METAPHOSPHATE SYNTHESIS BY AN ENZYME FROM *ESCHERICHIA COLI**

by

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During the half-century interval since the recognition of metaphosphate in cells[1], there have been sporadic but frequent discoveries in bacteria, molds and insects of unfamiliar phosphate fractions which proved to be long-chain polymers of inorganic phosphate and were termed metaphosphate**. Accumulations of basophilic material in yeast cells were identified, by WIAME, and SCHMIDT *et al.*[2], as metaphosphate, as were the metachromatic granules in corynebacteria, by EBEL[3]. In 1951, when SCHMIDT[4] reviewed the subject, there had been no reports of metaphosphate synthesis in cell-free systems, and no information concerning the mechanism of its synthesis or non-hydrolytic metabolism. Since then several significant reports have appeared, although the experimental basis for the claims has not yet appeared in any detail. YOSHIDA AND YAMATAKA[5] and HOFFMAN-OSTENHOF *et al.*[6] have reported the presence in yeast extracts of a reversible metaphosphate synthesis from ATP***; WINDER AND DENNENY[7] have described an apparent utilization of metaphosphate for glycerol phosphorylation by cell-free extracts of *Mycobacterium smegmatis*.

To study the mechanism of polyphosphate synthesis, we purified to a considerable extent an enzyme from *Escherichia coli* which catalyzes the production of metaphosphate, $(PO^-)_{n+x}$, according to the equation:

$$x\text{ATP} + \begin{bmatrix}(PO_3^-)_n \\ \text{primer}\end{bmatrix} \longrightarrow x\text{ADP} + (PO_3^-)_{n+x} \qquad (1)$$

The polyphosphate turns the color of toluidine blue from blue to pink (metachromasy)[8], is quantitatively converted to an acid-insoluble protein complex, and because of these and other properties may be considered a metaphosphate polymer of very long

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** The empiric formula for metaphosphate is $(x^+PO_3^-)_n$. At present there are only two cyclic polyphosphates of proved structure, tri- and tetrametaphosphate, and strictly speaking the term metaphosphate applies only to these. However, since the composition of a linear condensed phosphate and its cyclic homologue differ only by one molecule of water, it has not been possible to determine the structure of the very high molecular polyphosphates; these have been termed metaphosphates.

*** The abbreviations used are adenosine triphosphate, ATP or ARPPP; ^{32}P-labeled phosphate, P*; inorganic orthophosphate, P_i; inorganic pyrophosphate, PP; adenosine diphosphate, ADP or ARPP; tris(hydroxymethyl)aminomethane, Tris; adenosine-5′-phosphate, A5P; acetyl phosphate, acetyl P; counts per minute, c.p.m.

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chain length. This report is concerned with the purification and properties of the enzyme, the stoichiometry of the reaction and a preliminary characterization of the metaphosphate product.

MATERIALS AND METHODS

Crystalline A5P, crystalline sodium ATP and barium ADP were products of the Sigma Chemical Co.

ARP$\overset{*}{P}\overset{*}{P}$P was prepared in two ways: (a) by phosphorylation of A5P with rat liver mitochondria*, or (b) by exchange with $\overset{*}{P}\overset{*}{P}$ in the aceto-coenzyme A kinase system of BERG[9]. The product was purified either by (a) adsorption on acid-washed Norit[10]** at pH 2 and, after repeated water washing of the Norit, elution with a 0.05 *N* solution of NH_4OH containing 50% ethanol; or by (b) the ion-exchange chromatographic procedure of COHN AND CARTER[11] and KORNBERG AND PRICER[12].

ARP$\overset{*}{P}$P was prepared as follows: the terminal phosphate of ARP$\overset{*}{P}\overset{*}{P}$ was removed by means of hexokinase[13] and excess glucose. The ARP$\overset{*}{P}$, purified by the Norit procedure, was phosphorylated by the use of pyruvate phosphokinase with an excess of phosphopyruvate present. The final product was purified by the Norit procedure.

ARP$\overset{*}{P}\overset{*}{P}$ was prepared by phosphorylating ARPP by means of acetokinase[14] and acetyl $\overset{*}{P}$. It was purified by the Norit procedure. The concentration of labeled ATP determined by enzymic assay[15] agreed within 5% of that calculated from the density reading at 260 mμ.

ARPPP labeled with ^{14}C in the 8-carbon of adenine was prepared from ^{14}C-adenine (Isotope Specialties Company) by a sequence of previously described reactions[16].

Acetyl P and acetyl $\overset{*}{P}$ were prepared by a slight modification of the method of AVISON[17]. Pyridine (0.95 ml), K_2HPO_4 (1 *M*, 0.50 ml, containing $\overset{*}{P}$ when desired), and water (1.50 ml) were mixed in a 30 ml Erlenmeyer flask. The mixture was chilled to 0° or less, then kept in ice. Acetic anhydride in slight excess (0.11 ml) was added over a 3-minute period to the mixture which was constantly shaken. LiOH (4 *N*) was added 2 minutes later to adjust the pH to about 7.5 (0.45 ml was required). Ethanol (23 ml at −15°) was added slowly while agitating the reaction mixture. After 1 hour at 0°, the precipitate was collected by centrifugation, washed twice with cold ethanol and dried *in vacuo* over $CaCl_2$ and KOH. 416 μ*M* of acetyl P were recovered, a yield of 83% based on phosphate. Inorganic orthophosphate was not detectable in the product.

