Synthesis of High Molecular Weight Polyphosphate with a Partially Purified Enzyme from *Salmonella*

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SUMMARY

Polyphosphate (polyP) kinase was prepared from EDTA-lysozyme spheroplasts of a *Salmonella minnesota* rough mutant. The enzyme, purified about 80-fold, was free of ATPase and polyP-degrading activity and contained neither nucleic acids nor polyP which might act as primer. Using this enzyme, ^{32}P -labelled polyP was prepared from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The polyP was identified by the action of degrading enzymes, phenol extraction, adsorption characteristics on charcoal and hydrolysis kinetics.

Sedimentation data in sucrose gradients ($S' = 11.5$) and gel filtration on agarose indicated a molecular weight in the range 300,000 to 400,000 daltons.

INTRODUCTION

Enzymes synthesizing polyP have been detected in many biological systems ranging from bacteria to mammalian cell nuclei (for extensive reviews see Kuhl, 1960; Harold, 1966), but to our knowledge only two bacterial enzymes have been extensively purified, one from *Escherichia coli* (Kornberg, Kornberg & Simms, 1956), and the other from *Corynebacterium xerosis* (Muhammed, 1961).

Several reports have mentioned the high molecular weight of the polyP encountered in bacteria (Kornberg *et al.* 1956), yeast (Liss & Langen, 1960), slime moulds (Sauer, Babcock & Rusch, 1969), moulds (Ingelman & Malmgren, 1950), and blue-green algae (Correll, 1964). However, most isolation procedures (except those of Sauer *et al.* (1969) and of Ingelman & Malmgren (1950)) involve extraction steps with TCA or NaOH which will certainly degrade long-chain polyP to an extent that would make any estimate of the molecular weight unreliable. A better method of obtaining undegraded polyP is therefore by enzymatic synthesis using a purified enzyme.

During our studies on the biosynthesis of cell-wall constituents of *Salmonella* bacteria (Mühlradt, 1971) we encountered in protein extracts of these bacteria enzyme-catalysed transfer of phosphate from ATP to a substance which we have named X-P. This communication reports that X-P is polyphosphate of very high molecular weight.

METHODS

Reagents. The following reagents and enzymes were purchased from C. F. Boehringer & Söhne (Mannheim, Germany): ATP, ADP, phosphoenolpyruvate, NADH, pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27) from pig heart, egg-white lysozyme (EC 3.2.1.17), RNase from pancreas, trypsin (EC 3.4.4.4) from pancreas, and inorganic pyrophosphatase (EC 3.6.1.1) from yeast. DNase was purchased from Serva Entwick-

lungslabor (Heidelberg, Germany); *Escherichia coli* type III alkaline phosphatase (EC 3.1.3.1) was obtained from Sigma Chemicals Co. (St Louis, Missouri, U.S.A.). [32 P]ATP was prepared according to Glynn & Chappel (1964). The tRNA and ribosomal RNA used in the sucrose gradients as internal markers were prepared from EDTA-lysozyme spheroplasts which were lysed with Na dodecylsulphate (SDS). The preparation was extracted with phenol at room temperature, and the RNA was precipitated with ethanol from the aqueous phase.

Paper electrophoresis and autoradiography. High-voltage paper electrophoresis was carried out at 60 V/cm. in a buffer of pyridine + acetic acid + water (10:4:86, by vol.), pH 5.5. Radioactive zones were located on ADOX Doneo X-ray film, clear base.

Disc electrophoresis. Acrylamide-gel disc electrophoresis was carried out by the procedure of Davis (1964). No sample gel was used, but the samples were mixed with a small amount of glycerol and applied directly to the small pore gel. The electrophoresis was performed in the cold room and for efficient cooling of the electrophoresis tubes cold air was blown through the apparatus.

For determination of radioactive zones the gel was carefully removed from the tubes and cut in small sections. The sections were dissolved in the following scintillation fluid which was used throughout the experiments reported here: toluene-triton X-100 (2:1, v/v) containing 4% 'Omnifluor' (98% 2,5-diphenyl-oxazole plus 2% bis(*O*-methyl-steryl)benzene). Radioactivity was determined in a Packard liquid scintillation spectrometer.

Protein estimations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), bovine serum albumin (BSA) serving as standard.

