Purification and Properties of a Polymetaphosphatase from *Corynebacterium xerosis*

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SUMMARY: An enzyme which splits high molecular weight polymetaphosphate (PMP) to orthophosphate has been extracted from *Corynebacterium xerosis*, partially purified and some of its properties studied. A method for the preparation of bacterial PMP labelled with ^{32}P is described. The enzyme hydrolyses bacterial or synthetic PMP at the same rate. No formation of short-chain phosphate polymers was detected during the reaction and the enzyme did not attack metaphosphates smaller than hexametaphosphate. It does not transfer phosphate from PMP to ADP to form ATP, nor does it synthesize PMP from orthophosphate (Pi) in the presence of ATP or other nucleotides. All the metal ions tested, including Mg^{++} , inhibited the enzyme. Sodium ethylenediaminetetraacetate (EDTA; 0.2 mM) stimulated the rate of reaction; higher concentrations inhibited. The significance of polymetaphosphate and enzymes which split it in micro-organisms is briefly discussed.

Enzymes which split polymetaphosphate (PMP) to orthophosphate (Pi) occur in extracts of a variety of micro-organisms including bacteria, yeasts and moulds (Mann, 1944; Kitasato, 1928). Most of the earlier observations were, however, made with polymetaphosphatases which split metaphosphates of low molecular weight, e.g. trimetaphosphate, tetrametaphosphate and hexametaphosphate. More recently Malmgren (1952) studied the breakdown of very high molecular weight PMP preparations by extracts from *Aspergillus niger*, *Penicillium expansum* and some other organisms. By measuring the decrease in viscosity and the amount of orthophosphate formation for 2 days at pH 5.3 he concluded that low molecular weight compounds such as tetrametaphosphate and pentametaphosphate were the major end-products. More recently, Kornberg, Kornberg & Simms (1956) prepared an enzyme from *Escherichia coli* which could synthesize PMP from adenosine triphosphate (ATP); an unidentified primer was also necessary to initiate the reaction. Kornberg (1957) showed that this enzyme also catalysed the reverse reaction, i.e. synthesis of ATP from PMP and adenosine diphosphate (ADP). A similar enzyme has also been slightly purified from *Corynebacterium diphtheriae* (Kornberg, 1957). The aim of the present investigation was to purify the polymetaphosphatase (PMP-ase) from *C. xerosis* and to study some of its properties; a relatively simple assay system has been devised. We were also interested in finding out whether this enzyme catalyses the transfer of orthophosphate (Pi) from PMP to a suitable substrate such as ADP, besides breaking PMP to Pi, and whether PMP could directly phosphorylate other compounds, such as glucose, without the intermediation of the ADP-ATP system.

METHODS

Organism. The organism used, *Corynebacterium xerosis*, was originally National Collection of Type Cultures strain no. 7243. The organism was maintained on agar slopes of the medium used for growth (see below).

Growth of organism. *Corynebacterium xerosis* was grown in rectangular enamelled metal trays (15 in. $\times$ 10 in. $\times$ 1½ in.) with tight-fitting lids. The plates were sterilized in an oven at 110–150° for 3 hr. The medium added to each plate had the following composition: Tryptone (Oxo Ltd.) 10 g.; glucose 10 g.; Na₂HPO₄.2H₂O (British Drug Houses Ltd.) 8 g.; KH₂PO₄ (B.D.H.) 4 g.; NaCl (A.R.) 4 g.; yeast extract (Oxo Ltd.) 6 g.; water to 400 ml. The pH of the medium was 6.8–7.0. Eight g. of agar-agar (Bacto Difco Co.) were added, the medium sterilized in an Erlenmeyer flask by heating at 10 lb./sq.in. for 10 min. and then poured into the sterilized plate. Usually five plates were prepared in this way. The plates were allowed to cool until the agar solidified and the inoculum from agar slopes was spread on the medium with a sterilized stainless-steel spreader. The plates were incubated at 37° for 18 hr. After this time, the cells had fully developed metachromatic granules and were harvested from the plates with ice cold 0.15 M-NaCl; glass beads were used for loosening the organism from the agar. The suspension was passed through glass wool in order to remove lumps of agar, centrifuged in the International refrigerated centrifuge at 1000 g. for 20 min. and the organisms washed twice with cold 0.15 M-NaCl. The yield of organism prepared in this way was usually about 10 g wet wt./plate. The organisms were immediately processed for the extraction of the enzyme or stored in the deep freeze at –15°.

