A COBALT-ACTIVATED BACTERIAL PYROPHOSPHATASE

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Hydrolysis of inorganic pyrophosphate by magnesium-activated bacterial enzymes with pH optima from approximately 6.0 to 7.0 has been reported by Pett and Wynne (1933, 1938) and Cafiero (1948). The present paper describes the characteristics of a cobalt-activated acid pyrophosphatase obtained in the cell-free state from *Streptococcus faecalis* strain F24, and reports the presence of the enzyme in several other bacterial genera. The effect of cobalt addition to the *S. faecalis* growth medium on the pyrophosphatase (hereafter referred to as PPase) activity of the bacterial cells was explored and found to present an interesting type of enzyme activation.

METHODS

S. faecalis strain F24 was grown in broth containing 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent K_2HPO_4, and 0.1 per cent glucose for 16 hours at 37 C. The cells were removed by centrifugation and washed once with distilled water before further treatment. Three types of enzyme preparations were employed: (1) whole cell suspensions in water at 1 mg bacterial nitrogen per ml, (2) acetone dried preparations, and (3) cell-free extracts prepared either (a) by exposure of heavy cell suspensions to ultrasonic oscillation at 700 kc per second, at 300 watts input for 30 minutes or (b) by shaking a 5 per cent suspension of acetone dried preparation in water for 60 minutes at room temperature, then storing the suspension overnight in the refrigerator. The clear yellow supernatant obtained by centrifugation of suspensions (a) or (b) at 20,000 $\times$ G at 0 C for 15 minutes was then lyophilized and stored in the refrigerator. The PPase activity of such lyophilized extracts remained stable for several months. Since only a few milligrams were needed for each experiment, more consistent results were obtained by making a stock enzyme solution containing 600 mg of the lyophilized extract in H_2O at a concentration of 20 mg per ml, dispensing the solution in 1.5 ml aliquots in small tubes, and storing the tubes in deep freeze at -5 C until needed. Before use, 1.5 ml H_2O at room temperature was added to thaw the tube contents, which were further diluted with 10 parts of H_2O to give a working enzyme solution containing 1 mg extract per ml. Five-tenths ml of this latter solution was used per assay tube.

The substrates were employed as the commercially available products: sodium pyrophosphate (PP), $Na_4P_2O_7 \cdot 10H_2O$, Merck & Co., Inc.; adenosine diphosphate (ADP), Ba salt, Sigma Chemical Co.; adenosine triphosphate (ATP), Na salt, Sigma Chemical Co.; diphosphopyridine nucleotide (DPN), Pabst Laboratories; K glycerophosphate, Merck & Co., Inc., Na β-glycerophosphate, Nutritional Biochemicals Corp.; fructose-1,6-diphosphate (HDP), Ba salt, Schwarz Laboratories, Inc. Thiamin pyrophosphate was obtained from Merck & Co., Inc. Substrate solutions were adjusted to pH 6.0 before use. All metals were employed as the chlorides. Amino acids, and other compounds used in activation studies, were the commercially available products.

PPase activity, the formation of orthophosphate from pyrophosphate, was determined by incubating buffer, enzyme preparation, and varying amounts of cofactors in a volume of 2.5 ml for 5 minutes, in the 37 C water bath, and then adding 0.5 ml of substrate, to give a final volume of 3.0 ml. Sodium pyrophosphate (PP) was used as substrate for all experiments, except as otherwise noted. The tubes were mixed thoroughly, and further incubated with occasional shaking for 10 or 15 minutes. Immediately after addition of 0.3 ml 100 per cent trichloracetic acid to stop the reaction, the tubes were placed in cracked ice for a few minutes and then centrifuged at 4 C. The supernatants were assayed for orthophosphate by the method of Fiske and Subbarow (1925). The PPase values reported were corrected for zero time controls; under the experimental conditions employed, no orthophosphate was formed from PP in the absence of enzyme.

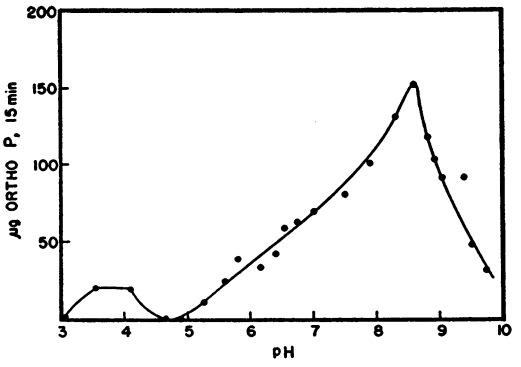

Figure 1. Effect of pH on Mg^{++} activation of *Streptococcus faecalis* PPase. 1.67×10^{-2} M buffer (pH 3.0–5.8, phthalate; pH 5.7–8.8, histidine; pH 7.9–9.75, borate), 1.67×10^{-3} M PP, 1 mg *S. faecalis* extract, 3.3×10^{-3} M Mg^{++}.