Phosphopyruvic acid was prepared by OHLMEYER's modification[18] of Kiessling's procedure, or the method of BAER AND FISCHER[19] with the chromatographic purification of UTTER AND KURAHASHI[20]. $\overset{*}{P}\overset{*}{P}$ was prepared as previously described[12] from labeled orthophosphate (Oak Ridge National Laboratory).

Crystalline inorganic pyrophosphatase was kindly furnished by Drs. M. KUNITZ AND G. PERLMANN. Crystalline pyruvate kinase was prepared by the method of BEISENHERZ *et al.*[21]; the suspension of crystals in 2 *M* ammonium sulfate contained $1.5 \cdot 10^5$ units per ml (specific activity $1 \cdot 10^3$ units per mg of protein). Ribonuclease and desoxyribonuclease were crystalline products of the Worthington Biochemical Corporation. Acetokinase (Fraction 3, 22 mg of protein per ml) was prepared according to ROSE[14], by the method of ROSE *et al.*

Protein was determined by the method of LOWRY *et al.*[22], phosphate by the method of FISKE AND SUBBAROW[23], and acetyl P by the method of LIPMANN AND TUTTLE using the $FeCl_3$ reagent of JONES *et al.*[24].

Radioactivity of labeled phosphate was measured in solution with a Geiger-Müller counter. ^{14}C-containing samples were plated in thin layers on metal disks and measured in a gas-flow counter.

Growth of cells. Escherichia coli strain B was grown in a medium containing 2% K_2HPO_4, 1.65% KH_2PO_4, 1% yeast extract (Difco, dehydrated), 1% glucose and about 20 mg per l of Antifoam A (Dow-Corning Company). The pH was 7.0–7.2. The glucose was autoclaved separately and added to the cooled medium. 15 l of the medium in a 20-liter Pyrex bottle were inoculated with 100 ml of an 8-hour broth culture and incubated at 34–37° for 16–20 hours with vigorous forced aeration. The yield of cells, harvested in a Sharples Super Centrifuge, was about 8 g (wet weight) per liter. The cells were washed with 2 volumes of cold 0.5% NaCl–0.5% KCl and extracted immediately or stored at −13°; active extracts were obtained from cells stored as long as 6 weeks.

* Furnished through the kindness of Dr. GEORGE DRYSDALE.

** Adsorption of nucleotides on Norit at acid pH has been used in these studies to separate nucleotides from non-nucleotides.

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Preparation of cell-free extract. 10 g of cells, suspended in 50 ml of glycylglycine buffer (0.02 *M*, pH 7.0), was treated in a Raytheon 10 KC Oscillator at 0–2° for 10 minutes. The suspension was centrifuged for 15 minutes at 10,000 × *g* and the residue was discarded. The supernatant extract (*E. coli* extract) was fractionated immediately or stored at —13°, with little loss in activity, for 6 weeks or longer.

Enzyme assay. The assay measured the production of acid-insoluble labeled phosphate derived from ARPP$\overset{*}{P}$. The incubation mixture contained 0.03 ml of ARPP$\overset{*}{P}$ (0.01 *M*, 10^5 c.p.m. per μM), 0.03 ml of acetyl $\overset{*}{P}$ (0.014 *M*, 10^5 c.p.m. per μM), 0.01 ml of $MgCl_2$ (0.1 *M*), 0.025 ml of glycylglycine buffer (0.5 *M*, pH 7.0, potassium salt), 0.01 ml of ammonium sulfate (1 *M*), 0.01 ml of acetokinase, 0.2 to 4 units of enzyme, and water to a final volume of 0.25 ml. After incubation for 15 minutes at 37°, the mixture was treated with 0.25 ml of cold 7% perchloric acid and then 0.5 ml of bovine serum albumin (Armour Company, crystalline; 1.6 mg per ml). The mixture was centrifuged at about 15,000 × *g* for 2 minutes. The precipitate was washed twice with 2.0 ml portions of 3.5% perchloric acid, dissolved in 0.4 ml of 0.5 *M* NaOH and an aliquot of 0.2 ml was assayed for radioactivity. One unit of enzyme is defined as the amount producing 0.01 μM of acid-insoluble P in 15 minutes. The extent of reaction was roughly proportional to the amount of enzyme added under these conditions; in assays of 0.01, 0.02, 0.04, and 0.08 ml of crude *E. coli* extract (diluted 5-fold), the radioactivity values of the aliquots were 219, 413, 750 and 1455 c.p.m., respectively.

Owing to the strong inhibitory action of ADP, the use of an ATP-regenerating system was essential. For readily interpretable results the donor system was adjusted to the same specific radioactivity as the terminal phosphorus atom of the ATP (see RESULTS).

RESULTS

Purification of the enzyme

See Table I. All operations were carried out at 0–3°.

