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Stimulation of Pyrophosphatase Activity in *Clostridium pasteurianum* by Reductants[1]

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Many biosynthetic reactions involving adenosine triphosphate (ATP) yield inorganic pyrophosphate as a by-product. Nitrogen fixation by extracts of *Clostridium pasteurianum* requires ATP as a source of energy and KBH_4, H_2, or pyruvate as a source of reducing power (Hardy and D'Eustachio, Biochem. Biophys. Res. Comm. **15**:314, 1964). While investigating the function of ATP in ammonia synthesis, we found that pyrophosphate accumulated only if the reducing agent was omitted. If one of the above reducing agents was present, neither pyrophosphate formation nor the inhibition of nitrogen fixation by pyrophosphate could be demonstrated. Further studies showed that a variety of reductants produced a 6- to 34-fold stimulation of the enzymatic hydrolysis of pyrophosphate to inorganic phosphate. Although the activity of some mammalian pyrophosphatases is occasionally enhanced by sulfhydryl compounds (Seal and Binkley, J. Biol. Chem. **228**:193, 1957; Racker, Proc. Nat. Acad. Sci. U.S. **48**:1639, 1962), the stimulation is much less than that observed with the clostridial enzyme. A brief examination of pyrophosphatase activity in several aerobes and anaerobes was done.

C. pasteurianum ATCC 6013 and *C. butyricum* were grown anaerobically and harvested, as described by Carnahan and Castle (J. Bacteriol. **75**:121, 1958). *Bacillus cereus* ATCC 9139 was grown in the modified "G" medium of Stewart and Halvorson (J. Bacteriol. **65**:160, 1953). *Escherichia coli* was grown in nutrient broth (Difco) supplemented with 1% yeast extract and 0.5% glucose. *Azotobacter vinelandii* was grown on Burk's nitrogen-free medium (Burk and Lineweaver, J. Bacteriol. **19**:389, 1930). *C. kluyveri*, *Micrococcus lysodeikticus*, and baker's yeast were obtained as dried commercial preparations. The clostridia were extracted by use of the method described by Carnahan et al. (Biochim Biophys. Acta **44**:520, 1960). The other organisms were suspended in water (1 g/2 ml) and treated in a 10-kc Raytheon sonic oscillator for 15 min. The disrupted cell suspensions were cooled to 1 C, clarified by centrifugation at 27,000 × *g* for 1 hr, and stored under an atmosphere of H_2.

Inorganic pyrophosphatase activity of all extracts was measured by use of a standardized assay. Reactions were carried out in 2-ml volumes in 25-ml anaerobic flasks equipped with a side arm. The standard reaction mixture contained: tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0), 100 μmoles; extract protein, 9 mg; Mg^{++}, 20 μmoles; pyrophosphate, 50 μmoles; reductant, 20 to 40 μmoles. The flasks were evacuated and filled with an atmosphere of argon. After pyrophosphate was tipped from the side arm, the flask was shaken at 30 C for 30 min. The incubation period was stopped by the addition of 1 ml of the reaction mixture to 1 ml of cold 10% trichloroacetic acid. After the precipitated protein was removed by centrifugation, samples of

TABLE 1. *Effect of various reductants on pyrophosphatase activity in extracts of Clostridium pasteurianum*

Reductant	Amt added	Specific activity*	Stimulation
	μmoles		
None		0.07	
KBH_4	20	2.36	34×
Cysteine	20	1.65	24×
Dithionite	20	1.43	20×
Ascorbate	20	1.39	20×
H_2	1 atm	1.39	20×
Pyruvate	40	1.31	17×
Formamidine sulfinic acid	20	1.0	14×
Mercaptoethanol	30	0.66	9×
Glutathione	20	0.55	8×
NADPH†	20	0.55	8×
$NADH_2$	20	0.50	7×

* Expressed as micromoles of pyrophosphate hydrolyzed per 30 min per milligram of protein.

† Reduced nicotinamide adenine dinucleotide phosphate.

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the supernatant fluid were assayed for inorganic phosphate by the method of Taussky and Schorr (J. Biol. Chem. **202**:675, 1953).

The reductants which stimulate pyrophosphatase activity are listed in decreasing order of effectiveness in Table 1. Potassium borohydride produced a 34-fold stimulation, whereas reduced nicotinamide adenine dinucleotide ($NADH_2$) raised the activity only 7-fold. Preincubation of the extract with KBH_4 or H_2 and immediate evacuation was as effective as a continuous supply of reductant.

TABLE 2. *Effect of reductant on pyrophosphatase activity in extracts of various organisms*

Organism	Specific activity*	
	$-KBH_4$	$+KBH_4$
Clostridium pasteurianum	0.05	2.16
C. kluyverii	0.02	0.19
C. butyricum	0.04	0.08
Baker's yeast	0.15	0.08
Bacillus cereus	4.25	3.97
Escherichia coli	0.56	0.55
Micrococcus lysodeikticus	0.42	0.42
Azotobacter vinelandii	0.13	0.07
Rat liver	0.68	0.58
Crystalline yeast pyrophosphatase	6.13	4.53

* Expressed as micromoles of pyrophosphate hydrolyzed per 30 min per milligram of protein.

Table 2 shows the inorganic pyrophosphatase activity in extracts of several organisms. The three anaerobic clostridia showed a 2- to 40-fold stimulation of pyrophosphate hydrolysis in the presence of KBH_4, whereas extracts from aerobic organisms showed no stimulation. Although this survey of organisms is limited, it suggests that the inorganic pyrophosphatases of anaerobic organisms require reductants for maximal activity, and that those from aerobic organisms do not. This phenomenon of a reductant-stimulated pyrophosphatase in extracts of clostridia should be considered in the study of reactions which eliminate pyrophosphate from ATP, such as amino acid activation.