RESULTS

Preliminary studies with acetone dried preparations of *S. faecalis* strain F24 indicated that sodium pyrophosphate was hydrolyzed to orthophosphate by this organism over a wide range of pH values (4 to 9), and that addition of various divalent metal ions to the reaction mixtures markedly stimulated PPase activity. Cell-free extracts of this organism were found to be almost completely incapable of hydrolyzing PP unless supplemented with divalent metal ions, of which Mg^{++} was the most effective in the alkaline pH range, and Co^{++} in the acid pH range.

The effect of pH on the activation by Mg^{++} of cell-free PPase from *S. faecalis* is shown in figure 1. This bacterial enzyme is, superficially at least, similar to the PPases of the erythrocyte (Naganna and Narayana Menon, 1948), firefly (McElroy, Coulombre, and Hays, 1951), bull seminal plasma (Heppel and Hilmoe, 1951), and liver (Lohmann, 1933) in the activation by Mg^{++} at optimal pH in the neighborhood of 8.0. With the cell-free extract of *S. faecalis*, divalent ions other than Mg^{++} also exerted stimulation of PPase activity, in the alkaline pH range, namely Co^{++}, Fe^{++}, and Mn^{++}, although these ions were far less effective than Mg^{++}. The requirement for ion activation in this pH range was shown more clearly after dialysis against distilled water, but even after prolonged dialysis, there was still some residual activity in the absence of added metal ion. For example, 1 mg of cell-free extract, dialyzed 96 hours and incubated for 15 min with PP at pH 8.6 in borate buffer, released 0.40 μM orthophosphate in the absence of metal ions, and the following amounts in the presence of 3.33×10^{-3} M metal ions: 3.31 μM with Mg^{++}, 1.22 μM with Co^{++}, 1.05 μM with Fe^{++}, and 0.55 μM with Mn^{++}.

The pattern of activation of the acid PPase proved to be quite different from any heretofore described, and detailed studies on this enzyme were carried out. The rest of this report is concerned solely with the characteristics of this acid PPase (pH optimum about 5.3–5.4), activated primarily by cobaltous ion, and more strongly so in the presence of histidine (figure 2). The addition of histidine alone has never produced any significant stimulation in enzymatic activity. The properties of the enzyme, as determined primarily with cell-free extracts obtained by the methods described above, are as follows:

I. *Specificity of the metal ion requirement.* Extracts of *S. faecalis* strain F24 as obtained by either method (a) or (b) described previously do not hydrolyze PP at acid pH in the absence of metal ion. As a matter of fact, neither intact cell suspensions nor acetone dried preparations

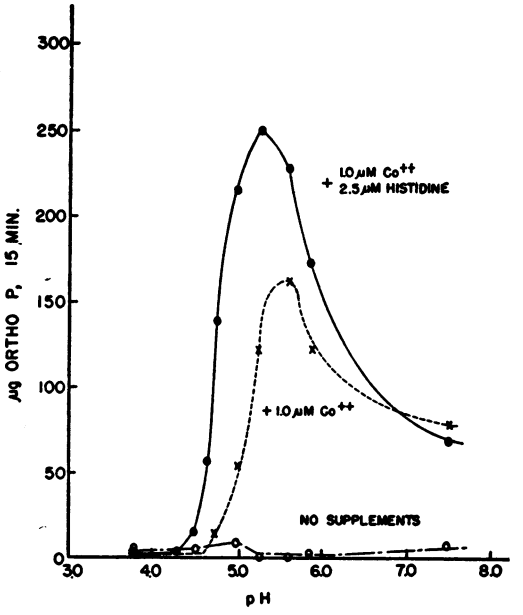

Figure 2. Effect of pH on Co^{++}-histidine activation of *Streptococcus faecalis* PPase. 3.3×10^{-2} M acetate buffer, 1.67×10^{-3} M PP, 500 μg *S. faecalis* extract. 3.3×10^{-4} M Co^{++}, 8.35×10^{-4} M histidine as indicated.