Sucrose gradient centrifugation. Sucrose gradient centrifugation was performed in a Beckman Spinco model L 2-65 B ultracentrifuge. PolyP samples (100 μ l.) containing about 5 n-equiv. of Pi (20,000 c.p.m.), with tRNA and ribosomal RNA from *Salmonella* as internal markers, were layered on top of a 30 to 8% (w/w) sucrose gradient in standard citrate buffered saline (0.15 M-NaCl + 0.015 M-citrate, pH 7.0). Usually, 4 h. runs were carried out at 5° and 50,000 rev./min. (280,000 *g* average) in a SW 56 rotor. The polyallomer tubes were punctured at the bottom and samples corresponding to 2 mm. segments were withdrawn. The samples were diluted with tris buffer, pH 7.0, and the extinction at 260 nm. measured. Either the whole fractions or portions thereof were then assayed in a scintillation counter.

Organisms and culture. *Salmonella minnesota* mR5 is a rough mutant with a defective heptose phosphorylating enzyme (Mühlradt, 1969); it was isolated by J. Schlosshardt (Zentrallaboratorium für bakterielle Darminfektionen, Berlin-Pankow, D.D.R.). Cultures were grown in 1 l. batches in Difco antibiotic medium 3 and harvested in the late exponential growth phase. The organisms were washed twice with 0.01 M-tris-chloride buffer, pH 8.0, and kept at -20°.

Preparation of enzyme. Protein extracts were prepared by repeated washing of EDTA lysozyme spheroplasts with 0.01 M-tris-Cl, pH 8.0, as previously described (Mühlradt, 1969). The extracts (usually from 5 to 6 g. wet wt bacteria) were centrifuged at 150,000 *g* for 3 h. The enzyme was exclusively in the sediment which was suspended in a few ml. 0.1 M-tris-sulphate buffer, pH 8.0, 1 mM-EDTA, 0.5 mM-2-mercaptoethanol buffer.

RNase, ammonium sulphate extraction step: after addition of 30 μ g. RNase/ml. the suspension was incubated at 25° for 20 min. The polyP kinase activity sedimented now at 1000 *g* in 10 min. The sediment was twice extracted at 25° with 2 ml. 0.02 M-tris-sulphate, pH 8.0, 0.2 M-(NH₄)₂SO₄, 1 mM-EDTA, 0.5 mM-2-mercaptoethanol buffer. The polyP kinase activity was in the extract.

Ammonium sulphate step: the extract was made 2 M in $(\text{NH}_4)_2\text{SO}_4$ and the protein precipitate containing the polyP kinase was obtained upon centrifugation. The sediment was dissolved in a minimum of 0.02 M-tris-sulphate, pH 8.0, 0.2 M- $(\text{NH}_4)_2\text{SO}_4$, 1 mM-EDTA, 0.5 mM 2-mercaptoethanol buffer.

DEAE-cellulose step: the fraction from the ammonium sulphate step was diluted to 0.05 M- $(\text{NH}_4)_2\text{SO}_4$ with 0.01 M-tris-sulphate buffer, pH 8.0, and immediately applied to a DEAE-cellulose column containing 10 ml. resin. The column was eluted with a 0 to 0.32 M- $(\text{NH}_4)_2\text{SO}_4$ gradient in 0.01 M-tris-sulphate buffer, pH 8.0. PolyP kinase was eluted in one peak at 0.15 M- $(\text{NH}_4)_2\text{SO}_4$. The active fractions were pooled and concentrated on a Sartorius protein filter no. 15100.

Enzyme assay. Precipitation test (Kornberg, 1956): the incubation mixture (250 μl .) was 0.1 M-tris-sulphate buffer, pH 8.0; 0.1 M- $(\text{NH}_4)_2\text{SO}_4$; 0.04 M- MgSO_4 ; 0.01 M- $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, specific activity 1000 to 10,000 c.p.m./nmole, and it contained 0.1 to 1×10^{-3} units polyP kinase.

After 30 min. incubation at 25°, 40 μl . 1.5 % BSA solution containing 2.5 μg ./ml. RNA was added as carrier, and the polyP was precipitated with 250 μl . 15 % trichloroacetic acid (TCA) in 0.1 M- KH_2PO_4 and centrifuged. The precipitate was dissolved in 0.1 N-NaOH and reprecipitated with TCA solution. The precipitate was washed again with 250 μl . TCA solution and was finally dissolved in 100 μl . 100 % HCOOH. Its radioactivity was counted in a scintillation spectrometer. Control mixtures without enzyme contained about 0.3 nmole $[\gamma\text{-}^{32}\text{P}]\text{phosphate}$. An enzyme unit is defined as the amount of enzyme which incorporates 1 μmole of TCA-precipitable $[\gamma\text{-}^{32}\text{P}]\text{phosphate}$ /min. under the above conditions.