Preparation of extracts containing enzymes. Organisms (50–60 g.) were made into a thick paste by adding 15 ml. cold 0.15 M-NaCl and crushed in a Hughes (1951) bacterial press with a cylinder of 1½ in. bore. The crushed material was homogenized with 60 ml. cold 0.15 M-NaCl in a Potter type glass homogenizer (Potter & Elvehjem, 1936). The final suspension, when stained with Albert stain (Mackie & McCartney, 1945) and examined under the microscope, showed very few intact cells.

Preparation of ³²P labelled PMP (PM³²P). *Bacterial polymetaphosphate.* PM³²P was extracted from *Corynebacterium xerosis* grown on the medium described above, to which 1 mc. of carrier-free ³²P orthophosphate (Radiochemical Laboratory, Harwell) was added. The washed organisms were extracted with 5 vol. 10 % (w/v) trichloroacetic acid (TCA) at room temperature and constantly shaken for 5 hr. The extraction of PM³²P was complete in that time (see Table 1). The suspension was centrifuged, the supernatant fluid neutralized with NaOH and brought to pH 3.5 by adding 5 M-acetate buffer (pH 3.5). PM³²P was precipitated by the addition of 1 ml. saturated BaCl₂ solution. The flask was thoroughly shaken and left at 0–2° overnight. The precipitate was collected by centrifugation and washed twice with acetate buffer (pH 3.5) and once with absolute ethanol. It was then treated with Amberlite-IR 120 resin (H⁺ form) in order to remove Ba⁺⁺, neutralized and dialysed against distilled water at 0–2°. The final solution was made up to

20 ml. giving 25,000 counts/min. (CPM) and containing $3.5 \mu\text{mole P/ml}$. Approximately 0.25 % of the ^{32}P added to the growth medium was recovered in PM^{32}P . The resulting product was very viscous. Mixed with solutions of toluidine blue, it showed metachromasy. The absorption maximum shifted from 625 to 530 $\text{m}\mu$. It was precipitated by albumin in 3.5 % (w/v) perchloric acid, and did not move on paper chromatography, with various solvents, and could not easily be removed from the anion exchange resins except by hydro-

Table 1. *The extraction of PMP from Corynebacterium xerosis by 10 % (w/v) trichloroacetic acid solution*

Organism (20 g. wet wt.) was washed, suspended in 5 vol. of 10 % (w/v) TCA and shaken at room temperature for the times stated. The precipitate was removed by centrifugation and metaphosphate determined in the supernatant fluids as described in the text.

Time of extraction (hr.)	Metaphosphate phosphorus in extract (mg.)
0.5	1.8
1.0	3.0
1.5	4.9
2.5	7.7
4.5	13.3
9.0	14.1

lysis. The Ba^{++} precipitate conformed to the formula $(\text{Ba}(\text{PO}_3)_2)_n$ when analysed for P and Ba^{++} . All the phosphorus in the preparation was hydrolysed when treated with N-HCl at 100° for 7 min. (We have used the term poly-metaphosphate in the usual sense to describe the high molecular weight phosphate polymers of this type, although no details are known about the precise structure.)

In contrast to the findings with yeast (Wiame, 1949; Katchman & Van Wazer, 1954; Juni, Kamen, Reiner & Spiegelman, 1948), moulds (Belozersky & Kulaev, 1957; Krishnan, Damle & Bajaj, 1957) and certain mycobacteria (Mudd, Yoshida & Koike, 1958; Winder & Denny, 1957) no 'acid-soluble' PMP was detected in *Corynebacterium xerosis*. When organisms were extracted with 5 % TCA at $0-2^\circ$ for 1 hr. and the extract was treated with Ba^{++} at pH 3.5, no precipitate was formed.