TABLE I

PURIFICATION OF THE ENZYME

Step	*Units per ml*	*Total units*	*Protein mg per ml*	*Specific activity, units per mg protein*	*Optical density* 260 mμ	280 mμ	*Enzyme units per optical density unit at 260 mμ*
1. *E. coli* extract	215	32,200	15.0	14.3	106.0	62.8	2.0
2. Streptomycin eluate I	95	28,500	1.82	52.1	27.0	14.9	3.5
3. Ammonium sulfate I	114	17,100	1.12	102	19.9	9.7	5.7
4. Ammonium sulfate II	262	9,850	0.98	265	21.4	10.4	12.2
5. Nuclease treatment	207	8,300					
6. Ammonium sulfate III	272	5,100	0.17	1600	0.81	0.72	328

Streptomycin eluate I

To 30 ml of *E. coli* extract in each of five 50-ml tubes was added, dropwise with stirring, an amount of streptomycin sulfate (5% solution) which left 5 to 10% of the enzyme in the supernatant fluid. The amount of streptomycin to be added was determined for each run by a small scale titration; 3.2 ml per tube was used for the preparation described here. After 5 minutes the precipitate was separated by centrifugation and the supernatant fluid discarded. The precipitate in each tube was thoroughly dispersed in 30 ml of potassium phosphate buffer (0.02 *M*, pH 6.5) with the aid of a pestle, and centrifuged after 5 minutes. To the combined supernatant fluids of the five tubes was added 20 ml of potassium phosphate buffer (0.1 *M*, pH 7.4) and water to bring the volume to 300 ml.

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Ammonium sulfate I

To 300 ml of the Streptomycin eluate I was added 60 g of ammonium sulfate. After 5 minutes, the precipitate was removed by centrifugation and to the supernatant fluid was added 31 g of ammonium sulfate. After 5 minutes, the precipitate was collected by centrifugation and dissolved in 150 ml of potassium phosphate buffer (0.02 *M*, pH 6.9).

Ammonium sulfate II

To 150 ml of the Ammonium sulfate I was added 33 g of ammonium sulfate. The precipitate was discarded after separation by centrifugation and 7 g of ammonium sulfate was added to the supernatant fluid. This precipitate was collected and dissolved in 37.5 ml of potassium phosphate buffer (0.02 *M*, pH 6.9).

Nuclease treatment and Ammonium sulfate III

To 37.5 ml of Ammonium sulfate II was added 1.87 ml of potassium phosphate buffer (1 *M*, pH 7.4), 0.93 ml of $MgCl_2$ (0.1 *M*), 0.375 ml of a desoxyribonuclease solution (0.3 mg per ml), and 0.375 ml of a ribonuclease solution (0.5 mg per ml). After incubation for 30 minutes at about 25°, the mixture was chilled in ice to 0–3° and treated with 8.4 g of ammonium sulfate. The precipitate was separated by centrifugation and discarded, and to the supernatant fluid was added 2.25 g of ammonium sulfate. This precipitate was collected and dissolved in 18.7 ml of potassium phosphate buffer (0.02 *M*, pH 6.9) After about 1 hour, a fibrous precipitate developed which was separated by centrifugation and suspended in 18.7 ml of the same buffer.

Streptomycin eluate II and Spinco residue

These purification steps were abandoned because of their poor reproducilibity but a description of the fractions is included since they were used in some of the experiments to be reported below. To 10.0 ml of Ammonium sulfate II in each of two tubes was added 8.0 ml of streptomycin sulfate (5%) (see the Streptomycin eluate I step above for details). The precipitate in each tube was collected by centrifugation and eluted three times with 10.0 ml of potassium phosphate buffer (0.02 *M*, pH 6.0). Generally the second elution resulted in a marked solution of the precipitate; the eluate contained 80–90% of the enzyme purified 2- to 2.5-fold. The active eluates were combined and adjusted with potassium phosphate buffer (1 *M*, pH 7.0) to neutrality, and the final volume brought to 25 ml. 1.0 ml of this Streptomycin eluate II fraction was diluted with an equal volume of water (0°) and centrifuged for 2 hours at 40,000 r.p.m. in the Spinco number 40 rotor. The pellet suspended in 0.5 ml of glycylglycine buffer (0.02 *M*, pH 7.4) contained 88% of the enzyme, 47% of the protein and 27% of the optical density at 260 mμ (based on the Streptomycin eluate II fraction).

Stability of the enzyme to storage, heating and pH

Enzyme fractions stored for weeks at —13°, often with repeated thawing and refreezing, showed no signs of deterioration. Fractions Streptomycin eluate I and Ammonium sulfate II retested after 8 and 6 weeks, respectively, showed no detectable loss in activity. The enzyme was inactivated by heating. The Ammonium sulfate II fraction, after 5 minutes at 55°, lost 27% of its activity; after 5 minutes at 60°, 93% was lost. Treatment at 0° with acetate buffer at pH 4.0 for only 10 minutes destroyed

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over 90% of the activity of Ammonium sulfate II; 70% of the activity was lost at pH 5.0.

Progress of the reaction with time

The extent of the reaction was proportional with time during a 60-minute period. At 15, 30, and 60 minutes, under standard assay conditions, 0.045, 0.085, and 0.18 μM of acid-insoluble P, respectively were produced. With twice the amount of enzyme 0.38 μM was produced in 60 minutes, representing a conversion of about 76% of the acetyl P added. Studies with prolonged incubation or the use of more enzyme have not as yet significantly increased the extent of reaction beyond this point.

Influence of Mg^{++}, potassium arsenate, pH, ammonium sulfate, F^- and dinitrophenol on the reaction rate

When Mg^{++} was omitted from the standard assay system, only 1% of the activity was observed. The rates at $1 \cdot 10^{-3}$, $2 \cdot 10^{-3}$, $10 \cdot 10^{-3}$, and $16 \cdot 10^{-3} M$ were 59, 86, 115, and 95%, respectively, of that at $4 \cdot 10^{-3} M$. Since acetokinase requires Mg^{++} for activity, it was necessary to study the reaction in the absence of the ATP-regenerating system. The rate without added Mg^{++}, and without acetyl P or acetokinase present, was 10% of that observed with $8 \cdot 10^{-3} M$ Mg^{++}.

