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Enzymatic Breakdown of Polymetaphosphate

B. INGELMAN and H. MALMGREN

The Institutes of Physical Chemistry and Biochemistry, University of Uppsala, Sweden

In the course of studies on phosphatases Kitasato found in 1928 that »taka-phosphatase» from *Aspergillus oryzae* was also capable of liberating orthophosphate from metaphosphate [1, 2]. This observation was subsequently confirmed by other workers, and it was also shown that yeast extract and some organ extracts also possess the power of liberating orthophosphate from metaphosphate and »triphosphate» [3, 4, 5].

While investigating phosphorus metabolism in moulds, Mann [6] observed powerful metaphosphatase activity in extracts from *Aspergillus niger*. Mann made at the same time the interesting observation that extracts from *A. niger* contained not only pyrophosphate but also metaphosphate.

Experiments have thus been described in the literature which demonstrate the existence of metaphosphatase. The activity has been demonstrated by determinations of the free orthophosphate liberated from the metaphosphate. There seem, however, to be no published experiments giving any indication as to how this enzymatic breakdown proceeds. We have now carried out some experiments, one of the objects of which was to ascertain that metaphosphatase breaks down polymetaphosphate of very high molecular weight. Another object of our experiments was to ascertain if the metaphosphatase breaks down high molecular polymetaphosphate by a random hydrolysis of -P-O-P- bonds, or whether the breakdown proceeds in such a way that one orthophosphoric acid residue at a time (or a few such residues. *e. g.* as pyrophosphoric acid) is split off from the ends of the metaphosphate chains. The postulated processes have an analogy in the breakdown of starch by the different amylases. We have also made preliminary experiments with the idea of finding out if any of the metaphosphate found by Mann in *A. niger* has such a high molecular weight as to be capable of determination in the ultracentrifuge, for comparison in this way with the polymetaphosphates of synthetic origin.

We have also attempted to demonstrate metaphosphatase activity in extracts of some micro-organisms other than *A. niger* and *A. oryzae.*

The substrate for our experiments has been the well-defined polymetaphosphate of very high molecular weight that has been described by Malmgren [7] and Malmgren and Lamm [8, 9]. These polymetaphosphates are built up from long chains which may be formulated as follows:

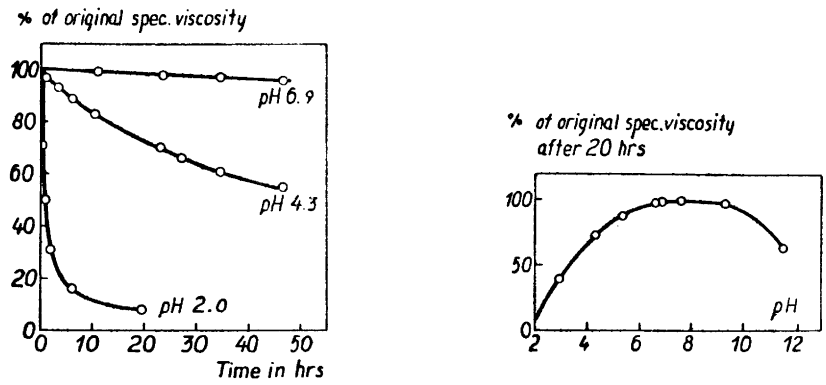
- - -O-PO(ONa)-O-PO(ONa)-O-PO(ONa)-O-PO(ONa)- - -

The molecular weight of these substances can be considerable, extending up to several millions. Two different preparations [7, 8, 9], designated as K11 and K14, have been employed. The molecular weight of K11 is about 2,000,000 and of K14 about 1,500,000 (as measured in buffer solutions of ionic strength 0.4). n in the formula $(NaPO_3)_n$ thus has magnitudes of the order 15,000—20,000. These substances were obtained by heating KH_2PO_4. The resulting potassium polymetaphosphate was dissolved, for the experiments, in buffer solutions with a large excess of sodium ions.

At low pH values the spontaneous breakdown velocity of the polymeta phosphate is quite considerable. We carried out a series of preliminary experiments to measure the velocity of breakdown of the polymetaphosphate in some buffers at different pH's. In this way it was hoped to ascertain in which pH region one should work to ensure that the spontaneous breakdown velocity of the polymetaphosphate is insignificant. Of course it might also be of interest to do experiments with metaphosphatase at pH values where the spontaneous breakdown of polymetaphosphate is considerable, but under these circumstances it would be necessary to make allowance for it. For the present purpose the viscosity at 25.0° C was determined after various intervals for three different solutions having the following composition (ionic strength of NaCl + the buffer substances = 0.3):

I. 0.5 % polyphosphate (K14) + 1.17 % NaCl in citrate buffer — pH 2.0.
II. 0.5 % polyphosphate (K14) + 1.17 % NaCl in acetate buffer — pH 4.3.
III. 0.5% polyphosphate (K14)+1.17% NaCl in phosphate buffer — pH 6.9.

