ADENOSINE TRIPHOSPHATE SYNTHESIS FROM POLYPHOSPHATE BY AN ENZYME FROM *ESCHERICHIA COLI*

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The synthesis of polyphosphate (metaphosphate)* by living systems and its metabolic function have interested many workers since the substance was discovered in the last century[1]. There have been a few brief accounts of polyphosphate synthesis and utilization by cell-free extracts[2-4] and in a recent publication from this laboratory[5] an enzyme purified from *Escherichia coli* was described in some detail which catalyzed the formation of highly polymerized inorganic polyphosphate from the terminal phosphate of ATP**. At the present time we are able to report that the reversal of this reaction is carried out by the enzyme according to the equation:

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

Polyphosphate synthesized chemically or enzymically is utilized quantitatively in the phosphorylation of ADP to ATP. Some kinetic data obtained in these studies may, together with observations on the synthetic reaction[5], provide an explanation for the physiologic accumulation and disappearance of polyphosphate in the cell.

MATERIALS AND METHODS

Crystalline sodium ATP and AMP, and the diphosphates of adenosine, uridine, cytidine, and guanosine were products of the Sigma Chemical Co. ^{32}P-Orthophosphate was purchased from the Oak Ridge National Laboratory, the Dowex resins from Dow Chemical Corp., and crystalline bovine serum albumin from Armour and Co. Glucose 6-phosphate was given us by Dr. B. L. HORECKER and glucose 6-phosphate dehydrogenase by Dr. D. E. BROWN. Ribonuclease was obtained from Worthington Biochemical Corp. and hexokinase from the Sigma Chemical Co.

Radioactivity was measured with a thin-window Geiger-Müller counter. Glucose 6-phosphate was determined by means of its enzymic oxidation reaction[6] or, in chromatographed fractions, by the reducing sugar method of NELSON[8]. Protein was assayed by the method of LOWRY *et al.*[8] and acid-labile phosphate according to FISKE AND SUBBAROW[9] after boiling the samples for 10 min in 1 *N* HCl. Norit was used at pH 2 to separate nucleotide from non-nucleotide phosphate[10].

The enzyme preparation was described in the earlier publication[5]. Further purification of the Ammonium Sulfate III, which is an insoluble fraction, was accomplished by adding 0.1 vol. of saturated ammonium sulfate (5.3 *M*) to the enzyme suspension. After centrifugation, the enzyme was recovered without loss in the supernatant fluid, whereas 70% of the protein was discarded in the precipitate. This preparation appears to have little or no adenylate kinase activity since after incubation with ^{32}P-polyphosphate and ADP, the ADP isolated by ion-exchange chromatography has very little radioactivity (see Table I, legend). It was also found that, in the absence of polyphosphate, the enzyme did not produce ATP from ADP.

* While the terms metaphosphate and polyphosphate have been used commonly to refer to high molecular condensed phosphates, the term polyphosphate is favored for precise usage.

** ATP refers to adenosine triphosphate, ADP to adenosine diphosphate, and AMP to adenosine 5'-monophosphate.

References p. 300.

The enzyme unit, previously defined, is the amount producing 0.01 μmole of acid-insoluble phosphate in the 15-min assay.

