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PROPERTIES OF POLYPHOSPHATE KINASE PREPARED FROM *MYCOBACTERIUM SMEGMATIS*

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SUMMARY

An ATP:polyphosphate phosphotransferase (EC 2.7.4.1) was partially purified from *Mycobacterium smegmatis* cells. Its K_m values for ADP, ATP and chemically prepared polyphosphate were $1.1 \cdot 10^{-3}$, $2.5 \cdot 10^{-3}$ and $1.0 \cdot 10^{-3}$ M, respectively. The reaction required Mg^{2+}, and was inhibited by AMP, ADP, ATP, orthophosphate and NaF. The enzyme converted 55% of ADP used to ATP, whereas it could dephosphorylate 25% of ATP to ADP under similar conditions. The biological role of this enzyme was discussed.

INTRODUCTION

There is an accumulation of data in favor of the hypothesis that the inorganic polyphosphate may serve as a microbial phosphagen[1,2]. The postulate is made that this substance may be synthesized from ATP under certain physiological conditions, and that it may provide the energy and phosphorus required for the synthesis of phosphate compounds under different conditions.

From enzymatic studies, it is evident that the synthesis of the polyphosphate is catalyzed by polyphosphate kinase (ATP:polyphosphate phosphotransferase, EC 2.7.4.1)[3-6]. How the polyphosphate is degraded *in vivo*, however, is still to be established. First, the same enzyme might transfer phosphate to ADP:

$$ADP + (PO_3^-)_n \rightleftharpoons ATP + (PO_3^-)_{n-1}$$

Kornberg *et al.*[3] have actually shown that this reaction, *in vitro*, is catalyzed by polyphosphate kinase from *Escherichia coli*. However, the enzyme purified from *Corynebacterium xerosis* did not catalyze the reaction in this direction[4]. Secondly, many organisms have been reported to have endopolyphosphatase (polyphosphate polyphosphohydrolase, EC 3.6.1.10)[1,2], which simply hydrolyzes the polyphosphate. Genetic studies[5,6] have suggested that this enzyme degrades intracellular polyphos-

Abbreviations: [^{14}C]AMP, [8-^{14}C]adenosine monophosphate, *etc.*

phate in *Aerobacter*. The third possibility is that other enzymes might transfer phosphate from polyphosphate directly to AMP[7] and/or hexose[8,9].

We expected that studies on mycobacterial polyphosphate kinase, whose presence has already been suggested[10], might provide some information relevant to this controversy. This organism has long been known to contain typical polyphosphate granules, and many preliminary data are available on the polyphosphate metabolism of this and related bacteria.

MATERIALS AND METHODS

Bacteria

Mycobacterium smegmatis ATCC 607 was grown for 70 h at 37 °C on a rotary shaker in a medium containing 2 g aspartic acid, 10 g glucose, 3 g yeast extract, 0.5 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7\ H_2O$ per l (pH 7.0). The cells were harvested and washed with distilled water on a filter paper, and stored at −20 °C.

E. coli, strain B, was grown overnight in 50 ml of the medium described by Kornberg *et al.*[3], collected by centrifugation, washed and transferred to 500 ml of the similar medium containing no added phosphates. After being shaken for 4 h at 37 °C, the cells were harvested and stored at −20 °C. The cells thus obtained were found to have more polyphosphate kinase activity than the late log cells, as it is expected from the experiments on *Aerobacter aerogenes*[5,11].

Chemicals

Polyphosphate was prepared by heating KH_2PO_4 (Junsei Chemical Co., Tokyo) in a furnace according to the method of Pfanstiel and Iler[12], and finely ground in a mortar. Its sodium salt was made by treating this potassium salt with Dowex-50 (Na^+ form) by the method of Kornberg *et al.*[3]. The concentration of sodium polyphosphate was measured by the method of Fiske and SubbaRow[13] after hydrolysis for 30 min at 100 °C.

Sodium pyro-, tripoly- and hexametaphosphates were gifts from the Taiyo Chemical Co., Tokyo. Concentrations of these polymers will be given as those of their total phosphate residues. ^{14}C-labeled AMP, ADP, ATP and GTP were purchased from the Radiochemical Center, Amersham, England.

Enzymes

Purification procedures for mycobacterial polyphosphate kinase will be given later; the *E. coli* enzyme was purified by the method of Kornberg *et al.*[3].

The sodium salt of polyphosphate has been widely used for enzyme studies, probably because it is readily soluble. However, the potassium salt was utilized in the present work because it gave better results in our preliminary tests, and because the solution of the sodium salt was too viscous for our chromatographic experiments.

