme, germination, and heat stability. We consider it important not only that the Mn^{++} activated pyrophosphatase content of the spore is reduced, but that changes in the character of the enzyme accompany germination. This seems to be in agreement with the ideas of Foster (1956) that spore and vegetative cell enzymes differ qualitatively and quantitatively from each other. Significantly, extracts of spores whose potential for germination has been greatly enhanced by sublethal heating (50 to 60 C for 5 to 15 min) always have greater pyrophosphatase activity per mg protein N than do extracts of unheated spores. The germinated spore has less Mn^{++} activated pyrophosphatase than does the supposedly metabolically inactive resting spore, and in the vegetative cell the need for Mn^{++} activation is no longer detectable. This situation is reminiscent of the requirement for Mn^{++} for spore germination and the loss of this requirement for growth once the spores have completed the germination process (Hyatt and Levinson, 1957). It is as if the vegetative cell, no longer having need for the Mn^{++} activated pyrophosphatase (for germination), has reduced the amount of this enzyme, perhaps to a level just high enough to insure genetic reproducibility, and the Co^{++} activated pyrophosphatase becomes dominant.

While bacterial spore enzymes are not rare (Spencer and Powell, 1952; Stewart and Halvorson, 1953; Lawrence and Halvorson, 1954; Lawrence, 1955; Levinson and Sevag, 1954*a*, *b*; Powell and Hunter, 1956; Krask, 1957), it is unusual for vegetative cells to possess less of an enzyme than the homologous spores (Hardwick and Foster, 1953). In this connection, however, it should be pointed out that in the fungus *Myrothecium verrucaria*, the mycelium had only

10 per cent of the ascorbic acid oxidizing ability of the spores (Mandels, 1953).

The intact spore is heat stable. The Mn^{++} activated, neutral pyrophosphatase extracted from the spore is quite sensitive to heat, and is, furthermore, somewhat protected from the effect of heat by Mn^{++}. Thus, as has been previously demonstrated (Murrell, 1952) the thermal stability of spore enzymes appears to depend on the integrity of the spore. As the spore germinates, this dependence of the enzyme on Mn^{++} for protection against heat inactivation decreases, and when the spores have finally completed the transition into vegetative cells, the dependence of the extract upon Mn^{++}, both for heat protection and for activation, has completely disappeared. It is interesting to note that as we go from extract of highest activity (heated spores) to extract of lowest activity (vegetative cells), the sensitivity of the enzyme to heat inactivation also decreases, and curves for enzyme inactivation as a function of temperature in the absence of Mn^{++} approach those in the presence of Mn^{++}. Significantly, Brown (1955) has shown that the Mg^{++} activated pyrophosphatases of *B. stearothermophilus* and of *B. subtilis* have greater heat stability with increased concentration of Mg^{++}.

The change in the character of the enzyme as it is extracted from spores and vegetative cells is consistent with the interpretation of sporulation as a period of new synthesis (Foster, 1956). The possibility that the enzymes in spores and vegetative cells are not identical is emphasized by the difference in character of the pH curve for pyrophosphatase from these two sources. Such a multiplicity of enzymes acting on a single substrate has previously been demonstrated; for example, protease (Crewther and Lennox, 1953), invertase (Cabib, 1952), amylase (Reid, 1950), cellulase (Gilligan and Reese, 1954), and catalase of *Bacillus cereus* spores (Lawrence and Halvorson, 1954).

The Mn^{++} stimulation of *B. megaterium* spore germination, (Levinson and Sevag, 1953; Hyatt and Levinson, 1957), as well as the specificity of Mn^{++} in activation of the spore pyrophosphatase, have led us to postulate that the Mn^{++} activated pyrophosphatase may function in spore germination (Levinson, 1957). It has been claimed that an acid and alkali insoluble phosphorus residue of spore coats constitutes about 60 per cent of the total phosphorus of *B. megaterium* spores, and that vegetative cells contain a much larger proportion of their phosphorus in soluble form than do spores (Fitz-James, 1955). Furthermore, from 12 to 20 per cent of the phosphorus of *B. cereus* spores exhibited some of the characteristics of a polymerized phosphate. It is possible that the insoluble residue forms a lattice work making the spore coats impermeable to nutrients. The breakdown, or partial breakdown, of this lattice work, through the mediation of the Mn^{++} activated pyrophosphatase, would permit nutrients to enter the spore and to participate in the biochemical events necessary for spore germination. While this may indeed be a mechanism for spore germination, we have been unable to demonstrate hydrolysis of spore coats or of vegetative cell walls by spore extract. However, spores of *B. cereus* do contain a Mn^{++} and Co^{++} stimulated enzyme which is capable of lysing cell walls and spore coats (Strange and Dark, 1957). The identity of the substrate for this enzyme is not known.

Inorganic pyrophosphate is a product in the reactions leading to synthesis of coenzyme A and in transacetylation (Lipmann, 1954). In the presence of pyrophosphatase, pyrophosphate will disappear, and production of coenzyme A and of acetyl coenzyme A will be increased as the result of a mass action phenomenon. One function of the spore pyrophosphatase may then be to remove the pyrophosphate formed in these reactions. If these reactions are important in spore germination, inhibition of the pyrophosphatase would result in decreased germination. It is well known that products of an enzymatic reaction can inhibit the specific enzyme involved in the reaction (Sevag, 1951), and it is therefore possible that the inhibition of spore germination by orthophosphate (Williams and Hennessee, 1956; Levinson and Sevag, 1953) is basically an inhibition of spore pyrophosphatase. These mechanisms are under investigation in our laboratory.

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to the manuscript through discussion and criticism.

SUMMARY

An inorganic pyrophosphatase has been demonstrated in extracts of spores of *Bacillus megaterium*. It is highly specific, requires Mn^{++} for activation, and is protected by Mn^{++} against heat inactivation. Extracted enzyme is quite heat labile, but enzyme in the intact spores is heat stable. In fact, heating of spores increases the activity of the extracted enzyme.

Metal activation and pH data strongly suggest that more than a single pyrophosphatase is active both in spores and in vegetative cells. As spores become vegetative cells, the Mn^{++} activated enzyme undergoes progressive quantitative and qualitative changes, decreasing in amount and becoming less sensitive to heat. Extract of vegetative cells exhibits none of the Mn^{++} requirement for activation shown by extract of spores, and the need for Mn^{++} for protection of the enzyme against heat inactivation decreases as the spores develop into vegetative cells. Moreover, a Co^{++} activated acid pyrophosphatase, a relatively minor component in spore extract, becomes dominant in vegetative cell extract.

There is a possibility that Mn^{++} activated pyrophosphatase may be involved in germination.

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