Chemical synthesis of PM^{32}P . The method of Pfanstiel & Iler (1952) as modified by Kornberg (1957) for small-scale preparation was used for preparing synthetic PM^{32}P . To 2 ml. of $\text{m-KH}_2\text{PO}_4$ in a platinum crucible 200 μc . of carrier-free ^{32}Pi was added and the pH was adjusted to 4.5 by adding N-KOH . The solution was evaporated gently on a sand bath, 2 ml. distilled water added from the sides of the crucible and the solution again evaporated. The crucible was then placed in a muffle furnace, the temperature of the furnace was raised to 770° over a period of 2 hr. and maintained at $770-780^\circ$ for 1 hr. The crucible was then removed from the furnace and allowed to cool. The residue was washed twice with ice-cold distilled water and then treated with Amberlite-IR 120 (Na^+ form). The mixture was stirred vigorously for 2 hr. at $0-2^\circ$. After

this, the resin was removed by centrifugation and the resulting viscous solution was dialysed against demineralized water at 0–2° for 24 hr. The yield of PM³²P on the basis of P content and radioactivity measurements was 50–60 %. The product had properties similar to those of bacterial PM³²P.

Chemicals. Adenosine triphosphate (sodium salt) and adenosine diphosphate (ADP) were products of the Schwarz Laboratories, Inc., U.S.A. ADP (Ba salt) was converted into the Na⁺ form by shaking with Amberlite IR-120 (H⁺ form) and neutralized with NaOH. Adenosine-5'-monophosphate (AMP; acid) was a Waldhof Type I product of Zellstoffabrik, Wiesbaden, Germany, and was neutralized before use. Potassium polymetaphosphate, sodium hexametaphosphate, sodium tetrametaphosphate and sodium trimetaphosphate were kindly given by Messrs Albright and Wilson Ltd. Pancreatic ribonuclease, deoxyribonuclease and protamine sulphate were obtained from Messrs Light and Co. Ltd.

As the mol. wt. of PMP was not known, the molarity of PMP solutions is given in terms of orthophosphate (Pi) liberated after hydrolysis with N-HCl at 100° for 7 min. The concentration of hexametaphosphate is expressed similarly for the same reason.

Chemical estimations. Phosphorus was estimated by the method of Berenblum & Chain (1938). The sample was extracted as usual in isobutanol and the volume made up to 10 ml. with ethanol. Before developing the colour with SnCl₂ the sample was counted in a GM liquid counter tube (type M6) to estimate ³²P. A suitable number of counts was taken to ensure a standard error of ± 2 %.

Barium was estimated gravimetrically as BaSO₄, protein by the biuret method (Gornall, Bardawill & David, 1949) and also by the spectrophotometric method of Kalckar (1947) when the nucleic acid content of the solution was low. Total N was estimated by the micro-Kjeldahl method.

Measurement of enzyme activity. The assay measured the amount of ³²Pi liberated from PM³²P under standard conditions. In the initial work the assay system contained 0.1 ml. MgCl₂ (2.0 mM), 0.1 ml. 2-amino-2-hydroxymethyl propane-1:3 diol (tris) + maleate buffer (0.1 M, pH 7.0), 0.1 ml. PM³²P (0.5 μmole, 10,000–20,000 CPM/μmole), enzyme, and water to final volume 0.5 ml. This system was later altered by omitting MgCl₂ and substituting 0.1 ml. of sodium ethylenediaminetetra-acetate (EDTA; 1 mM). The mixture was incubated at 37° for 20 min. and then 0.5 ml. of cold 7 % (w/v) perchloric acid and 1.0 ml. 0.1 % (w/v) bovine serum albumin (Armour) was added. The mixture was stirred and placed on ice for 5 min., centrifuged at 0–2° at 10,000 g for 5 min., and a suitable sample of the supernatant fluid taken for extraction of ³²Pi by the method of Berenblum & Chain (1938) and counted. One unit of the enzyme is defined as that amount which catalyses the breakdown of 0.001 μmole of non-dialysable PMP to orthophosphate in 20 min. at 37°.

Purification of the enzyme. Step I. Nuclease treatment. The crushed organisms gave a very viscous suspension after homogenizing. Before attempting any purification, the suspension was treated with nucleases, which resulted in a decrease in viscosity. To 100 ml. of the thick suspension was added approximately

1 mg. each of ribonuclease and deoxyribonuclease (Light and Co. Ltd.), 20 μ mole-MgCl₂ and tris buffer (pH 7.0). The suspension was incubated at 37° for 20 min. with occasional shaking, after which it was immediately cooled in ice and centrifuged in the Spinco Model 'L' ultracentrifuge at 50,000 *g* for 20 min.; 60–70 % of the activity of original extract was then present in the supernatant fluid. The residue was discarded.