The standard incubation mixture for polyphosphate kinase contained 150 nmoles of [^{14}C]ADP (0.2–0.4 μCi/μmole), about 1 mg or 8.5 μmoles of ground potassium polyphosphate, an appropriate amount of enzyme, 0.4 μmole of $MgSO_4$, and 20 μmoles of succinic acid–NaOH buffer (pH 6.0) in 0.2 ml. The mixture was incubated for 30 min at 37 °C and the reaction terminated by the addition of 0.02 ml of 0.2 M EDTA. Aliquots from the mixture were placed on a Whatman No. 1 filter

paper with 5 or 10 μl micropipets together with authentic AMP, ADP and ATP, and chromatographed for 20 h using *n*-butyric acid–NaOH–water[14] as a solvent. In some experiments, a paper electrophoretic method was used instead. Spots were marked under an ultraviolet lamp and cut out, and their radioactivities were determined in a Beckman liquid scintillation system. At least 95% of the initial radioactivity were recovered in the three spots of AMP, ADP and ATP, even when crude cell extracts were used as the enzyme.

One unit of polyphosphate kinase was defined as the amount catalyzing the transfer of 1 nmole phosphoric acid residue during the 30-min incubation under the standard conditions. For quantitative experiments, the amount of enzyme used per 0.2 ml of mixture should be less than 60 units. Contamination with other enzymes sometimes made it almost impossible to estimate the activity of a crude preparation.

Desiccated firefly tails were a product of the Sigma Chemical Co., St. Louis, U.S.A., whereas yeast hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49) and adenylate kinase (ATP: AMP phosphotransferase, EC 2.7.4.3) were obtained from the Boehringer Mannheim Japan Co., Tokyo.

Glucose 6-phosphate, and sometimes ATP, were determined enzymatically. A reaction mixture for the determination of ATP contained 30 μmoles of D-glucose, 0.056 unit of hexokinase, 0.07 unit of glucose 6-phosphate dehydrogenase, 6 μmoles of $MgCl_2$, 0.5 μmole of $NADP^+$, 50 μmoles of Tris buffer (pH 7.4), and an aliquot of sample in 1 ml. It was prepared in a spectrophotometric cell at room temperature, and the change in absorbance at 340 nm was recorded using a Hitachi Spectrophotometer 124-recorder QPD_{54}. ATP content of the sample was estimated from an experimental calibration curve obtained with authentic ATP. A very similar method was employed for the determination of glucose 6-phosphate.

RESULTS

Purification of the enzyme

All procedures were conducted at 4 °C or in an ice bucket.

Frozen mycobacterial cells, 100 g, were ground in a mortar with 200 g of sea sand and the enzyme was extracted with 500 ml of 0.1 M KH_2PO_4–K_2HPO_4 buffer (pH 6.9). Sand and cell debris were removed by centrifugation at 10 000 $\times$ *g* for 10 min (Fraction I).

To this fraction, 450 ml, 79 g of ammonium sulfate were added, and the precipitate formed was collected by centrifugation at 23 000 $\times$ *g* for 10 min .The precipitate was then homogenized with 30 ml of 0.1 M phosphate buffer (pH 6.9) in a Teflon homogenizer and divided into two parts by centrifugation. The precipitable fraction was treated again with 20 ml of buffer and centrifuged in the same way. The supernatants from these two centrifugations were combined, brought to 100 ml with the same buffer, and centrifuged at 49 000 $\times$ *g* for 30 min.

To the almost transparent supernatant (Fraction II) 9.8 g ammonium sulfate were added, and centrifugation was carried out at 23 000 $\times$ *g* for 10 min. The supernatant (Fraction IIa), being heavily contaminated with phosphatase(s), was discarded, and the precipitate was dissolved in 20 ml of 0.1 M phosphate buffer (Fraction III).

Fraction III was dialyzed against 2 mM phosphate buffer (pH 6.9) overnight,

concentrated in a rotary evaporator to about 2.0 ml, and loaded on a Sephadex G-200 column (2.5 cm × 40 cm). When eluted with 2 mM phosphate buffer, polyphosphate kinase was recovered in fractions immediately after the void volume with a rather broad peak, followed by adenylate kinase, and then by phosphatase activities. To avoid the interference by undesirable enzymes, the first fractions were taken, concentrated, and dialyzed as before to give 2.0 ml of the purified enzyme in 2 mM phosphate buffer.