TABLE 1

Effect of divalent metal ions on acid PPase of Streptococcus faecalis

Metal Ion 3.3×10^{-4} M	μg Ortho P, 10 Min		
	(a) Alone	(b) With 1.67×10^{-3} M histidine	(c) With 1.67×10^{-3} M histidine + 3.3×10^{-4} M Co^{++}
Co^{++}	67	150	150
Ni^{++}	0	2	126
Zn^{++}	11	23	43
Cu^{++}	0	1	37
Mg^{++}	0	4	131
Mn^{++}	10	46	190
Fe^{++}	0	0	60
Ca^{++}	0	2	147
Hg^{++}	0	1	1

3.3×10^{-2} M acetate buffer pH 5.3, 1.67×10^{-3} M PP, 500 μg *S. faecalis* extract obtained by H_2O extraction of acetone dried cells.

exhibit PPase activity without supplementation unless a considerable amount of cellular material is employed, that is, about 15 mg dry weight of acetone dried preparation. The cell-free extracts could therefore be tested for ion activation without dialysis. A series of divalent metal ions was tested for their effect on the PPase, at 3.33×10^{-4} M concentration, under three conditions: (a) with no further supplementation, (b) with 1.67×10^{-3} M histidine, and (c) with 3.33×10^{-4} M Co^{++} plus 1.67×10^{-3} M histidine. The results, given in table 1, show that only Co^{++}, Zn^{++}, and Mn^{++} were capable of activating the PPase, particularly so in the presence of histidine; however, the effect of Co^{++} was far more striking than the effects of either Zn^{++} or Mn^{++}. Inhibition of the Co^{++}-histidine activated enzyme was observed when Zn^{++}, Cu^{++}, Fe^{++}, or Hg^{++} was added. It is interesting that Zn^{++} both stimulated and inhibited the PPase; at one-tenth the concentration, i.e., 3.33×10^{-5} M Zn^{++}, the stimulatory effect disappeared, but the inhibitory effect was still quite marked (34 per cent inhibition of Co^{++}-histidine activation). On the other hand, the activation by Mn^{++} occurred even in the presence of the optimum concentration of Co^{++} and thus appeared to be an additive function. Stimulation by Zn^{++} in low concentration was reported by Pett and Wynne (1933, 1938) for PPase activity, tested at or near pH 7, of *Bacillus subtilis*, *Alcaligenes faecalis*, *Clostridium acetobutylicum*, and *Propionibacterium jensenii*. These authors did not test the effects of Co^{++} or Mn^{++}.

The protein fraction precipitating between 60 and 75 per cent ammonium sulfate contained most of the pyrophosphatase activity of the crude cell-free extracts, and some increase in PPase activity per mg protein was obtained. This fraction contained both the Mg^{++} and Co^{++} activated enzymes. We have been unable thus far to separate the two types of enzymes by further fractionation, and therefore can only presume that the two metal ions activate different protein entities.

II. *Specificity of the histidine requirement.* In early studies on the enzyme, considerably higher activity was obtained in histidine buffer than in acetate, phthalate, or borate buffers. Investigation revealed that only a few μM of histidine were needed to increase PPase activity in the presence of Co^{++}, and succeeding experiments were carried out in acetate buffer. The histidine solutions employed throughout were adjusted to pH 6.0. At very low concentrations of Co^{++} and histidine, the amount of buffer was adequate to maintain the pH, whereas at the concentrations ordinarily employed, the addition of Co^{++} to histidine at pH 6.0 brought the pH down to the optimum, 5.3. Quantitative determinations of the amount of histidine required are described in the following section.

Histidine could be replaced with almost equal effectiveness by the amino acid cysteine; histamine and lysine were also quite active (table 2). The following compounds were inactive: DL-α-alanine, L-glutamic acid, L-proline, L-tryptophan,

TABLE 2

Effect of amino acids on Co^{++} activation

Amino Acids	μg Ortho P, 10 Min
None	20
L-Histidine	79
Histamine	40
L-Cysteine	71
L-Lysine	61
Glycine	28
DL-Aspartic acid	29
L-Arginine	28
L-Methionine	28

3.3×10^{-2} M acetate buffer pH 5.3, 1.67×10^{-3} M PP, 500 μg *S. faecalis* extract, and 1.67×10^{-4} M Co^{++}. Amino acid concentration 1.67×10^{-3} M.

L-tyrosine, imidazole, and adenine. Ethylenediaminetetraacetic acid (EDTA) completely abolished the effect of Co^{++}. The molar concentration of Co^{++} employed in these experiments was one-half that used in the experiments reported in table 1, to allow for maximal stimulation by amino acid.