Potassium arsenate, at a level of 0.04 M, did not affect the rate.

The optimum pH of the reaction, determined in Tris buffers in the presence of a several-fold excess of acetokinase, was found to be at 7.2. The rates at pH 6.2, 6.7, 7.5, and 8.2 were 45, 75, 83, and 73%, respectively, of the rate at pH 7.2. At pH 7.2 the rate in phosphate buffer was about 25% slower than in Tris buffer. Ammonium sulfate, as well as other salts, stimulated the rate; its absence from the assay system resulted in a reduction of about 30%. Fluoride ($5 \cdot 10^{-3} M$) completely inhibited the enzyme; 2,4-dinitrophenol ($3 \cdot 10^{-4} M$) did not inhibit.

The terminal phosphate of ATP as the reactive group

With ATP containing $\overset{\bullet}{P}$ in the terminal P atom only (ARPPP$\overset{\bullet}{P}$), or with ATP containing the same amount of $\overset{\bullet}{P}$ in both the terminal and middle P atoms (ARPP$\overset{\bullet}{P}\overset{\bullet}{P}$), the same amount of labeled product was produced (Table II). A second demonstration that the middle phosphate of ATP makes no contribution to the product was the result obtained with ARPP$\overset{\bullet}{P}$P as the substrate; no $\overset{\bullet}{P}$ was detectable in the acid-insoluble phosphate product.

Stoichiometry of the reaction

In the absence of an ATP-regenerating system, the inhibitory action of the ADP formed (see section below) generally limited the extent of reaction to a conversion of only about 8% of the ATP. In a balance study (Table III, Expt. 1), the production of acid-insoluble P was matched by the disappearance of radioactivity in the acid-soluble fraction and the appearance of chromatographically separated ADP in equivalent amounts.

Balance studies in the presence of acetyl P and acetokinase to regenerate ATP (Table III, Expts. 2, 3) showed that the concentration of ATP (Norit-adsorbable radioactivity) remained essentially unchanged, while the amount of acid-insoluble

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TABLE II

TERMINAL PHOSPHATE OF ATP AS THE REACTIVE GROUP

Labeling of the substrate	$\overset{*}{P}$ *in the product* μM
ARPP$\overset{*}{\underset{*}{P}}$ (prepared by aerobic phosphorylation)	0.0121
ARPP$\overset{*}{\underset{*}{P}}$ (prepared by PP exchange)	0.0147
ARPP$\overset{**}{P}$	0.0131
ARPP$\overset{**}{P}$	< 0.0001

* Specific radioactivity calculated as c.p.m. per μM of ATP × ½.
** Specific radioactivity calculated as c.p.m. per μM of ATP.

The reaction mixtures contained 0.25 μM of ATP (of accurately determined specific radioactivity in the neighborhood of $2 \cdot 10^5$ c.p.m. per μM, labeled as stated above), and 0.10 ml Streptomycin eluate II in the standard assay mixture from which the ATP-regenerating system was omitted.

TABLE III

BALANCE STUDIES OF THE REACTION

Expt. No.	*Fraction*	*Control*		*Experimental*		△	
		c.p.m.	μM	*c.p.m.*	μM	*c.p.m.*	μM
1	Acid-insoluble	300		54,200		+53,900	
	Acid-soluble						
	("ATP + ADP")	940,000		880,000			
	ADP	12,100		74,500		+62,400	
2	Acid-insoluble	220	0.000	122,000	0.168		+0.168
	Acid-insoluble		0.059*		0.255*		+0.196
	Acid-soluble						
	Norit-adsorbable ("ATP")	241,000	0.332	236,000	0.325		
	Norit-nonadsorbable ("Acetyl-P")	237,000	0.326	94,000	0.129		−0.197
3	Acid-insoluble	500	0.04	152,500	1.28		+1.24
	Acid-insoluble		0.19*		1.53*		+1.34
	Acid-soluble	1,160,000		1,010,000			
	Norit-adsorbable ("ATP")	341,000		350,000			
	Acetyl-P		5.08		3.83		−1.25

* Determined as orthophosphate after hydrolysis in 1 N HCl (100°, 7 minutes).

In the control experiments the enzyme was omitted during the incubation period (30 minutes) and added after the reaction was stopped with perchloric acid.

In Expt. 1, the incubation mixture contained 0.40 ml of ARPP$\overset{**}{P}$ (0.0023 M, $1.6 \cdot 10^6$ c.p.m. per μM), 0.10 ml of glycylglycine buffer (0.5 M, pH 7.0), 0.01 ml of $MgCl_2$ (0.1 M), 0.04 ml of ammonium sulfate (1 M), 0.08 ml of Ammonium sulfate III, and water to a volume of 1.0 ml. The acid-insoluble P fraction was obtained as in the standard assay procedure; the acid soluble fraction was made up of the combined perchloric-acid supernatants. An aliquot, to which unlabeled carrier ADP was added, was chromatographed on a Dowex-1 column (Cl^- form, 2% cross-linked, 5 cm in height, 1 cm in diameter). The ADP fraction appeared between 7 and 14 resin-bed volumes of eluent (0.01 N HCl, 0.04 M KCl).