The final preparation, Fraction IV, was practically free from nucleic acid and its derivatives, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), adenylate kinase and phosphatases. It was stored at −20 °C for a month without loss of activity.

The results are summarized in Table I. However, the enzyme activity presented

TABLE I

PURIFICATION OF THE POLYPHOSPHATE KINASE

[^{14}C]ADP was incubated with enzyme samples (50 μl each of Fractions I to III, or 5 μl of Fraction IV) in the presence and absence of polyphosphate under otherwise standard conditions. Protein was determined by the method of Lowry *et al.*[15].

Fraction	*Total volume (ml)*	*Protein (mg)*		*Poly-phosphate*	*Products* (nmoles)*		*Total activity** ($\times 10^3$ units)*
		Per ml	*Total*		*ATP*	*AMP*	
Fraction I	452	9.1	4 111	+	12.6	92.4	359
				−	4.6	133.8	
Fraction II	89	5.6	498	+	35.7	49.3	281
				−	3.0	135.9	
Fraction IIa	86	3.4	302	+	49.8	38.1	370
				−	6.8	108.8	
Fraction III	20	4.1	82	+	33.4	42.5	31.8
				−	17.5	87.2	
Fraction IV	2.0	12.7	25.4	+	81.8	4.2	137
				−	2.2	10.6	

* Calculated from the radioactivity on an assumption that the total recovered radioactivity corresponds to 150 nmoles of ADP used as the substrate.

** Corrected for ATP production in the absence of polyphosphate.

is only apparent. At least, that of Fraction III is unreasonably poor, possibly because adenylate kinase, whose presence in this fraction is shown later, limited the accumulation of ATP in the reaction mixture.

Evidence for ATP synthesis

Although routine assays were made as described in Materials and Methods, some experiments were performed with other enzymes to confirm that one of the reaction products is really ATP.

Semi-quantitative determination of ATP was made with firefly tails. A reaction mixture containing non-radioactive ADP and 590 units of Fraction IV was incubated under the standard conditions. Aliquots, 0.1 ml each, of the mixture before and after incubation were added to another prewarmed mixture consisting of 15 mg powdered firefly tails, 1 ml of 0.1 M Tris buffer (pH 7.4), and 1 ml of 0.16 M

$MgSO_4$. Chemiluminescence was observed with Hitachi Fluorescence Spectrophotometer 204 connected with a recorder. The results clearly showed the increase of ATP by the action of our enzyme. It was also confirmed that ATP synthesis depends on ADP and polyphosphate, as well as on the enzyme.

An attempt to determine ATP using the hexokinase–glucose 6-phosphate dehydrogenase system was also successful, though the turbidity of the reaction mixture limited the accuracy. Thus it was estimated that 96 nmoles of ATP were formed from 150 nmoles of ADP with 826 units of Fraction IV under standard conditions (30-min incubation). The presence of ADP, polyphosphate and an enzyme preparation was again required for the synthesis.

Effect of pH and temperature

Effect of pH on the reaction is shown in Fig. 1. The standard experiments were carried out at pH 6.0 to reduce the possible effect of adenylate kinase, though the optimum pH was found to be a little higher.

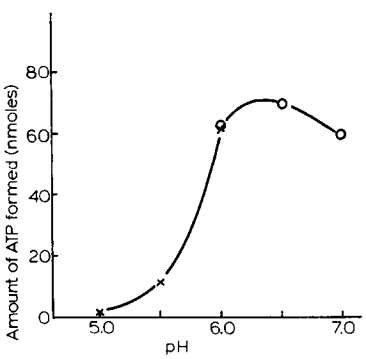
The enzyme activity at 37 °C was a little higher than that observed at 30 °C.

Effect of divalent cations

The presence of Mg^{2+} was essential for the reaction, its optimum concentration being about 2 mM. $MnCl_2$ had a small, but $CaCl_2$ had no stimulatory effect at 2 mM. $MgSO_4$ at 8 mM as well as EDTA at 10 mM completely inhibited the reaction.

Effect of enzyme concentration

Fig. 2 shows the effect of enzyme concentration on the formation of ATP. When the enzyme concentration is lower, the amount of ATP produced is proportional to that of the enzyme used. The activity of this enzyme preparation, Fraction IV, was estimated to be 68 500 units per ml, and the specific activity 5400 units per mg protein. At higher enzyme concentration, however, the ATP level remained essentially constant, suggesting an equilibrium was established.