Spectrophotometric assay: the incubation mixture (300 μl .) contained 0.1 M-tris-sulphate buffer, pH 8.0; 0.1 M- $(\text{NH}_4)_2\text{SO}_4$; 0.04 M- MgSO_4 ; 0.01 M-ATP; 0.33 mM-phosphoenolpyruvate; 0.33 mM-NADH, with 8 μg . lactate dehydrogenase, 3.5 μg . pyruvate kinase, and about 10^{-3} units of polyP kinase. The decrease in extinction at 340 nm. was followed for 30 min. at 25°. The reaction rate remained linear with time for more than 30 min.

Preparation of ^{32}P -labelled polyP. A sample (0.75 ml.) of the assay mixture described above was incubated for 45 min. at 25°. The mixture was filtered through a 0.9×22 cm. Sephadex G-75 column with 0.01 M-tris buffer, pH 8.0, as eluant. PolyP was eluted in the exclusion volume and was thus separated from the remaining ATP.

RESULTS

Purification of the enzyme. The crude protein-containing extract was prepared by repeated washing of cells which had been partially lysed by EDTA and lysozyme (Mühlradt, 1959). This procedure was more time-consuming than ultrasonication, but yielded a preparation of higher specific activity. The further purification of the enzyme was somewhat similar to the procedure of Kornberg *et al.* (1956) except that the last step employed chromatography on DEAE-cellulose. When the RNase step was omitted, a second peak of polyP kinase activity in addition to the one eluted at 0.15 M- $(\text{NH}_4)_2\text{SO}_4$ was eluted at 0.3 M-salt. RNase treatment resulted in the loss of this second peak of activity and a concomitant increase of activity in the first peak. The purification steps are outlined in Table 1. The polyP kinase obtained after the DEAE-cellulose chromatography contained neither polyP nor nucleic acids. These substances were eluted from the column at considerably higher salt concentrations than the enzyme. Activity determinations on this enzyme preparation using the precipitation test and the spectrophotometric assay under identical conditions – i.e. under ATP-regenerating conditions – gave the same results. Any ATPase activity would have resulted

in seemingly higher activities in the spectrophotometric assay. No polyPase activity could be detected in this enzyme preparation.

Properties of the enzyme. The *Salmonella* polyP kinase appeared to be very similar to the *Escherichia coli* enzyme described by Kornberg (1956). It was soluble when nucleic acid was present and sedimented only upon ultracentrifugation for 3 h. at 150,000 g. After

Fig. 1. ADP-inhibition of polyP kinase activity. Incubation mixture (250 μ l.) contained: 100 mM-tris-HCl, pH 8.0; 40 mM-MgCl₂; 1.6 mM-[³²P] _{γ} -ATP (sp. act. 8500 c.p.m./nmole); 12.5 μ g. enzyme protein, and ADP as indicated. Incubation time: 30 min. at 25°.

Table 1. *Purification procedure of polyP kinase*

Preparation	Volume (ml.)	Protein (mg./ml.)	Activity (units/ml. $\times 1000$)	Spec. activity (units/mg. protein $\times 1000$)	Yield (%)	Purification (-fold)
EDTA-lysozyme lysate	50	0.8	0	0	—	—
Tris-extract	100	0.8	0.47	0.6	100	1
150,000 g Sediment	4	5.2	10.0	1.93	86	3
(NH ₄) ₂ SO ₄ —extraction after RNase treatment	2.8	3.2	19.0	5.94	114	10
(NH ₄) ₂ SO ₄ —fractionation	4	0.4	6.1	15.2	51	25
DEAE-cellulose eluate after concentration	1.7	0.08	3.7	46.6	14	78

RNase treatment, which rendered the polyP kinase insoluble, the enzyme could be redissolved in 0.2 M-ammonium sulphate. The enzyme is activated by potassium and ammonium sulphate, but not by ammonium chloride. Both *Escherichia coli* and *Salmonella* polyP kinases were inhibited by ADP (Fig. 1). The K_m value for ATP determined by the spectrophotometric assay was $(1.25 \pm 0.1) \times 10^{-3}$ M.

Identification of the reaction product as polyphosphate. Prior to identification, the reaction product formed on incubation of ATP with extract was named X-P. The high molecular

nature of X-P soon became apparent because it could be co-precipitated with BSA and separated from ATP by gel filtration on Sephadex G 200.