In Expt. 2, the standard assay system with 0.02 ml of Streptomycin eluate II was used.

In Expt. 3, the incubation mixture contained 0.42 ml of ARPP$\overset{*}{P}$ (0.008 M, $1.2 \cdot 10^5$ c.p.m. per μM), 0.125 ml of acetyl $\overset{*}{P}$ (0.044 M, $1.2 \cdot 10^5$ c.p.m. per μM), 0.03 ml of acetokinase, 0.28 ml of Ammonium sulfate III, 0.35 ml of glycylglycine buffer (0.5 M, pH 7.0), 0.28 ml of $MgCl_2$ (0.1 M), 0.14 ml of ammonium sulfate (1 M), and water to a volume of 3.50 ml.

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P formed, equalled the amount of acetyl P removed (estimated chemically or as a Norit nonadsorbable fraction).

In order to determine whether any acid-soluble intermediates were produced, the acid-soluble fraction (Expt. 3) was chromatographed on an anion-exchange resin. The fraction was adsorbed on a Dowex-1 column (Cl^-, 2% cross-linked, 5.0 cm in height, 1 cm in diameter) and eluted first with 0.01 *N* HCl – 0.04 *M* KCl, and then with 0.02 *N* HCl – 0.15 *M* KCl. With the first eluent, 91% of the acetyl P calculated to be present was recovered in an orthophosphate fraction (3 to 8 resin-bed volumes), and with the second, 92% of the calculated ATP content was recovered (3 to 9 resin-bed volumes). The ADP area (9 to 14 resin-bed volumes of the first eluent) contained less than 1% of the radioactivity found in the ATP area. No radioactivity appeared in the effluent beyond ATP, even up to 38 resin-bed volumes. The column of resin, when extruded and counted as 7 separate sections, contained, evenly distributed among them, 0.15% of the radioactivity found in the acid-insoluble P fraction. While the significance of this small amount of radioactive material is questionable, it is clear that low molecular weight polyphosphates were not present in any appreciable amounts.

Influence of ATP and ADP on the reaction rate

ADP is a very strong inhibitor of the reaction (Table IV), preventing any detectable reaction at a level of $8 \cdot 10^{-5} M$, when the ATP concentration was 6-fold higher. Even at an ATP:ADP ratio of 24 the inhibition was 57%. Since attempts to demonstrate reversibility of the reaction have thus far been unsuccessful, the inhibition by ADP is apparently not due to its reversing the reaction. With an ADP-phosphorylating system present, a clear dependence of the reaction rate on ATP concentration was observed. From a LINEWEAVER AND BURK[25] plot, a dissociation constant (K_m) of $1.4 \cdot 10^{-3} M$ was calculated. In the absence of added ATP, no reaction was detectable.

The strong inhibitory action of ADP and the rather high K_m for ATP suggested the possibility that some nucleoside diphosphate impurity in the ATP might be the reactive component. However, crystalline ATP preparations were as reactive as amorphous ones in producing acid-insoluble phosphate.

TABLE IV

INFLUENCE OF ATP AND ADP ON THE REACTION RATE

ATP, $M \cdot 10^3$	0.48	0.48	0.96	0.96	1.92	1.92	3.84	3.84	5.76	5.76
ADP, $M \cdot 10^3$	0	0.08	0	0.08	0	0.08	0	0.08	0	0.08
Rate, mμmoles	7.5	0.0	12.0	0.7	16.0	6.9	21.0	12.4	17.4	12.3
Inhibition, %		100		94		57		42		29

The rate is expressed as mμmoles of acid-insoluble P produced in the 15-minute incubation. The mixture contained ARPPP (1.1 · 10^6 c.p.m. per μM), 0.02 ml of the Ammonium sulfate III fraction, and the other components of the standard assay system except acetyl P and acetokinase.

Incorporation of $\dot{P}\dot{P}$ into acid-insoluble phosphate

Incorporation of $\dot{P}\dot{P}$ into acid-insoluble phosphate was observed consistently (Table V, Expts. 1, 2a, 2b, 4a, 4d, 5); this radioactivity was equivalent to one PP residue for

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every 2,000 to 4,000 P residues in the product. This incorporation could be prevented by preincubation of the $\overset{\bullet\bullet}{PP}$ with PPase (Expt. 4b), or obscured by dilution with unlabeled PP (Expt. 2b). The product of the reaction was not degraded by PPase to any significant extent (Expt. 3b, 4c). (The production of acid-insoluble phosphate from ARPP$\overset{\bullet}{P}$ under standard assay conditions was not inhibited by PPase (<5%) and PP at the level used in this experiment (0.0016 *M*) produced only a 20% inhibition.)