The chemical synthesis of polyphosphate described by PFANSTIEL AND ILER[11] was adapted for small-scale use to prepare ^{32}P-labeled material. In a typical preparation, 0.5 ml of Oak Ridge ^{32}P-labeled orthophosphate (2 mc) was placed in a platinum crucible and dried down in a sand bath. Water (2 ml) was added and evaporated to dryness; this was repeated once; 0.5 ml of 1 *M* KH_2PO_4 (500 μmoles) was added and the pH of the solution was adjusted to 4.5 with 0.1 *N* KOH. After drying, the crucible was placed in a muffle furnace and the temperature was raised to 775° over a period of 4 h. The temperature was maintained at 775° for 40 min and then the crucible was removed and held in ice water to cool. The residue in the crucible was first washed with 3 portions of ice water, and then converted to the soluble sodium salt by treatment with 4.5 ml of a suspension of Dowex 50 (Na^+ form) (1 vol. resin in 1 part of water). After vigorous stirring, the resin was removed by centrifugation, and a highly viscous product resulted. The yield of polyphosphate was about 65%, determined both by acid-labile phosphate analysis and by radioactivity. (The yield may be improved by carefully recovering all fine particles from the ice-water washes, and by washing the resin several times with cold water after removal of the main product.) The product was insoluble in the presence of albumin and perchloric acids under the assay conditions described below, only 1 part in 1,600 remaining in the supernatant fluid. The same results were obtained with 200 μmoles of KH_2PO_4 and 5 mc of ^{32}P as starting materials. Enzymically synthesized polyphosphate was prepared as described before[5]. Molar values for polyphosphate are expressed as phosphate residues determined as acid-labile phosphate.

Enzymic formation of ATP from polyphosphate and ADP was measured by determining the acid-soluble ^{32}P released from ^{32}P-polyphosphate. The routine assay mixture was as follows: glycylglycine buffer (pH 7.0). 12.5 μmoles; $MgCl_2$, 1 μmole; ammonium sulfate, 10.5 μmoles; ADP, 0.17 μmole; ^{32}P-polyphosphate, 0.056 μmole; enzyme; and water to 0.25 ml. Following a 15 min incubation at 37°, 0.25 ml of 7% perchloric acid and then 0.50 ml of a 0.15% serum albumin solution were added. The mixture was stirred and set in ice for 2 min, then centrifuged for 2 min, and a suitable aliquot of the clear supernatant fluid was used to measure radioactivity. ADP was omitted in control runs.

RESULTS

Stoichiometry of the reaction

The enzyme converts polyphosphate and ADP to ATP, and the terminal phosphate of the ATP formed is derived exclusively from the polyphosphate. The following evidence may be cited:

1. The formation of ATP was matched by the disappearance of ADP and of polyphosphate in stoichiometric amounts. Table I shows the determination by ion-exchange chromatography of the ATP formed and the ADP that remained after a 15-minute reaction period.

2. The acid-soluble ^{32}P produced was Norit-adsorbable. Under the assay conditions, and employing an enzymically formed sample of ^{32}P-polyphosphate (0.05 μmole, $6.3 \cdot 10^5$ c.p.m. per μmole), 6,550 and 12,500 c.p.m. became acid-soluble in the presence of 0.01 and 0.02 ml of enzyme, respectively. 98% of the radioactivity was adsorbed by the Norit. The two large-scale experiments that are described in detail in Table I also illustrate the Norit-adsorbability of the product.

3. When the reaction was coupled with hexokinase action the ATP formed could be utilized to phosphorylate glucose. Table II shows that the utilization of acid-insoluble ^{32}P results in the appearance of an equivalent amount of glucose 6-phosphate, identified chromatographically (see Fig. 1). The radioactivity was recovered quantitatively in the glucose 6-phosphate.

4. No reaction occurred in the absence of ADP (see Fig. 2). Incubation mixtures yielded the same blank values when ADP or enzyme was omitted or when the incubation was treated with acid at zero time.

References p. 300.

TABLE I

STOICHIOMETRY OF THE REACTION

Expt.	*Time (min)*	*ADP**	*^{32}P-Polyphosphate*	*^{32}P-Nucleotide (Norit)*	*ATP**	*^{32}P-ATP*
		μmoles	*μmoles*	*μmoles*	*μmoles*	*μmoles*
1	0	8.1	9.8	0.0	—	—
	15	3.0	(3.7)	5.7	4.8	4.9
	Δ	—5.1	—6.1	+5.7	+4.8	+4.9
2	0	0.89	1.11	0.0	—	—
	15	0.32	(0.51)	0.54	0.58	0.59
	Δ	—0.57	—0.60	+0.54	+0.58	+0.59

* Values were calculated from the optical density readings at 260 mμ ($\varepsilon = 14.7 \cdot 10^3$).