Action of degrading enzymes on X-P. Samples of X-P prepared enzymatically, containing 0.5 n-equiv. phosphate (5000 c.p.m.), were incubated with deoxyribonuclease, ribonuclease, inorganic pyrophosphatase, trypsin or alkaline phosphatase at 30° for 5 min., while a control sample was incubated in 0.05 M-tris buffer, pH 7.5. Sucrose gradient centrifugation and paper electrophoresis indicated some degradation with alkaline phosphatase but none with trypsin, like the control. Pyrophosphatase degraded the X-P to yield products too small to sediment in the sucrose gradient but of too high molecular weight to migrate in the electrophorogram. Deoxyribonuclease and ribonuclease had the strange effect of increasing the adsorption of the radioactive material to the glass test tubes. However, no degradation was noted.

Phenol extraction, adsorption on charcoal. A sample of X-P containing 57 n-equiv. of phosphate (250,000 c.p.m.) in 250 μ l., 1 mM-EDTA, 0.01 M-tris buffer, pH 8.0, containing 1 % SDS, was extracted three times with an equal volume of buffer-saturated phenol at 5°. All of the radioactivity remained in the aqueous phase. The material was undegraded according to sucrose gradient centrifugation and gel filtration on Sephadex 4B.

Muller-Felter & Ebel (1962) separated polyP and nucleic acids by selective adsorption on charcoal which, with 0.5 M-NaCl, selectively adsorbs high molecular weight substances such as proteins, lipids, polysaccharides, nucleic acids but not polyP. X-P containing 50 n-equiv. phosphate (200,000 c.p.m.) was therefore mixed with 125 μ g. yeast RNA and 55 μ g. commercial polyP (degree of polymerization about 8) as carrier, made up to 0.5 ml. in 0.5 M-NaCl and shaken with 10 mg. charcoal for 5 min. at room temperature. The charcoal was removed by centrifugation and the supernatant solution was assayed for radioactivity and for nucleic acid. Ninety-eight % of the nucleic acid had been adsorbed but 84 % of X-P was still present in the supernatant. Again no degradation of X-P had taken place.

Kinetics of hydrolysis in 0.1 N-HCl at 60°. X-P containing 90 n-equiv. of phosphate (200,000 c.p.m.) was hydrolysed in 3 ml. 0.1 N-HCl at 60°. Samples (250 μ l.) were withdrawn at various times and immediately frozen at -80°. Residual undegraded X-P was assayed by (a) precipitation with 7 % TCA after addition of 500 μ g. of BSA/sample as carrier, (b) as the fraction which did not migrate upon paper electrophoresis. Both assay methods gave first-order kinetics of hydrolysis with half-times of 9 and 37 min. respectively. This difference in half-time is easily explained, since assay (a) measured material of much higher molecular weight than did assay (b). Thilo (1959) reported a half-time of 23 min. for acid hydrolysis of polyP under comparable conditions. Paper electrophoresis of partially hydrolysed X-P revealed a number of spots migrating both faster and slower than inorganic phosphate. They represented polyP fractions of different chain lengths. On total hydrolysis these spots disappeared, yielding a single zone of inorganic phosphate as the product. The above results indicate that X-P is a polyP of high molecular weight.

Sedimentation data. Because enzymatic preparation of sufficient material for analysis in the analytical ultracentrifuge was not feasible, the sedimentation coefficient had to be determined from sedimentation data using radioactive polyP in a sucrose gradient (Fig. 2). In this experiment ribosomal RNA and tRNA from *Salmonella* served as internal markers. The sedimentation properties of the polyP did not change after phenol extraction and the polyP was prepared using enzyme fractionated on DEAE-cellulose.

Occasionally, when rather crude enzyme had been used to prepare the polyP sample, a faster sedimenting peak was observed in addition to that shown in Fig. 2. This material

Fig. 2. Sucrose gradient centrifugation of polyP. A 100 μ l. sample containing 2 n-equiv. [32 P] polyP (sp. act. 6500 c.p.m./n-equiv. Pi), with tRNA and ribosomal RNA from *Salmonella* as markers, was layered on top of a 30 to 8% (w/w) sucrose gradient in 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0. Centrifugation was performed in a Spinco SW 56 rotor at 280,000 g (average) for 4 h. at 5°. The tube was punctured at its base and fractions corresponding to 2 mm. segments were withdrawn. $\times-\times-\times$, Counts $\times$ min. $^{-1}$; $\bullet-\bullet-\bullet$, extinction at 260 nm.