Vitamin B_{12} was completely ineffective in the stimulation of PPase activity, even at molar concentrations equivalent to those of Co^{++} and histidine.

III. *Quantitative studies of the two requirements.* The requirement of the enzyme for both Co^{++} and histidine suggested that the activation was accomplished, not by the two compounds acting individually, but rather by a Co^{++}-histidine coordination complex (Hearon, Burk, and Schade, 1949; Hearon, 1948). If the latter were the true case, one would expect that the ratio of histidine to cobalt producing maximal stimulation of the PPase would remain constant over a series of cobalt concentrations, and also that such a ratio would be indicative of the proportion of cobalt and histidine in the complex. However, the experimental data presented in figure 3 show that the ratio of cobalt to histidine did not remain constant. It is apparent that with a constant amount of enzyme, addition of increasing amounts of cobalt alone resulted in increasing stimulation (up to an optimum at 1 μM Co^{++}, or a concentration of 3.33×10^{-4} M). Further addition of histidine raised the enzymatic activity by a constant amount, independent of the concentration of cobalt present. One thus obtained varying ratios of histidine to cobalt (8 to 1 at 0.1 μM Co^{++}, 4 to 1 at 0.2 μM, 2.5 to 1 at 0.4 μM). Moreover, the *amount* of histidine required for maximal activation at any particular level of Co^{++} was approximately the same as that at any other level of cobalt tested. The optimum amount of histidine appeared to be more directly related to the amount of enzyme employed since, at identical levels of Co^{++}, increasing the amount of enzyme increased the amount of histidine required for maximal activation, although a definite ratio of histidine: enzyme was not obtainable experimentally.

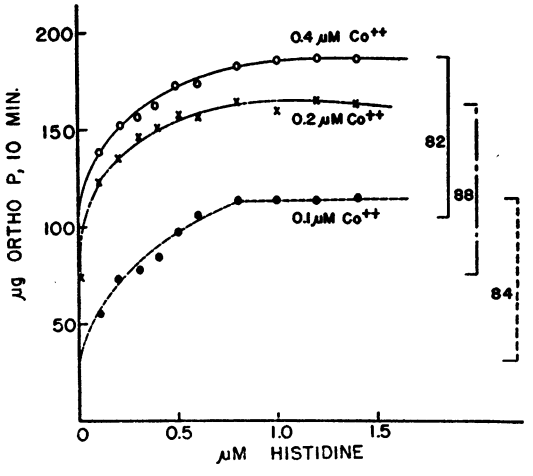

Figure 3. Effect of histidine concentration on Co^{++} activation of *Streptococcus faecalis* PPase. 3.3×10^{-2} M acetate buffer pH 5.3, 1.67×10^{-3} M PP, 500 μg *S. faecalis* extract. Co^{++} and histidine concentrations as indicated.

IV. *Concentration and specificity of substrate.* The PPase was found to have a K_m of about 2×10^{-4}. However, high concentrations of substrate, above 3×10^{-3} M, were inhibitory, probably because of binding of the Co^{++} by precipitation at these high levels of PP.

No orthophosphate was liberated by the enzyme, in the presence of both Co^{++} and histidine, from the following compounds: DPN, thiamin pyrophosphate, HDP, or glycerophosphate as the β-isomer or the α-, β-isomer mixture. Liberation of orthophosphate was consistently obtained from the commercial preparations of ADP and ATP employed, although at a far lesser rate than from PP. With 1×10^{-3} M substrate and 500 μg of enzyme preparation, on 15 minutes' incubation, 25 μg of ortho P were liberated from ATP, 27 μg from ADP, and 198 μg from PP. Incubation of enzyme preparation with ATP over a range of pH values from 5.3 to 8.6 in the absence of Co^{++} and histidine did not result in liberation of any orthophosphate. Aliquots of such ATP-enzyme mixtures were removed at 0, 10, and 20 minutes of incubation, supplied with adequate Co^{++}, histidine, and buffer at pH 5.3, and returned to the water bath for 10 minutes. The amount of orthophosphate released was the same with both zero-time controls and preincubated mixtures. This finding suggested that the orthophosphate was released directly from ATP by the Co^{++}-histidine requiring enzyme, rather than with the intermediate formation of inorganic PP by another enzyme independent of Co^{++} and histidine. Definitive proof of PPase activity directly on ATP would require further purification of both the enzyme preparations and the ATP.