Step II. Ammonium sulphate treatment I. Saturated (NH₄)₂SO₄ solution (neutralized with NaOH) was added to the supernatant fluid from step I at 0–2°. The (NH₄)₂SO₄ addition was done with constant mechanical stirring until a final concentration of 60 % saturation was reached. The mixture was kept in ice for 10 min. and centrifuged at 0–5° in the International refrigerated centrifuge. The supernatant fluid, which was inactive, was discarded and the bulk of the activity was recovered in the residue which was dissolved in tris + maleate buffer (pH 7.0) to give a final volume of 23 ml.

Table 2. *Summary of the enzyme-purification procedure*

Step no.	Volume (ml.)	Enzyme activity (units/ml.)	Protein concn. (mg./ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. protein)
1. Nuclease-treated crushed organisms	90	1260	42.20	113,400	3798	29
50,000 <i>g</i> supernatant fluid	75	1080	20.10	81,000	1507	54
2. (NH ₄) ₂ SO ₄ I	23	3360	40.90	77,280	941	82
3. Protamine sulphate (NH ₄) ₂ SO ₄ II	28	2560	4.90	71,680	137	522
4. (NH ₄) ₂ SO ₄ III	25	2344	2.02	58,600	51	1132
5. Ethanol	20	1073	0.38	21,460	7.6	2823

Step III. Protamine sulphate treatment. Protamine sulphate solution (20 mg./ml.; pH 5.0) was slowly added to the solution from step II in the cold and with constant stirring. The precipitate was centrifuged off and the supernatant fluid examined for absorption at 260 and 280 m μ . The addition of protamine sulphate was continued until the E 280/E 260 ratio was more than 0.75, the reason being that at ratios higher than this the amount of nucleic acid remaining in the supernatant fluid was very small and did not interfere with further fractionation. A total of 15 ml. of the solution of protamine sulphate (equiv. 300 mg.) was added and the precipitate was removed by centrifugation. The supernatant fluid was diluted with cold distilled water to 400 ml. with constant stirring, and the bulky precipitate formed was centrifuged off and discarded. To the supernatant fluid was added 152 g. solid (NH₄)₂SO₄ and the precipitate was collected and dissolved in 20 ml. water ((NH₄)₂SO₄(II)). About 90–95 % of the activity from step II was present in the final solution.

Step IV. (NH₄)₂SO₄ fractionation III. The above solution was subjected to (NH₄)₂SO₄ fractionation. Saturated (NH₄)₂SO₄ solution was added to give a saturation of 28 % and the precipitate was centrifuged off and discarded. Saturated (NH₄)₂SO₄ was added to the supernatant fluid to bring the con-

centration to 54 % and the precipitate which formed was collected and redissolved in 25 ml. water ($(\text{NH}_4)_2\text{SO}_4$ (III)).

Step V. Ethanol fractionation. The $(\text{NH}_4)_2\text{SO}_4$ (III) fraction was further purified by reprecipitation with absolute ethanol at -15° . The fractionation was carried out in a salt ice bath maintained at $-15 \pm 1^\circ$, and the fractions were collected in the refrigerated centrifuge at -15° . The fraction appearing between 60 and 75 % (v/v) ethanol in water contained most of the activity and was collected. Active material was extracted with tris buffer (pH 7.0). In some experiments, where concentrated enzyme preparation was required, the final solution was brought to a 50 % saturation with $(\text{NH}_4)_2\text{SO}_4$ and the residue dissolved in a small amount of water without loss of activity. The enzyme preparation thus obtained was dialysed against 0.1 M- $(\text{NH}_4)_2\text{SO}_4$ at pH 7.0 and $0-2^\circ$ for 24 hr. The overall purification, on a protein basis, was approximately 100 times.

RESULTS

Specificity. The purified enzyme did not hydrolyse trimetaphosphate, tetrametaphosphate, pyrophosphate, ATP or glucose-6-phosphate. Hexametaphosphate (Graham's salt) was broken down almost as rapidly as PMP. Bacterial PMP was hydrolysed as rapidly as the synthetic PMP (Table 3).

Table 3. *Action of PMP-ase on various substrates*

The reaction mixture consisted of MgCl_2 solution 0.1 ml. ($0.2 \mu\text{mole}$); tris + maleate buffer (pH 7.0) 0.1 ml.; enzyme solution 0.1 ml. (780 units); substrate solution, 0.1 ml.; water, 0.1 ml. The mixture was incubated at 37° for 20 min. and the reaction stopped by adding 0.5 ml. 7 % (w/v) perchloric acid. The mixture was centrifuged and orthophosphate (Pi) estimated in the supernatant fluid by the method of Berenblum & Chain (1938). A blank was run for the enzyme and for each substrate.