TABLE V

INCORPORATION OF PP INTO METAPHOSPHATE

Expt. No.	*Additions*	$\overset{\bullet\bullet}{PP}$ *incorporation into the*					
		Acid-insoluble phosphate fraction			*ATP fraction*		
		Exptl. c.p.m.	*Control c.p.m.*	*Specific radioactivity c.p.m./μM P*	*Exptl. c.p.m.*	*Control c.p.m.*	*Specific radioactivity c.p.m./μM terminal P*
1	None	475	9	3320	308	43	630
2a	None	364	40		350	85	
b	PP (0.4 μ*M*)	40	54		74	34	
3a	None	640	72	4230	405, 350	273, 283	765, 665
b	PPase (10γ) 25 min later	506	0				
4a	None	900	174	5600	220	66	410
b	PPase (10γ) 5 min before	0	0		3	14	
c	PPase (10γ) 25 min later	628					
d	None	670	62		244	65	
5	Amounts of $\overset{\bullet\bullet}{PP}$ varied:						
a	0.01 μ*M* $\overset{\bullet\bullet}{PP}$	322	12				
b	0.02 μ*M* $\overset{\bullet\bullet}{PP}$	684	22				
c	0.04 μ*M* $\overset{\bullet\bullet}{PP}$	1190					

The incubation mixtures contained 0.03 ml of ATP (0.01 *M*), 0.02 ml of phosphopyruvate (0.025 *M*), 0.02 ml of $\overset{\bullet\bullet}{PP}$ (0.001 *M*, $1.3 \cdot 10^7$ c.p.m./μ*M*), 0.025 ml of glycylglycine buffer (0.5 *M*, pH 7.0), 0.01 ml of $MgCl_2$ (0.1 *M*), 0.01 ml of ammonium sulfate (1 *M*), 0.01 ml of pyruvate phosphokinase, 0.03 ml of the Streptomycin eluate II fraction (except in Expt. 4-d, in which 0.02 ml of Spinco residue fraction was used) and water to a volume of 0.25 ml. The time of incubation was 30 minutes, except in Expt. 2 when it was 15 minutes. The acid-insoluble metaphosphate fraction was the washed acid-insoluble precipitate. The ATP was estimated by treating the acid-soluble supernatant fluid and wash, combined, with 0.1 ml of Norit suspension (10% dry weight), washing the Norit with water, and measuring the radioactivity of the Norit suspended in water. In the control, the enzyme was added after the addition of perchloric acid at the end of the incubation period.

In these experiments a small amount of radioactivity was found in ATP. This radioactivity was distributed equally between the two terminal phosphates, as shown by the equal distribution of counts between ADP and glucose-6-phosphate when the ATP was converted to these products by the use of hexokinase. However, the specific radioactivity of the terminal P of the ATP, even at the end of the incubation, was in no case more than one-fifth that of the acid-insoluble phosphate fraction, thus showing that the $\overset{\bullet\bullet}{PP}$ in the product could not have arisen from ATP except to a minor extent.

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It may be noted that the incorporation of $\overset{\bullet\bullet}{PP}$ increased proportionately with the concentration of PP in the range of $4 \cdot 10^{-5} M$ to $1.6 \cdot 10^{-4} M$ (Expt. 5). Neither P_i, when added to the incubation mixture, nor the adenine of ATP was detected in the product. In two experiments in which the specific radioactivity of the $\overset{\bullet}{P}_i$ was the same as that of the $\overset{\bullet\bullet}{PP}$ used in Table V, the radioactivity incorporated into the acid-insoluble phosphate fraction did not exceed the control value (enzyme omitted). Also in one experiment with 8-^{14}C adenine-labeled ATP ($3 \cdot 10^5$ c.p.m. per μM), the radioactivity in the product was 15 c.p.m. in both the control and experimental reactions. With ARPP$\overset{\bullet}{P}$ of one-third the specific radioactivity in an otherwise identical reaction mixture the control and experimental values were 10 and 8100 c.p.m. respectively. These values indicate that incorporation of ^{14}C-ATP, were it demonstrable with more strongly labeled material, would be less than one residue per 3000 P residues.

Properties of the product

The product was prepared by use of reaction mixtures 5–10 times the scale of the standard assay mixture, with incubation periods of 30 or 60 minutes. The perchloric acid-washed precipitate, sometimes washed with cold water, was dissolved in one of two ways: (1) water was added and dropwise 0.1 *N* KOH to bring the pH to neutrality; at this point the precipitate was completely dissolved; or (2) cold saturated ammonium sulfate (adjusted to pH 8.25 with ammonium hydroxide) was added, bringing the precipitate readily into solution. The phosphate product has been termed a metaphosphate on the basis of the following properties: (1) induces metachromasy; (2) is nondialyzable; (3) forms an acid-insoluble complex with protein; (4) is labile to acid; (5) is labile to alkali; (6) binds tenaciously to anion exchange resins, and (7) is not attacked by nucleases.

(1) *Metachromasy.* The enzymic metaphosphate added to toluidine blue (Toluidine Blue 0, National Aniline and Dye, 30 mg per liter), following the procedure of WIAME[8], converted the color of the dye from blue to pink, and produced a marked shift in absorption to the shorter wavelengths (Table VI). The change in the optical density ratio 530 mμ/630 mμ was within a narrow range (at ratios of 0.5 to 2.0) roughly proportional to the metaphosphate concentration.

TABLE VI

METACHROMASY OF ENZYMIC METAPHOSPHATE

Enzymic metaphosphate μM *P/ml*	*Optical density*		
	530 mμ	*630 mμ*	*Ratio* $\frac{530\ m\mu}{630\ m\mu}$
0.0000	0.244	0.985	0.247
0.0054	0.302	0.895	0.337
0.0108	0.330	0.860	0.384
0.0216	0.429	0.458	0.935
0.0432	0.508	0.236	2.15
0.0864	0.511	0.209	2.44

The enzymic metaphosphate was obtained from a 60-minute incubation of the standard assay mixture. The perchloric acid-washed precipitate was washed once with 0.01 *N* HCl before it was dissolved in water by adjusting the pH of the solution to 7 to 8 with KOH.