Expt. 1: The following reagents, in a final vol. of 50 ml, were incubated at 37°C for 15 min: 2.5 mmoles glycylglycine buffer (pH 7.0), 0.2 mmole $MgCl_2$, ADP and synthetic polyphosphate as shown (the latter, $7 \cdot 10^6$ counts/min/μmole), and 1,650 units of enzyme (0.30 mg protein). The mixture was then chilled in ice, and 6 ml of cold albumin solution (50 mg/ml) and 1 ml of cold 70% perchloric acid were added. An aliquot of the supernatant fluid was treated with acid-washed Norit and centrifuged. The precipitate was washed once with cold water, and then eluted with 3 portions of ammoniacal ethanol (50 parts water: 50 parts 95% ethanol: 0.3 parts concentrated NH_4OH). The radioactivity of the eluate neutralized with KOH was measured. Another aliquot of the supernatant fluid, after centrifugation and neutralization with KOH, was chromatographed on Dowex 1 resin (10% cross-linked, Cl^- form, 4 cm × 1 cm). Elution with 0.01 *N* HCl removed 2.1% of the ^{32}P and 15% of the optical density units adsorbed. Fractions eluted with 0.01 *N* HCl–0.02 *M* KCl contained 4.3% of the ^{32}P; ADP values were calculated from these fractions. Values for ATP were obtained from the fractions eluted with 0.01 *N* HCl–0.10 *M* KCl.

Expt. 2: 250 μmoles of glycylglycine buffer (pH 7.0), μmoles $MgCl_2$, ADP and ^{32}P-polyphosphate ($3.34 \cdot 10^5$ counts/min/μmole) as shown, 165 units of enzyme (0.03 mg protein) and water to 5 ml were incubated for 15 min at 37°C. Fifteen ml of ice water and 1 drop of 2 *N* KOH were added and 18 ml of the mixture was chromatographed as above.

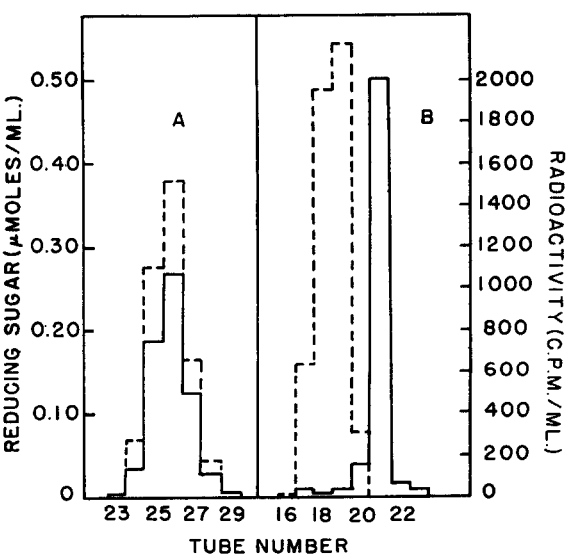

Fig. 1. Identification of ^{32}P-glucose 6-phosphate. Chromatography of enzymically synthesized ^{32}P-glucose 6-phosphate with authentic, unlabeled glucose 6-phosphate. – – – Reducing sugar; ——— Radioactivity. A. Aliquots of the incubation mixtures described in Table II were mixed with 8 μmoles of glucose 6-phosphate, adsorbed on Dowex 1 resin (10% cross linked, Cl^- form, 12 cm in height × 1 cm in diameter), and eluted with a gradient of 0.1 *N* HCl dropping into 1 l of water. Fractions (8 ml) were assayed for reducing sugar and ^{32}P. In the control column, no radioactivity was detected in the fractions containing reducing sugar. B. Chromatogram showing the separation of orthophosphate and glucose 6-phosphate. A similar but shorter column (7 cm) was used with identical elution conditions. Glucose 6-phosphate (15 μmoles) and 34,000 c.p.m. as orthophosphate were applied to the column.