Substrate	Amount added		Pi formed (μmole)		
	Substrate (μmole)	Total Pi in substrate ($\mu\text{mole P}$)	Pi formed (μmole)		
			Observed	Blank	Net
Trimetaphosphate	0.5	1.5	0.14	0.14	0
Tetrametaphosphate	0.5	2.0	0.14	0.14	0
Pyrophosphate	1.00	2.0	0.22	0.23	0
ATP	1.00	3.0	0.25	0.26	0
Glucose-6- PO_4	0.50	0.5	0.08	0.08	0
Hexametaphosphate	—	1.58	1.29	0.46	0.83
Polymetaphosphate (synthetic)	—	1.01	0.86	0.08	0.78
Polymetaphosphate (bacterial)	—	0.05	0.46	0.07	0.39

Addition of ATP, ADP or AMP to the complete reaction mixture did not significantly affect the breakdown of PMP to orthophosphate (Table 4). When ADP, glucose and hexokinase were added, there was no decrease in the counts appearing in the orthophosphate fraction and no incorporation into the acid stable P fraction. This clearly indicates that the enzyme is not capable of transferring phosphate from PMP to ADP or AMP (cf. Kornberg, 1957).

Table 4. *Effect of adenine nucleotides on PMP-ase activity*

The reaction mixture consisted as usual of tris buffer, Mg^{++} , $PM^{32}P$ and enzyme solution. Following additions were made and enzyme activity measured as usual.

Addition	Final concn. (mM)	Counts/min. in $^{32}P_i$
None	—	3491
ATP	2.0	3532
ADP	4.0	3505
AMP	2.0	3545
Glucose	10.0	3502
ADP	2.0	
Hexokinase	0.05 mg. protein	

Kinetics of the enzyme

pH effect and stability. The pH dependence of the enzyme was studied in 0.1 M-tris + maleate buffer at pH 5–8. The optimum pH value was at pH 7.0 and there was very little activity at pH 6.0 (Fig. 1). The enzyme was completely

Fig. 1. Effect of pH value on enzyme activity. The reaction mixture contained purified dilute enzyme 50 units; $PM^{32}P$, 0.5 μ mole-P; $MgCl_2$, 0.2 μ mole; tris + maleate buffer (10 μ mole); water to 0.5 ml. Enzyme activity was measured as usual after 20 min. incubation at 37°.

Fig. 2. Effect of temperature on enzyme activity. Assay mixture contained: purified enzyme 200 units; $PM^{32}P$, 0.5 μ mole; tris + maleate buffer (pH 7.0), 10 μ mole; $MgCl_2$, 0.2 μ mole; water to 0.5 ml. Before adding to the assay system the enzyme preparation was first incubated for 20 min. at the different temperatures and then assayed for activity as usual.

Fig. 3. Rate of enzyme reaction with time. The reaction mixture contained: purified enzyme 320 units; $PM^{32}P$, 0.5 μ mole; tris + maleate buffer (pH 7.0), 10 μ mole; $MgCl_2$, 0.2 μ mole; water to 0.5 ml. Reaction was stopped after different intervals of time and activity measured as usual.

inactivated by incubation at 50° for 20 min. Incubation at 37° for 20 min. resulted in a 10 % loss in activity as compared with the activity at 0° (Fig. 2). There was no loss in activity when the purified enzyme was stored at –15° from 5 to 6 weeks.

Effect of substrate concentration and rate of reaction. The progress of the reaction was proportional with time during a 30 min. period (Fig. 3). The rate of reaction increased approximately linearly with low substrate concentrations up to a final concentration of 0.30 mM (Fig. 4a). Further increase in substrate concentration did not increase the rate of orthophosphate formation linearly. The K_m value for the PMP-ase as determined from the Burk-Lineweaver plot is $7.7 \times 10^{-4} M$ (Fig. 4a, b), the molarity being expressed as inorganic orthophosphate added as PMP.

Fig. 4a, b. Initial rate of reaction at different substrate concentrations. The reaction mixture contained: purified enzyme previously dialysed against EDTA, 80 units; tris + maleate buffer (pH 7.0), 10 μ mole; EDTA, 0.2 μ mole; different amounts of $PM^{32}P$; water to 0.5 ml. Enzyme activity was measured as usual after 20 min. incubation at 37°. The data is plotted according to Lineweaver & Burk (1934) in b.