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A 530 mμ/630 mμ ratio of 0.94 was obtained at a P residue concentration of $2.2 \cdot 10^{-5} M$ (Table VI); WIAME[8], working with synthetic metaphosphate, found a ratio of 1.24 at a P residue concentration of $2.4 \cdot 10^{-4} M$; with an acid-soluble yeast metaphosphate fraction (prepared according to WIAME[26]) at a concentration of $4.7 \cdot 10^{-5} M$, the ratio was 0.86. It appears then that the enzymically formed metaphosphate is at least as effective in inducing metachromasy as samples of metaphosphate synthetically prepared or isolated from a natural source.

(2) *Nondialyzability.* A solution of the product was passed through a cation exchange resin (Dowex-50), and dialyzed for 22 hours in the cold against distilled water; of 17,500 c.p.m. at the start of dialysis, 16,100 c.p.m. were recovered in the cellophane dialysis sac. In another trial, a 2 ml solution of the enzymic metaphosphate at 3° was brought to 85% saturation with solid ammonium sulfate (pH about 5) and the precipitated protein was removed. The supernatant fluid, containing 38,800 c.p.m., was dialyzed for 20 hours against two 5-liter quantities of distilled water at 3°; 35,200 c.p.m. were recovered from the sac.

(3) *The acid-insoluble metaphosphate-protein.* The metaphosphate product, in the presence of protein in the incubation mixture, was precipitated by the addition of acid. The metaphosphate-protein was soluble in cold water at neutral pH and in cold, saturated ammonium sulfate of pH 8.25. It could be quantitatively reprecipitated from neutral solution by acetate buffer at pH 4; it was 75% precipitable at pH 5 (acetate buffer), but remained completely soluble at pH 6 (phosphate bufler), It was only partly soluble in cold, saturated ammonium sulfate at pH 5, while cold, half-saturated ammonium sulfate (pH 5) dissolved 90%. Calcium and zinc salts partially dissolved the metaphosphate-protein product; barium acetate (0.05 *M*) did not.

(4) *Lability in acid.* The phosphate in the acid-insoluble product was readily and completely hydrolyzed to P_i. A sample that contained 11,500 c.p.m.* was heated with 1 *N* HCl in a boiling-water bath for 6 minutes and, after cooling and neutralization, mixed with 20.8 μ*M* of KH_2PO_4 and chromatographed on Dowex-1 resin (chloride form, 2% cross-linked, 2 cm in height, 1 cm in diameter). 21.2 μ*M* of P_i and 11,200 c.p.m. were eluted between 10 and 20 resin-bed volumes of eluent (0.01 *N* HCl).

(5) *Lability in alkali.* After treatment with alkali (0.1 *N* NaOH, or 0.1 *N* NH_4Cl buffer, pH 10.2) at 37° for 45 minutes, 22–26% of the acid-insoluble P was no longer precipitable by acid. At 100°, treatment for 4–8 minutes at these pH levels rendered 82–86% of the P no longer acid insoluble. The major products of alkaline hydrolysis have not been determined, but on the basis of their elution from Dowex-1 resin (eluted by 1 *N* HCl, but not by 0.025 *N* HCl–0.1 *M* KCl), they appear to be polymers longer than triphosphate and shorter than the starting material.

(6) *Binding to resins.* The metaphosphate compound was held fast by the anion-exchanger Dowex-1, 10% cross-linked. A sample containing 100,000 c.p.m. was adsorbed on a column of this resin (Cl^-, 10 cm in height, 1 cm in diameter), and eluted first with 0.25 *M* KCl (pH 7.28), which readily removes P_i, PP, and tripoly- and trimetaphosphate[27]. One liter of eluent removed only 5,000 c.p.m., and subsequent elutions with 500 ml of 1 *M* KCl and 20 ml of 2 *M* KCl removed 20,000 c.p.m. When the column of resin containing 75% of the radioactivity was then extruded and cut up, the bulk of the radioactivity was found in the topmost 0.8 cm.

After adsorbing a sample on a column of resin 2 cm high, and then passing

* Approximately 0.2 μmole P.

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through 450 ml of 0.5 *M* potassium salicylate, 40% of the radioactivity was eluted 30% was then removed with 10 ml of 20% $CaCl_2$ and 30% was still bound.

Ecteola resin* also retained the metaphosphate after attempts to elute it with 1 *M* KCl and with 2 *M* $ZnSO_4$, eluents which readily remove nucleic acids.

The cation exchanger Dowex-50 did not retain the $\dot{P}$ product. When 1 ml of solution containing 60,000 c.p.m. was passed through a Dowex-50 column, 53,400 c.p.m. were recovered.

In an attempt to study the behavior of the metaphosphate by paper electrophoresis, the material remained at the origin and, furthermore, could not be removed by elution with water or with 20% $CaCl_2$.

(7) *Resistance to nucleases.* In an experiment in which the dissolved product (5200 c.p.m., 0.11 μmole P) was treated for 15 minutes at 37° with ribonuclease (25γ) and desoxyribonuclease (9γ), reprecipitation with acid left behind a solution with 170 c.p.m. (3%). In a control experiment without the nucleases, 214 c.p.m. (4%) remained unprecipitated.