References p. 300.

TABLE II

FORMATION OF GLUCOSE 6-PHOSPHATE IN A COUPLED REACTION

	Glucose-6-P (*μmoles*)	*Acid-soluble* ^{32}P (*μmoles*)	*Acid-insoluble* ^{32}P (*μmoles*)
Experimental	0.190	0.198	0.002
Control	0.000	0.018	0.198

Control and experimental vessels contained, in 5.0 ml, 125 μmoles glycylglycine buffer (pH 7.0); 250 μmoles glucose; 210 μmoles ammonium sulfate; 0.225 μmole ADP; 0.2 μmole synthetic ^{32}P-polyphosphate containing $3 \cdot 10^5$ counts/min/μmole; 20 μmoles $MgCl_2$; 1 mg hexokinase; and, in the experimental vessel only, 40 units of enzyme (0.035 mg protein)). After 60 min at 37°C, the mixtures were chilled in ice and diluted with 5.0 ml ice-water. Glucose 6-phosphate was measured in a 0.5-ml aliquot. To another 0.5-ml aliquot were added 0.05 ml of 70% perchloric acid and 0.5 ml of 0.15% albumin. Radioactivity of the supernatant fluid was measured after centrifugation; after washing the precipitate with 3.5% perchloric acid twice and dissolving it in 0.5 *M* NaOH, acid-insoluble ^{32}P was measured. The remainders of the incubation mixtures were analyzed by chromatography (see Fig. 1).

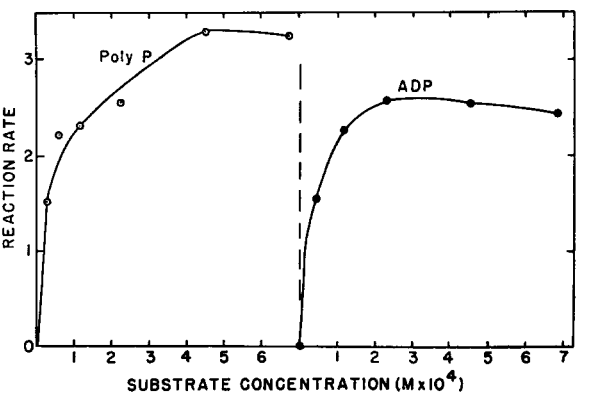

Fig. 2. Influence of substrate concentration on rate of reaction. Reaction rate values are mμmoles of acid-soluble ^{32}P produced by 0.4 unit of enzyme under standard assay conditions.

Influence of concentrations of substrates on the rate, and substrate specificity

The K_m for ADP calculated from a LINEWEAVER-BURK plot[10] of the data in Fig. 2 is $4.7 \cdot 10^{-5}$ *M*, and that for chemically prepared polyphosphate is $2.6 \cdot 10^{-5}$ *M*.

All the chemically prepared samples of polyphosphate were attacked by the enzyme at quite comparable rates; on the other hand, not all enzymically prepared polyphosphates were equally good substrates. The utilizability of the latter ranged from samples which reacted at rates quite similar to chemical samples to some which were highly inhibitory. For example, at levels of $0.22 \cdot 10^{-4}$, $1.0 \cdot 10^{-4}$, and $2.0 \cdot 10^{-4}$ *M*, the rates were 25 to 30 % lower with an enzymic sample than with chemical polyphosphate. Another sample of enzymically prepared polyphosphate gave a rate 130% the rate found with a chemically prepared sample when both were tested at $1.6 \cdot 10^{-4}$ *M*. Still another enzymically prepared sample which was tested at 4 levels from $0.6 \cdot 10^{-4}$ *M* to $3.3 \cdot 10^{-4}$ *M* showed progressive decline in reaction rate (until at the highest concen-

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tration the rate was one-third the rate at $0.6 \cdot 10^{-4}$ *M*). At these concentrations, Fig. 2 shows that the reaction rate with a chemically prepared sample is increasing. Rarely, samples made enzymically showed no reactivity at all, which, as suggested before[5], may account for our early failures to demonstrate reversal of polyphosphate formation.