When ^{32}Pi was incubated in the presence of ATP instead of $PM^{32}P$, all the radioactivity was recovered in the acid soluble inorganic phosphate fraction. As such, no $PM^{32}P$ was synthesized and the reaction was not reversible under these conditions.

Effect of metal ions and EDTA on PMP-ase activity. The effect of metal ions, EDTA and some inhibitors on the purified enzyme is shown in Table 5. EDTA stimulated the enzyme appreciably at a final concentration of 0.5 mM. Ammonium molybdate and ammonium vanadate had a slightly stimulatory effect. All the other metal ions tried, namely Mg^{++} , Ca^{++} , Fe^{++} , Mn^{++} , Co^{++} , Zn^{++} and Cu^{++} inhibited the enzyme to different extents. Uranyl acetate (0.1 mM) almost completely inhibited the polymetaphosphatase activity.

Dialysis against water, tris buffer (0.1 M), pyrophosphate buffer (0.1 M) or EDTA (0.02–0.05 M) at pH 7.0 and 0–2° completely inactivated the enzyme. Dialysis against 0.1 M- $(NH_4)_2SO_4$ under the above conditions did not affect the enzyme activity. In an attempt to remove metal ions the enzyme was dialysed, with continuous stirring, against 0.1 M- $(NH_4)_2SO_4$ + 50 mM-EDTA (pH 7.0) and 0–2° for 24 hr. EDTA was removed by dialysis against 0.1 M- $(NH_4)_2SO_4$ for 40 hr. The effect of different concentrations of Mg^{++} and EDTA was tried on

Table 5. *Effect of metal ions and inhibitors on the PMP-ase activity*

The reaction mixture contained: tris + maleate buffer (pH 7.0) 0.1 ml.; purified enzyme solution 0.1 ml.; PM³²P, 0.1 ml.; other additions as shown below.

Addition		
Substance	Final concentration (mM)	Counts/min. in Pi fraction
No addition	—	3102
EDTA	0.4	3807
Ammonium molybdate	0.4	3422
Ammonium vanadate	0.1	3218
MnCl ₂	0.58	2737
FeSO ₄	0.55	2722
CuCl ₂	0.58	2642
MgCl ₂	0.4	2516
CaCl ₂	0.4	2232
ZnCl ₂	0.4	1123
Co(NO ₃) ₂	0.4	609
Uranyl acetate	0.1	151
Sodium arsenate	1.00	3152

Fig 5

Fig. 6

Fig. 5. The effect of EDTA on the activity of purified enzyme. The reaction mixture contained: purified enzyme previously dialysed against EDTA 105 units; PM³²P, 0.5 μ mole; tris + maleate buffer (pH 7.0), 10 μ mole; different concentrations of EDTA; water to 0.5 ml. Enzyme activity was measured as described in the text.

Fig. 6. The release of orthophosphate by PMP-ase action on PM³²P compared to non-dialysable PM³²P remaining. Composition of the reaction mixture was as given in the text (p. 491). ●—●, ³²Pi formed; ○—○, non-dialysable ³²Pi left.

the enzyme preparation dialysed in this way. It was clear (Table 6) that EDTA at low concentration stimulated the enzyme, whereas when the concentration was increased, EDTA had an inhibitory effect (Fig. 5). Mg⁺⁺ inhibited the purified enzyme even at low concentrations. When EDTA was added in concentrations sufficient to complex added metals it annulled their inhibitory effects.

Mechanism of PMP-ase action

Estimation of orthophosphate and non-dialysable radioactivity in the reaction mixture. Seven tubes were set up as follows: MgCl_2 0.1 ml. ($0.2 \mu\text{mole}$); tris-maleate buffer 0.1 ml. (0.1 M, pH 7.0); PM^{32}P , 0.1 ml. (3062 CPM); purified enzyme 0.1 ml. (600 units); water, 0.1 ml. The reaction was stopped after suitable intervals by adding 0.5 ml. cold 7 % (w/v) perchloric acid, the mixture

Table 6. *Effect of different concentrations of EDTA and Mg^{++} on PMP-ase activity*

The reaction mixture contained: tris + maleate buffer (pH 7.0) 0.1 ml.; purified enzyme solution, 0.1 ml.; PM^{32}P , 0.1 ml.; and varying amounts of EDTA or Mg^{++} as shown below.