DISCUSSION

While many aspects of the chemical structure of metaphosphate are still unknown, there has been progress in recent years in eliminating a number of incorrect notions. It has become clear that the only characterized and authentic metaphosphates are the tri- and tetrametaphosphates[4,28]. What has been called hexametaphosphate (Graham's salt) is now recognized as a mixture of polyphosphates of varying chain lengths, preparations of average chain lengths from 85 to 230 phosphate groups having been described[29]. The development of techniques for obtaining homogeneous preparations of a polyphosphate and for its accurate analysis are obviously essential for further progress.

In the present work only a gross and qualitative identification of the enzymically synthesized metaphosphate has been attempted. It has been shown to be a nondialyzable, phosphorus-rich substance which yields inorganic orthophosphate on acid hydrolysis and is effective in inducing metachromasy. The fact that it is quantitatively precipitated by albumin at acid pH suggests that it is a polymer of very long chain length. KATCHMAN AND VAN WAZER[29] examined a number of synthetic polymers and observed quantitative precipitability by albumin only with a preparation which reached an average chain length of 1,600 P residues.

A remarkable feature of the enzymic synthesis is the formation of such long chain molecules without the production in detectable amounts of short or intermediate chain length polymers. There is thus an analogy with the synthesis of glycogen, fatty acids, nucleic acids and protein in which low molecular weight intermediates rarely, if ever, accumulate. Unfortunately, we have no information as yet on the need for, or identity of, a primer. While PP can be incorporated into metaphosphate by the enzyme, the results do not suggest its function as the true primer. Thus many questions remain regarding the initiation and also the progress of metaphosphate synthesis.

Reports of large amounts of metaphosphate in a variety of cells have led to considerable speculation regarding the physiologic function of metaphosphate. Anaerobiosis, phosphate starvation followed by exposure to phosphate, poisons and

* Kindly supplied by Dr. HERBERT SOBER.

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unfavorable media have been shown to favor accumulation of metaphosphate; a source of energy for the cell has been found essential. A hypothesis, already suggested[30], and in keeping with the enzymic mechanism thus far elucidated, is that metaphosphate production supervenes in the absence of effective utilization of ATP for protein, nucleic acid, and other major synthetic processes. The function and disposition of the accumulated metaphosphate are not clear. It may serve as a reservoir of phosphate or of energy, or may function in some specific reactions. While the reversibility of the reaction with the purified enzyme described in this report was not demonstrable, this may be due to experimental inadequacies rather than an inherent property of the system. For example, the metaphosphate, being a large polyanionic polymer, may be complexed in forms that are not enzymically reactive. The demonstration that the product can induce metachromasy was made only after impurities and complexing substances were eliminated. The phosphoanhydric nature of the linkages in the metaphosphate and reported statements of reversibility with other systems suggest that further work with the *E. coli* enzyme may be fruitful.

The observation has been made by WIAME[26] and JUNI *et al.*[31] that there are two physically and metabolically distinct metaphosphate fractions in yeast cells, one extractable and the other not extractable by acid. KATCHMAN AND VAN WAZER[29] have shown that only very high molecular polyphosphates, in the presence of protein, are completely precipitated by acid and this would suggest that the acid-soluble fraction obtained from cells is of relatively lower molecular weight. Since we have found that acid-insoluble metaphosphate is formed from ATP without the accumulation of detectable amounts of acid-soluble metaphosphate, a separate pathway of acid-soluble metaphosphate production is likely. This is indicated also by the studies of JUNI *et al.* that resting yeast cells incorporated labeled orthophosphate in the acid-insoluble, but not in the acid-soluble, metaphosphate. It is possible that these yeast cultures consisted of a heterogeneous population of which some cells were actively synthesizing acid-insoluble metaphosphate, while others in a phase of decline were breaking down old stores of the acid-insoluble material to the acid-soluble level.

The purification of the *E. coli* enzyme was carried to a point where its content of protein and nucleic acid, from the standpoint of furnishing substrate, were negligible when compared to the molar quantities of metaphosphate it formed. The purity of this preparation is unknown and may prove to be rather low. Of interest in the purification procedure is that after treatment with nucleases the protein was insoluble in water. Prior to this the enzyme activity was either soluble in water or was sedimented only after centrifugation at high speeds. The possibility of K^+ activation of the enzyme system deserves investigation in view of the interesting findings of SCHMIDT *et al.*[32] of a K^+ requirement for metaphosphate accumulation by yeast cells.

SUMMARY

1. An enzyme that synthesizes metaphosphate has been purified more than 100-fold from extracts of *Escherichia coli*. The reaction may be described by the equation:

$$x\ \text{ATP} + \left[\begin{matrix}(PO_3^-)_n \\ \text{primer}\end{matrix}\right] \longrightarrow x\ \text{ADP} + (PO_3^-)_{n+x}$$

2. Only the terminal phosphate group of ATP was found in the polymer. Since ADP, the other product of the reaction, proved to be a very potent inhibitor, an ATP-regenerating system was generally coupled to the reaction.

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3. Inorganic pyrophosphate (PP), but not inorganic orthophosphate or adenine nucleotide, was incorporated into metaphosphate. However, the lack of a requirement for PP and the absence of any inhibition by PPase leaves the nature of the true primer $(PO_3^-)_n$ in doubt.

4. The phosphate polymer has been characterized as a long-chain metaphosphate because it (a) induces metachromasy, (b) forms an acid-insoluble complex with protein, (c) is nondialyzable, (d) is labile to acid and alkali, (e) binds tenaciously to anion exchange resins, and (f) is not attacked by nucleases.

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