It should be emphasized that the stated molarities give total phosphate residues and that the molecular size of the polyphosphate samples is not known. Thus, not only is the true molar concentration of substrate unknown but also it is not known whether all samples prepared are of the same size.

Cytidine, uridine, and guanosine diphosphates, as well as adenosine 5′-monophosphate, showed little or no reactivity in this system. Under conditions where, ADP gave a conversion of 35 μmoles, guanosine diphosphate produced 0.9 mμmoles and the others were inert.

$MgCl_2$ was essential to the reaction. In the absence of $MgCl_2$ no reaction was observed, while at $2 \cdot 10^{-3}$ and $8 \cdot 10^{-3}$ *M* the rates were 45 and 82%, respectively, of the rate at $4 \cdot 10^{-3}$ *M*.

Ammonium sulfate stimulated the reaction rate of the enzyme fraction Ammonium Sulfate III; the rate in the presence of 0.04 *M* ammonium sulfate was maximal and almost 3 times the rate in its absence. When the enzyme solubilized by ammonium sulfate was studied, the addition of ammonium sulfate did not stimulate the rate, although the concentration of the salt was only 0.0017 *M*.

DISCUSSION

In earlier studies of the synthesis of polyphosphate from ATP, a striking inhibition by ADP was observed[5]. For example, ADP inhibited the reaction completely at a concentration of $8 \cdot 10^{-5}$ *M* with ATP present at 8 times that level. Since attempts to demonstrate a reversal of the reaction at that time were complicated by an inability to isolate an active polyphosphate substrate (see above), the basis for this ADP inhibition was left in doubt. From the kinetic results of the current investigation it now appears quite clear that the diminished rates of polyphosphate synthesis in the presence of ADP can be explained by reversal of the reaction. The value determined for the dissociation constant of the ADP–enzyme complex, $4.7 \cdot 10^{-5}$ *M*, indicates that essentially maximal rates of polyphosphate breakdown are attainable even at the ADP level used in the earlier study. Since the dissociation constant of the polyphosphate–enzyme complex, $2.6 \cdot 10^{-5}$ *M*, is also very small and since the opposing maximal reaction rates are nearly equal*, the suppression of polyphosphate synthesis by low concentrations of ADP is to be expected.

These kinetic data also offer some basis for speculating about a possible mechanism for the accumulation and utilization of polyphosphate in cells. Polyphosphate is

* An enzyme preparation which per mg of protein formed 44 μmoles of polyphosphate from ATP in 15 min, produced 42 μmoles of ATP from polyphosphate and ADP. Polyphosphate formation was measured with incubation mixtures (0.25 ml) which contained glycylglycine, $MgCl_2$, acetokinase[5], 0.3 μmole of ADP, 0.38 μmole of ^{32}P-labeled acetyl phosphate[5], and 0.7 to 1.5 units of enzyme (4,400 units/mg protein). The reverse reaction was measured in 0.25 ml assay mixtures containing 2.5 μmoles glycylglycine (pH 7.0), 1 μmole $MgCl_2$, 0.20 μmole ADP, 0.11 μmole of chemically produced polyphosphate, and 0.7 unit enzyme.

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accumulated when cells are aged[13] or exposed to a poison such as tetrahydrofurfuryl alcohol[13], or to phosphate after prolonged phosphate starvation[1]. Under these and other conditions unfavorable for growth and for synthetic activities which normally consume ATP, the latter would begin to rise in concentration until levels were reached sufficiently high to satisfy even the rather large dissociation constant ($1.4 \cdot 10^{-3}$ *M*) of the polyphosphate-synthesizing enzyme. The extent of polyphosphate accumulation, as has already been stated, is in turn sensitive to the concentrations of ADP and polyphosphate and low levels of these substances are sufficient to saturate the enzyme for polyphosphate removal and ATP resynthesis. Thus when the cell resumes growth processes which demand ATP and consume it effectively at relatively low concentrations, the level of ADP would rise and then be phosphorylated by polyphosphate. As an example, SCHMIDT *et al.*[14] may be cited in which the correlation between polyphosphate depletion and nucleic acid synthesis in phosphate-starved yeast cells may be mediated through nucleoside di- or triphosphates in just such a process.