Substance	Addition	
	Final concentration (mM)	Counts/min. in Pi fraction
None	—	940
EDTA	0.05	1287
EDTA	0.20	1350
EDTA	0.50	1175
EDTA	1.00	684
EDTA	2.00	326
EDTA	4.00	199
Mg^{++}	0.05	802
Mg^{++}	0.20	613
Mg^{++}	0.50	425
Mg^{++}	2.00	307

Table 7. *The amounts of ^{32}Pi formed and non-dialysable radioactive material (as counts) left after PMP-ase action for different times*

Composition of the reaction mixture as given in the text.

Period of incubation (min.)	a. Counts in Pi fraction (calculated in the total reaction mixture) (CPM)		b. Counts after dialyses (CPM)		Recovery	
	Observed	% of total in PM^{32}P	Observed	% of total in PM^{32}P	Total CPM (a + b)	% of total counts in PM^{32}P taken
0	60	1.9	2939	95.9	2999	97.9
0.25	115	3.7	2830	92.4	2945	96.1
1	240	7.8	2737	89.4	2977	97.2
15	2032	66.8	942	30.9	2972	97.2
30	2832	92.1	128	4.1	2951	96.2
60	3053	99.7	16	0.5	3069	100.2
300	3070	100.2	0	0	3070	100.2

centrifuged and the clear supernatant fluid taken in another tube kept in ice. A sample (0.4 ml.) of the supernatant fluid was taken for ^{32}Pi estimation and another sample (0.25 ml.) for dialysis. The samples were dialysed overnight in 15 l. of demineralized water at $0-2^\circ$ and the radioactivity remaining in the non-dialysable solution was counted. The results are given in Table 7 and Fig. 6.

It is clear from the results of the above experiment that when PMP was broken down no dialysable intermediates other than Pi were formed. Very little PMP was left after incubation for 1 hr. and almost all of it was recovered as Pi.

Chromatography of the reaction mixture. To confirm the conclusion of the above experiment, the reaction mixtures after different intervals of time were chromatographed. Five tubes were assembled as shown in Table 8. The reaction was stopped by adding 0.1 ml. cold 21 % (w/v) perchloric acid and the mixture centrifuged. A sample of the supernatant fluid (0.1 ml.) was spotted on Whatman no. 4 filter-paper for ascending chromatography as described by Berg (1958). Trimetaphosphate, tetrametaphosphate, PMP and Pi were run as markers. The composition of the solvent was methanol 50 ml., *n*-butanol 10 ml., water 22 ml., aqueous concentrated ammonia (sp.gr. 0.880) 18 ml., titrated with 25 % (w/v) formic acid to pH 11.4. The solvent was run for 16 hr. at room temperature, dried and sprayed with Hanes & Isherwood phosphate spray reagent (Hanes & Isherwood, 1949). The spot with the same R_f value as tetrametaphosphate was present in the enzyme blank and samples 3–5, and did not have any radioactivity. This spot was light blue as compared to the phosphate spots (including tetrametaphosphate) which were dark blue. The spot did not contain any phosphorus, as determined by the method of Berenblum & Chain (1938) after ashing, and has not yet been identified.

Table 8. *Chromatography of the reaction mixtures*

The tubes had MgCl_2 solution, 0.1 ml. ($0.2 \mu\text{mole}$); tris + maleate buffer (pH 7.0), 0.1 ml., PM^{32}P , 0.1 ml. (2980 CPM, none in No. 1); enzyme solution, 0.1 ml. (none in no. 2); water to 0.5 ml. total volume. Details of chromatography are given in the text.

Description	Period of incubation (min.)	Counts at origin	Counts in Pi spot	Total
Without PM^{32}P	5	—	—	—
Without enzyme	5	483	10	493
Complete mixture	0.7	452	38	490
Complete mixture	5	320	185	505
Complete mixture	60	12	447	459

There was no evidence of any other compounds on the chromatogram except PMP and Pi. The spots were counted on the paper with an end-window counter tube and the results, summarized in Table 8, confirm that no intermediates of low molecular weight other than orthophosphate were formed during the enzymic hydrolysis of PMP.