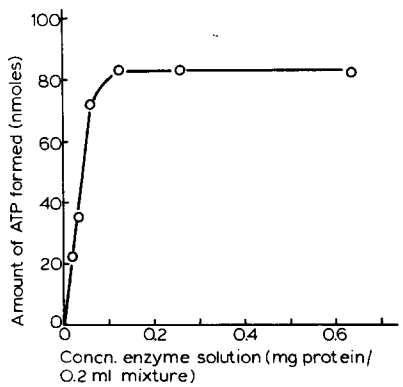


Fig. 1. Effect of pH on the polyphosphate kinase activity. Data were obtained with 345 units of the enzyme, Fraction IV, under the standard conditions, except that 0.1 M succinic acid–NaOH (×—×) or 0.1 M Tris–maleate (○—○) buffers at various pH values were used.

Fig. 2. Effect of the polyphosphate kinase concentration on the formation of ATP. Various amounts of the enzyme, Fraction IV, were used under the standard conditions.

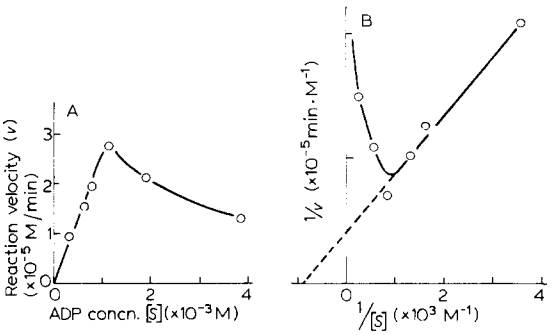


Fig. 3. Effect of ADP concentration on the velocity of ATP formation. Various amounts of ADP and 685 units of Fraction IV were incubated for 10 min under otherwise standard conditions.

Effect of ADP concentration

Fig. 3A represents the relationship between the initial concentration of ADP and the reaction velocity, and Fig. 3B shows its Lineweaver–Burk plot. The velocity increased normally up to a certain ADP concentration. The K_m was estimated to be about $1.1 \cdot 10^{-3}$ M from the figure. At higher concentrations, however, the velocity decreased, suggesting a substrate inhibition.

With *E. coli* polyphosphate kinase, whose K_m had been reported to be $4.7 \cdot 10^{-5}$ M for ADP[16], inhibition by ADP was not observed at $6.8 \cdot 10^{-3}$ M under our standard experimental conditions.

No synthesis of GTP was detectable when [^{14}C]GDP, instead of ADP, was used in a reaction mixture.

Effects of phosphate polymers

A comparison of various phosphate polymers as the substrate is summarized in Table II. Besides potassium polyphosphate, sodium polyphosphate and hexametaphosphate were utilized by the enzyme. Another "sodium polyphosphate",

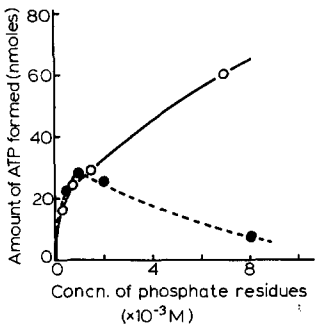
TABLE II

EFFECT OF VARIOUS PHOSPHATE POLYMERS ON ATP FORMATION

Each polymer, instead of potassium polyphosphate, was incubated with [^{14}C]ADP and 590 units of Fraction IV under the standard conditions.

Polymer	*Concentration (μmoles/ml)*	*Products* (nmoles)*	
		ATP	*AMP*
Pyrophosphate, sodium salt	2.0	0.9	3.0
Tripolyphosphate, sodium salt	2.0	1.3	4.8
Hexametaphosphate, sodium salt	2.0	25.2	2.5
Polyphosphate, sodium salt	1.4	29.0	7.6
Polyphosphate, potassium salt	(100)	68.6	2.4
None	—	1.2	4.5

* Calculated from the radioactivity on an assumption that the total recovered radioactivity corresponds to 150 nmoles of ADP used as the substrate.



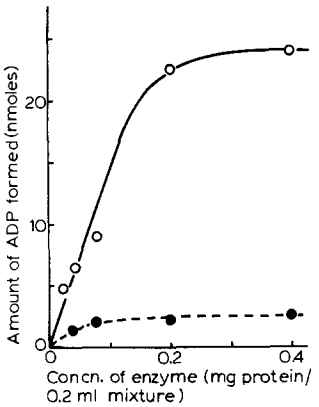


Fig. 4. Effect of the concentration of phosphate polymers on the formation of ATP. Various amounts of sodium polyphosphate (○—○) or sodium hexametaphosphate (●---●), instead of potassium polyphosphate, and 685 units of the enzyme, Fraction IV, were used under the standard conditions.