TABLE 3

Distribution of cobalt-activated acid PPase in bacteria

Organism	μg Ortho P Formed/Hr					
	Cell suspension			Acetone dried preparation		
	μg P/mg bacterial N			μg P/mg dry weight		
	No additions	+Co^{++}	+ Co^{++} + Histidine	No additions	+ Co^{++}	+ Co^{++} + Histidine
Escherichia coli, Gratia	—	—	—	75	94	92
Escherichia coli, Murray	17	39	23	2	31	18
Escherichia coli, Crookes	—	—	—	0	808	372
Aerobacter aerogenes, 174	—	—	—	0	14	12
Proteus vulgaris, WC83	73	165	209	10	109	122
Pseudomonas aeruginosa	27	40	45	—	—	—
Lactobacillus lactis, Dorner	—	—	—	80	248	408
Lactobacillus arabinosus, 17-5	—	—	—	38	65	189
Streptococcus faecalis, F24	0	256	405	4	520	808
Streptococcus faecalis, 10Cl	—	—	—	30	59	75
Streptococcus faecalis, R	83	1,890	2,000	—	—	—
Staphylococcus aureus, MB283	92	133	160	—	—	—
Leuconostoc mesenteroides, MB224	17	59	72	—	—	—
Bacillus subtilis	0	102	109	—	—	—

3.3×10^{-2} M acetate buffer pH 5.3, 1.67×10^{-3} M PP, 3.3×10^{-4} M Co^{++}, 1.67×10^{-3} M histidine. With cell suspensions, 0.3 mg bacterial N was used per assay tube. With acetone dried preparations, 5 mg dry weight per tube was used, except for the very active preparations, of which 1 mg was used per tube.

V. *Effect of inhibitors.* The inhibition by Hg^{++}, Fe^{++}, Cu^{++}, and Zn^{++} has been described in section I.

No effect on PPase activity was observed in the presence of 1×10^{-3} N concentrations of the following common enzyme inhibitors: arsenate, arsenite, fluoride, cyanide, semicarbazide, hydroxylamine, or azide. Even a tenfold increase of the fluoride concentration, to 1×10^{-2} M, did not result in PPase inhibition. The lack of sensitivity of this enzyme to fluoride contrasts markedly with the strong inhibition by fluoride of the Mg^{++}-activated PPases of yeast (Bailey and Webb, 1944), erythrocyte (Naganna and Narayana Menon, 1948), and firefly (McElroy, Coulombre, and Hays, 1951).

VI. *Distribution of cobalt-activated acid PPase.* While the enzyme content of *S. faecalis* strain F24 is particularly high, acid PPase appears to be present in a wide variety of microorganisms. Indeed, no bacterium tested thus far has been devoid of activity. Table 3 presents results obtained with resting cell suspensions and acetone dried preparations of various gram positive and gram negative bacteria. The cell suspensions were tested for activity within 24 hours after harvesting from the tryptone-yeast extract medium described in the "Methods" section. However, the acetone dried preparations of the different organisms varied in age from 1 day to 2 years, and direct comparison of PPase activity of such aged preparations would not be valid. Nevertheless, whether the cells were fresh or aged, acetone dried or intact, there was observed a distinct difference in the response to histidine supplementation (in addition to Co^{++}) by the colon-aerogenes bacteria, on the one hand, and the lactic acid bacteria, on the other. The PPase activity of the colon-aerogenes organisms was in some cases unaffected by histidine, in others inhibited, but never was the activity in the presence of Co^{++} further stimulated by histidine addition. In all lactic acid bacteria tested, on the other hand, PPase activity was always increased when histidine was present in addition to Co^{++}. Other bacterial genera, gram negative or gram positive, generally were stimulated by histidine, but the effect was far less pronounced than with the lactic acid bacteria. Histidine alone, in the absence of Co^{++}, exerted no effect at all on any of the bacteria tested.