DISCUSSION

Three types of enzymes which break down metaphosphate have previously been described. The first type consists of a number of metaphosphatases isolated from various sources, all of which are hydrolytic enzymes which attack only relatively short chain molecules of the size of trimetaphosphate. An example of this type described recently is the enzyme isolated from pea seedlings by Pierpoint (1957); we tested samples of the enzyme which this

author kindly supplied and found that it did not attack either the bacterial PMP or the synthetic PMP. The second type of polymetaphosphatase attacks long-chain metaphosphates of the molecular size of those isolated from bacteria and moulds as well as the smaller metaphosphates like tri- and hexametaphosphate. Malmgren (1952), working with an enzyme of this type isolated from *Aspergillus niger*, found that no inorganic phosphate was formed and from the results of kinetic studies has suggested that it can be described as a depolymerase. The third type of metaphosphatase is a transferase described by Kornberg *et al.* (1956), which in the presence of an unidentified primer transfers terminal orthophosphate from ATP to form high molecular weight PMP. This transferring enzyme is reversible and will split PMP by transferring Pi to form ATP from ADP. The enzyme which we have isolated from *Corynebacterium xerosis* appears to differ from these three types of metaphosphatases in that it attacks long-chain metaphosphates, with the formation of inorganic orthophosphate; as far as could be detected there was no formation of intermediate short-chain molecules and the enzyme does not attack metaphosphates of shorter chain length than hexametaphosphate. When tested under the conditions described by Kornberg (1957) for transferase activity there was no transfer of phosphate from PMP to ADP or AMP, neither did the addition of ATP or ADP affect the rate of enzyme reaction. The enzyme from *C. xerosis* thus appears to be a hydrolytic enzyme.

The significance of the enzymes which break down metaphosphate in micro-organisms must be related to the role which metaphosphate plays in the economy of the organism. Two different views have been expressed recently about this. Russian workers (Belozersky, 1958; Kulaev & Belozersky, 1957) regard the metaphosphates, because they are energy-rich compounds, as energy stores which can phosphorylate other compounds without the usual intervention of ATP or other nucleotides. In support of this idea it is claimed that metaphosphates are in combination with ribonucleoproteins, and together are concerned with nucleic acid and protein synthesis. In view of the rather drastic isolation procedures involved in the separation of the PMP complexes it is doubtful whether they exist as such in the cell. An alternative suggestion is that PMP may be regarded simply as a source of phosphate. This conception fits with the finding of many laboratories that polymetaphosphates accumulate when cells are given ample phosphate and energy supplies but growth retarded either by nitrogen or other starvation (Winder & Denneny, 1954; Sall, Mudd & Davis, 1956). Metaphosphate rapidly disappears from cells once normal growth is started after a period of starvation and the phosphate from PMP appears rapidly in most phosphate fractions of the cell (Mudd *et al.* 1958; Kulaev & Belozersky, 1957; Winder & Denneny, 1957). If, therefore, metaphosphate is regarded as a phosphate store the various metaphosphatases would presumably serve to break down the polymers to orthophosphate.

In connexion with the distribution of the various types of metaphosphatases in micro-organisms it is of significance that we have found an enzyme which splits the polymers to orthophosphate in *Corynebacterium xerosis* and which does not attack the short-chain molecules, since we found no short-chain

metaphosphates to be present in the organism. In moulds and yeasts in which the depolymerase type of enzyme occurs, not only do the shorter chain compounds occur but enzymes which split them to orthophosphate have also been isolated.

Activation by EDTA and Mg^{++} . In the early experiments Mg^{++} was added to the enzyme assays because of the general finding that Mg^{++} stimulates this type of enzymes (Mattenheimer, 1951; Kornberg, 1957), and a small (20 %) stimulation was found with crude concentrated enzyme preparations; in higher concentrations however, Mg^{++} inhibited even in these crude preparations. With the purified enzyme, Mg^{++} did not stimulate and a higher concentration of Mg^{++} was markedly inhibitory. All the other metal ions tried were inhibitory. The addition of EDTA to the purified preparation stimulated in low concentration and inhibited in higher concentrations. A mixture of divalent metals did decrease the inhibitory effect of high concentration of EDTA, probably removing its effect by chelation. The inhibitory effect of Mg^{++} and divalent metals might be explained by their combination with the PMP in competition with the enzyme because PMP is a powerful chelating agent. The inhibition by high concentration of EDTA is possibly due to the chelation of activating metal bound to the enzyme.

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