Fig. 5. Effect of the polyphosphate kinase concentration on the formation of ADP. Various amounts of the enzyme, a Fraction IV (4 630 units/mg protein), and 102 nmoles of [^{14}C]ATP (0.3 μCi/μmole) instead of [^{14}C]ADP, were incubated with (○—○) and without (●---●) potassium polyphosphate under otherwise standard conditions.

a product for industrial use, did not serve as a substrate, probably because it contained some oligomers.

The effect of concentration of sodium polyphosphate and hexametaphosphate on the ATP formation are indicated in Fig. 4. Sodium polyphosphate, for which the K_m was found to be about $1.0 \cdot 10^{-3}$ M, was confirmed to be a good substrate. In fact, at higher concentrations of the sodium salt, as much ATP was synthesized as from the potassium salt, which was always used in a large excess in the present work. On the other hand, sodium hexametaphosphate was as effective as the polyphosphate only at lower concentrations. The chemical nature of this hexametaphosphate preparation remains obscure, although it was confirmed not to contain a large amount of oligomers.

Reverse reaction

To study whether the enzyme catalyzes the reaction in the reverse direction, experiments were performed with the use of ATP instead of ADP as the substrate. Since this reverse reaction by *E. coli* enzyme has been reported to be inhibited by a small amount of ADP[3], both non-radioactive and radioactive ATP were purified by paper chromatography before use.

Fig. 5 shows that almost 25 out of 102 μmoles of ATP were converted to ADP by our enzyme, when ATP was substituted for ADP in the standard method. Data on the effects of ATP, presented in Fig. 6, were remarkably similar to those of ADP on the forward reaction. The K_m for ATP was around $2.5 \cdot 10^{-3}$ M, and the reaction was inhibited by high concentrations of the substrate.

Inhibitors

Not only ADP and ATP, but AMP was also found to be inhibitory for the for-

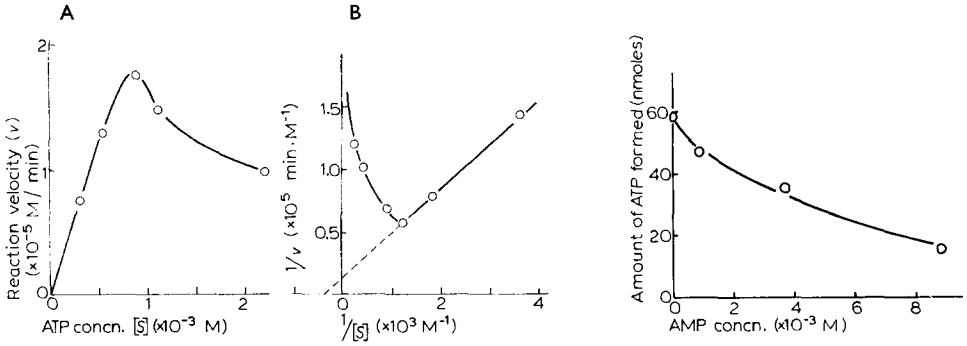


Fig. 6. Effect of ATP concentration on the velocity of ADP formation. Various amounts of [^{14}C]ATP (0.3 μCi/μmole) and 685 units of Fraction IV were incubated for 10 min under otherwise standard conditions.

Fig. 7. Effect of AMP on the formation of ATP. Various amounts of AMP, 73.5 nmoles of [^{14}C]ADP, and 343 units of Fraction IV were incubated under otherwise standard conditions.

ward reaction. Fig. 7 shows that the reaction was inhibited by 15 and 70% when the AMP/ADP ratios were 1 and 10, respectively.

Attempts to study the possible effects of some other nucleoside phosphates were unsuccessful, apparently because of the contamination with some non-specific nucleoside diphosphate kinase.

Orthophosphate was also inhibitory. Thus KH_2PO_4–K_2HPO_4 buffer (pH 6.0) inhibited the forward reaction by 33 and 49% at $5.5 \cdot 10^{-3}$ and $11 \cdot 10^{-3}$ M, respectively. The data shown in Fig. 4 also suggest that hexametaphosphate and presumably some other oligomers inhibit the reaction.

NaF, a less specific inhibitor, was also effective. It inhibited the ATP formation by 75% at a concentration of $5.0 \cdot 10^{-3}$ M.

Other enzyme activities

Data presented in Table I suggest that the crude cell extracts contained some adenylate kinase and dephosphorylating enzymes.