Studies of cell-free enzyme, prepared by water

TABLE 4

Effect of Co^{++} incorporation in culture medium on PPase activity

μg Co^{++} per ml Medium	Yield of Bacterial N per 250 ml Medium	μg Ortho P/15 Min			
		No additions	+Co^{++}	+Histidine	+Co^{++} +Histidine
0	21.7	0	6	2	10
1	17.7	0	8	3	11
5	16.7	0	16	2	23
10	15.6	3	29	2	38
30	13.5	2	25	2	34
60	no growth	—	—	—	—

2×10^{-2} M acetate buffer pH 5.3, 1.67×10^{-3} M PP, 0.3 ml cell suspension containing 1 mg bacterial N per ml; 3.3×10^{-4} M Co^{++}, 1.67×10^{-3} M histidine where indicated.

extraction of the acetone dried Crookes strain of *Escherichia coli*, showed that here also histidine depressed the PPase response to Co^{++} addition. The same degree of inhibition was observed when either enzyme or histidine was preincubated with Co^{++} for 10 minutes prior to the addition of the other component. The reasons for the difference in the effects of histidine on this enzyme and on the enzyme of *S. faecalis* are not known.

VII. *Effect of cobalt addition during growth to PPase content.* Resting cell suspensions of *S. faecalis* strain F24, grown in tryptone-yeast extract medium, exhibited little or no PPase activity unless Co^{++} was added in the assay procedure. Since the enzyme thus would not be capable of function in cells grown in customary medium, it was of interest to determine whether the PPase activity of the cells could be increased by addition of Co^{++} to the culture medium. Varying amounts of Co^{++} were added aseptically to tryptone-yeast extract medium, and *S. faecalis* strain F24 cells were grown and harvested in these media according to the procedures previously described. The data obtained in a typical experiment are presented in table 4. It was found that the addition of Co^{++} during growth resulted in cells which had only very slight PPase activity when tested in the absence of supplements, or in the presence of histidine alone. However, a pronounced effect on the level of enzyme activity was observed when Co^{++} was added in the PPase assay. There was a marked increase in the amount of what one might call "activatable" or "potential" enzyme in bacteria grown with the metal ion. This is indeed a curious phenomenon and superficially appears to be an induction of enzyme formation by the necessary cofactor, in this case a metal ion. The mechanism by which this increase in enzyme activity occurs has not been investigated; one could propose several alternative mechanisms—net increase in enzyme synthesis, activation of preformed but inert enzyme, or stabilization of enzyme in the presence of Co^{++}—but which of these may be involved is pure conjecture. Quantitative comparison of the amount of Co^{++} required in the PPase assay for maximal stimulation of control cells and that required in the growth medium for maximal enzyme "potentiation" resulted in approximately the same figure: about 20 to 25 μg Co^{++} per 0.3 mg bacterial N. This agreement of course may be fortuitous since the range of concentrations employed was rather narrow; nevertheless, the fact that the limiting activation concentrations are of the same order may bear directly on the mechanism of "potentiation". It is curious that cells grown with Co^{++} did not possess PPase activity in the absence of further ion stimulation. There was the possibility that the washing procedure employed in preparation of the suspensions removed the Co^{++} from these cells; however, further experiments showed that the activation by Co^{++} added to resting cell suspensions was not markedly decreased by the washing procedure.

No increase in PPase activity was observed when the cells were grown in medium containing 100 μg per ml of sodium pyrophosphate, nor was the increase in enzyme activity produced by Co^{++} addition to the medium further stimulated by the simultaneous incorporation of this amount of pyrophosphate.

SUMMARY

Cell-free extracts of *Streptococcus faecalis* strain F24 contain two enzymes hydrolyzing inorganic pyrophosphate: one with a pH optimum of about 8.5 activated primarily by Mg^{++} and the other enzyme with a pH optimum of about 5.3 activated primarily by Co^{++}.

Mn^{++} and Zn^{++} were also capable of activating this acid pyrophosphatase, but their effect was much smaller than that of Co^{++}. The enzyme was inhibited by Hg^{++}, Cu^{++}, Fe^{++} and Zn^{++}.

The Co^{++} activation was further stimulated by the presence of histidine; the histidine requirement could be replaced, in decreasing order of

effectiveness, by cysteine, lysine, and histamine. The ratio of histidine:Co^{++} producing maximal pyrophosphatase activity did not remain constant over a range of Co^{++} concentrations.

The cell-free extracts also released orthophosphate from ADP and ATP, although at a far lower rate than from inorganic pyrophosphate. No evidence was obtained for intermediate formation of inorganic pyrophosphate from ATP.

Acid pyrophosphatase, activated by Co^{++}, was found to be present in all bacteria tested. The marked stimulation by histidine was observed only in the lactic acid bacteria; the amino acid actually depressed the response to Co^{++} in some colon-aerogenes bacteria.

The addition of Co^{++} to the culture medium of *S. faecalis* strain F24 increased the level of acid pyrophosphatase activity as measured in the presence of Co^{++} and histidine.

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