It is interesting that the polyphosphate-synthesizing enzyme occurs in *E. coli*, an organism which had never been observed to accumulate cytochemically detectable deposits of polyphosphate. Large masses of polyphosphate are, however, found in *Corynebacterium diphtheriae* in the form of intracellular metachromatic-staining granules[15]. Sonic extracts which we have prepared from two strains of diphtheria bacillus contained from 20 to 100 units of the enzyme per mg of protein*. Although one of the strains, C_7 (β_1), was lysogenic and toxin-producing and the other, C_7, was not[16], extracts of both had quite similar metaphosphate-forming activities. It is worth noting that the *Corynebacterium* enzyme is markedly inhibited by ammonium sulfate, in contrast to the stimulatory effect of this salt on the enzyme from *E. coli*.

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SUMMARY

An enzyme previously purified from *Escherichia coli* which catalyzes the conversion of the terminal phosphate of ATP to a highly polymerized polyphosphate has now been shown to catalyze a reversal of this reaction. The enzyme was also obtained from *Corynebacterium diphtheriae*. Chemically or enzymically synthesized polyphosphate is quantitatively converted to ATP with ADP as the specific acceptor. The reaction may, therefore, be formulated as:

$$(PO_3^-)_n + n\text{ADP} \rightleftharpoons n\text{ATP}.$$

When this reaction is coupled with hexokinase action, polyphosphate is quantitatively converted to glucose 6-phosphate. The purified enzyme is free of adenylate kinase and ATP is formed exclusively by a reaction of polyphosphate with ADP.

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* Purification of the enzyme was carried out by means of the following steps:
(1) Streptomycin sulfate addition, which left the enzyme entirely in the soluble fraction;
(2) Protamine treatment which precipitated the activity, extractable with phosphate buffer;
(3) Ammonium sulfate sulfate fractionation; and
(4) Calcium phosphate gel addition to adsorb inactive protein, but not the activity.
The enzyme had an activity of 1,750 units/mg of protein.

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ETUDE DU MÉTABOLISME DE LA TAURINE CHEZ LE RAT. FORMATION DE SULFATE

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INTRODUCTION

En dehors de son incorporation dans les acides biliaires tauroconjugués, la taurine a été pendant longtemps considérée, avec l'acide cystéique et le sulfate, comme un des termes finaux du catabolisme des acides aminés sulfurés et de la cystéamine. D'autre part, l'opinion qui a toujours prévalu est que, ni l'acide cystéique, ni la taurine, ne donnent naissance à du sulfate. Il a en effet été démontré que ce dernier se forme principalement à partir d'un dérivé intermédiaire, l'acide cystéine-sulfinique[1].

Au cours de ces dernières années, la taurine a été l'objet d'un intérêt accru. C'est ainsi que chez le mammifère, diverses études analytiques ont révélé sa présence, en quantités relatives parfois très importantes à l'état libre et combiné, dans divers organes[2,3]. Chez le rat, par exemple, AWAPARA *et al.*[3] ont montré que, dans le muscle cardiaque, la quantité de taurine présente équivaut presque au total des acides aminés libres. Il nous paraît difficile d'admettre que la taurine n'y serait qu'une substance de déchet. La biosynthèse de taurine à partir de sulfate minéral a pu être mise en évidence chez l'embryon de poulet[4,5,6].

A l'exception des acides biliaires déjà cités, les seules formes conjuguées de taurine trouvées

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