In fact, Fractions IIa and III clearly manifested their adenylate kinase activity, producing ADP from a mixture of AMP and ATP (see Table III).

To determine the possible activity of polyphosphate–AMP phosphotransferase,

TABLE III

ADENYLATE KINASE ACTIVITY OF SOME FRACTIONS

The reaction mixture contained 142 nmoles of [^{14}C]AMP (0.5 μCi/μmole), 192 nmoles of ATP, 0.4 μmole of $MgCl_2$, 0.10 ml of enzyme solution, and 20 μmoles of succinic acid–NaOH buffer (pH 6.0) in 0.2 ml. After incubation at 37 °C for 30 min, the distribution of radioactivity was determined as in the standard polyphosphate kinase assay.

Fraction	^{14}C *distribution (%)*		
	ATP	*ADP*	*AMP*
Fraction IIa (≥430 units)	29.0	32.7	38.3
Fraction III (>159 units)	27.0	32.8	40.2

some fractions were incubated with polyphosphate and [^{14}C]AMP under the conditions described by Winder and Denneny[7] and the distribution of radioactivity was determined by our standard method. Fractions IIa and III showed some activity, while Fraction IV did not. This could mean that polyphosphate–AMP phosphotransferase present in crude fractions was removed by gel filtration, or that formation of ATP from AMP was due to a co-operative action of polyphosphate kinase with adenylate kinase.

The polyphosphate hexokinase activity of Fraction IV was not detectable. Thus, a reaction mixture containing glucose, polyphosphate and the polyphosphate kinase preparation was incubated as reported by Szymona and Ostrowski[9], and it was found that an aliquot of this mixture was unable to reduce $NADP^+$ when incubated with glucose 6-phosphate dehydrogenase. As expected, the positive result was easily obtained with the above mixture supplemented by ADP and hexokinase.

DISCUSSION

Polyphosphate kinase from *M. smegmatis* has now been found to dephosphorylate almost 25% of ATP and to phosphorylate about 55% of ADP. The *E. coli* enzyme has been reported[16] also to catalyze the reversible reaction, though the yield of ADP from ATP was only about 8%. However, Muhammed[4] described the enzyme from *C. xerosis* as catalyzing only the synthesis of polyphosphate. According to Harold and Harold[6], polyphosphate is synthesized, but not degraded by polyphosphate kinase in *Aerobacter*.

Of course, the possibility that different bacteria might have different pathways can hardly be ruled out. However, it does not seem reasonable to suppose that *E. coli* and *M. smegmatis* are similar in metabolism, but different from *A. aerogenes* and *C. xerosis*, for bacteriologists would classify *Escherichia* and *Aerobacter* into one group and *Corynebacterium* and *Mycobacterium* into another.

Comparing the three polyphosphate kinases so far purified, we would like to point out that the enzymes from different bacteria differ in their K_m values for their substrates and in their sensitivity to the inhibition by ADP. The mycobacterial enzyme was found to be inhibited by relatively low concentrations of AMP, ADP, ATP, orthophosphate and some other phosphate polymers. These findings suggest the action of polyphosphate kinase may be measurable only under restricted experimental conditions, and we would consider that there appears to be little reason to doubt the essential reversibility of the reaction.

If the synthesis of ATP by polyphosphate kinase does not take place in nature, the next problem will be whether the bacteria are able to utilize the high energy of polyphosphate by transferring its phosphoric residue to AMP and/or hexose. We found that our crude preparations apparently have polyphosphate–AMP phosphotransferase activity. We would suppose, however, that these fractions were contaminated with a catalytic amount of ADP, and that a combined action of polyphosphate and adenylate kinases could synthesize ATP under the condition employed. Actually we synthesized some [^{14}C]ATP using a model mixture containing the Fraction IV, myokinase, [^{14}C]AMP and a small amount of ADP. Thus, further studies seem to be required to determine whether the enzyme, polyphosphate-AMP phosphotransferase, does in fact exist. Similarly, it is unfortunate that some critical experiments would

be needed on polyphosphate hexokinase, especially on its polyphosphate kinase activity.

In conclusion, we would like to consider that polyphosphate would be synthesized and utilized by one and the same polyphosphate kinase, at least in *Mycobacteria*. It is of interest to speculate that the action of this enzyme and the solubility of polyphosphate in the cells may be controlled by the concentration of a variety of phosphate compounds and by cationic environment, respectively. Although there remain some other possibilities, the data reported here certainly support the phosphagen hypothesis on microbial polyphosphate.

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