Fig. 3. Gel filtration of polyP on Sepharose 4B. A 350 μ l. sample containing 14 n-equiv. phenol extracted polyP (sp. act. 4100 c.p.m./n-equiv. Pi), with tRNA and ribosomal RNA from *Salmonella* as markers, was filtered through a 1.4 $\times$ 35 cm. Sepharose 4B column. Elution buffer: 10 mM-tris-HCl, pH 7.0; 1 mM-EDTA; 0.1% SDS. Elution rate: 0.9 ml./h. Elution was carried out at 5°. $\Delta-\Delta-\Delta$, Counts $\times$ min. $^{-1}$; $\times-\times-\times$, extinction at 260 nm. (RNA); $\bullet-\bullet-\bullet$, extinction at 260 nm. (blue dextran and adenosine).

disappeared upon phenol extraction, and was possibly an aggregate of the more slowly sedimenting material.

The integrating tables of McEwen (1967), which only apply to particle densities up to 1.9, indicated an $S_{W,20}$ value of 11.5 ± 1.5 % for the peak fraction in three different experiments. The actual $S_{W,20}$ value must be somewhat lower because polyP has a particle density of 3.2 (Malmgren, 1952), but the influence of the particle density is not very great in these calculations.

Gel filtration on agarose. A sample of polyP prepared using DEAE-treated enzyme free of degrading activities, was extracted with phenol at 0° and filtered through an agarose gel (sepharose 4B) in 0.01 M-tris buffer, pH 8.0, containing 1 mM-EDTA, plus 0.1 % SDS to prevent aggregation. *Salmonella* ribosomal RNA and tRNA were reference substances (Fig. 3). PolyP was eluted as a broad, asymmetrical peak somewhat earlier than 16 S and 23 S RNA. As already reported by Öberg & Philipson (1967), 16 S and 23 S RNA were eluted in one peak.

Disc electrophoresis on acrylamide gel. A sample of polyP was subjected to disc electrophoresis on 5 % acrylamide gel in a glycine buffer of pH 8.3 containing 1 mM-EDTA and 1 % SDS. The substance did not penetrate the gel. The experiment had to be performed at low temperature because otherwise some degradation of the polyP took place during the electrophoresis.

DISCUSSION

Long-chain linear polyP was extremely labile. It was broken down even if enzymatic degradation was prevented by the use of a purified polyP kinase for its preparation and when it was isolated under very mild conditions. For instance, mere treatment of polyP at pH 8.3 and 40° for only 1 h. caused some cleavage, as became evident in our first attempts to subject it to disc electrophoresis. Only when the temperature was lowered to 5° was this breakdown negligible.

PolyP kinase synthesized polyP of considerable chain length. The appearance of a small fraction of lower molecular weight polyP in the gel filtration experiment was probably due to its instability.

PolyP was eluted earlier from agarose gel than were the ribosomal RNAs, yet it sedimented slower than these substances in the sucrose gradients. These findings are seemingly contradictory. Although nothing is known of the shape of the polyP molecule in solution, nor of its tendency to form aggregates, one might reasonably assume that aqueous polyP behaves similarly to denatured, single-stranded nucleic acid since both compounds are long-chain polyanions. If this assumption is correct, our gel filtration and sedimentation data become consistent, because a given length of linear polyP is only about one-third as heavy as single-stranded nucleic acid of comparable chain length (computed from values given for $\phi \times 174$ phage DNA by Freifelder, Kleinschmidt & Sinsheimer (1964) and for polyphosphate by Thilo (1959).

Malmgren (1952) has shown a pronounced dependence of the sedimentation values of chemically synthesized polyP on polyP concentration as well as on the composition of the solvent. He reported an S_0 value of 14 for a polyP of 480,000 mol. wt when sedimented in 0.1 M-NaCl. Our enzymatically prepared polyP had at negligible concentrations an S value of 11.5 in 0.15 M-NaCl and would thus have a molecular weight in the range of 300,000 to 400,000.

Several authors have suggested that RNA-polyP complexes exist (Correll, 1964; Wang & Mancini, 1966). We found no evidence for such a complex in our system. The polyP kinase

from *Salmonella* required neither RNA nor a polyP primer for activity, though the enzyme was associated with RNA, as was evident from its altered solubility and changed behaviour on DEAE-cellulose after RNase treatment. This, and the fact that it could be solubilized by a mild procedure known to release enzymes from membranes (Munoz, Nachbar, Schor & Salton, 1968; Mühlradt, 1969) suggests that the polyP kinase in its natural state is bound to the cytoplasmic membrane.

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