In fig. 1 the y axis shows the percentage of the original specific viscosity $(\eta/\eta_0\text{-}1)$ and the x axis the time in hours. In fig. 2 the percentage of the original specific viscosity reached after 20 hours is plotted against the pH of the solutions. (The curve has been supplemented with 5 extra points obtained in rther experiments.) It is evident from the curves that one should avoid caroying out enzyme experiments at pH values that are too low; under these



Figs. 1 and 2. Breakdown of polymetaphosphate at different pH values.

conditions the polymetaphosphate undergoes spontaneous decomposition quite rapidly.

All the enzyme experiments were done at 25.0° C (except those with *P. expansum*). The enzymatic breakdown of the polymetaphosphate was followed by means of viscosity measurements, ultracentrifuge studies, dialysis experiments and orthophosphate determinations.

The viscosity measurements were made by determining the running time in ordinary Ostwald viscosimeters. As these polymetaphosphate solutions have a comparatively high viscosity, it is easy to follow the first phase of the breakdown by viscosity measurements. The viscosity of the polymetaphosphate solutions has been measured relative to that of the buffer solutions, and from the relative viscosity (η/η_0)the specific viscosity (η/η_0-1) has been calculated. In the graphs the % of the original specific viscosity has been plotted against the time elapsing after the enzyme was added to the polymetaphosphate solution. For present purposes it can be assumed that the specific viscosity provides a qualitative measure of the degree of polymerisation of the polymetaphosphate.

The centrifugations were done in the ultracentrifuge of Svedberg. In these experiments both sedimentation constants and the quantities of sedimenting polymetaphosphate were determined (the latter from the area under the »peak» in the sedimentation diagram). For details of the experimental procedure in the ultracentrifugations, reference is made to [10].

For determining the liberated orthophosphate we have used a method of determination recently published by Lowry & Lopez [11]. The reaction is based on the reduction of phosphomolybdate by ascorbic acid at pH 4. This

method has the advantage that the determination of orthophosphate can be done in such conditions that easily hydrolysable phosphorus compounds (*e. g.* labile esters of phosphoric acid) do not split off orthophosphate, that would interfere with the analytical results. To give a suitable pH to the solutions for analysis, they were diluted with an acetate buffer of ionic strength 0.2 and pH 4.0. The readings were taken at a wave-length of 720 mμ. To convince ourselves that pyrophosphate and metaphosphate do not, in this method, interfere with the determination of orthophosphate, we analysed for orthophosphate known mixtures of orthophosphate, pyrophosphate and polymetaphosphate. These experiments showed that we could determine orthophosphate with satisfactory accuracy in the presence of 100 times as much pyrophosphate or polymetaphosphate.

A number of experiments have been done with extracts from *Aspergillus niger* and with »clarase» (enzyme preparation from *A. oryzae*). As they all gave results pointing towards the same conclusion, we will confine ourselves to describing a few of these experiments.

Experiment with »clarase»

For this experiment a »clarase» preparation was used (»taka-diastase» from *A. oryzae*, commercial preparation), which we purified by dialysis against distilled water and then dried *in vacuo* from the frozen state. In the experimental series (no. I) a solution was used containing 0.44 % polymetaphosphate (K14) and 0.12 % »clarase» in a maleic acid sodium maleate buffer to which had been added 1.17 % NaCl (ionic strength of NaCl + buffer substance = 0.3, pH 6.6). In the blank series (no. II) a solution of the same composition was used, except that here the enzyme solution had been kept for 30 min at 100° C before it was added to the polymetaphosphate solution.

The breakdown of the polymetaphosphate in the two solutions was followed by viscosity measurements, orthophosphate determinations and dialysis experiments. The viscosity fell markedly faster in the solution containing the active enzyme. After 48h the specific viscosity of solution no. I had sunk to 47 % of its original value: for solution no. II the corresponding value was 92 %. During the same period only traces of free orthophosphate-P had been formed, corresponding to less than 0.5 % of the total P present in the solution. Thus at the beginning only insignificant amounts of orthophosphate are split off from the ends of the polymetaphosphate chains.

To show that it is not either the case that the first phase of the breakdown proceeds in such a way that two (pyrophosphate) or a few phosphoric acid residues at a time are split off from the ends of the polymetaphosphate chains,

a dialysis experiment was done (also at 25.0° C) in parallel with the above and employing the same solutions. 10 ml of each of the polymetaphosphate solutions (the one containing unheated, the other heated enzyme) were transferred to cellophane sacs; the two sacs were then placed each in a separate cylinder containing 25 ml of the buffer solution. At suitable intervals phosphate determinations were carried out on the liquid surrounding the dialysis sacs. In these the phosphate was determined both directly and after boiling with dilute sulfuric acid, under such conditions as to break down any pyrophosphate or polymetaphosphate of low molecular weight that had dialysed out. This experiment showed that after 48 h only quite insignificant amounts of pyrophosphate or low-molecular, dialysable polymetaphosphate had been formed (less than 1 % of the total P present).

It is thus apparent that the first phase of breakdown due to the action of this enzyme does not involve the splitting off of one, two, or a few phosphoric acid residues from the ends of the polymetaphosphate chains. (If this were the case, the marked fall in viscosity should be accompanied by the liberation of considerable quantities of orthophosphate). It seems that the splitting occurs here and there, probably at random along the chains, leading chiefly at first to the formation of comparatively large fragments.

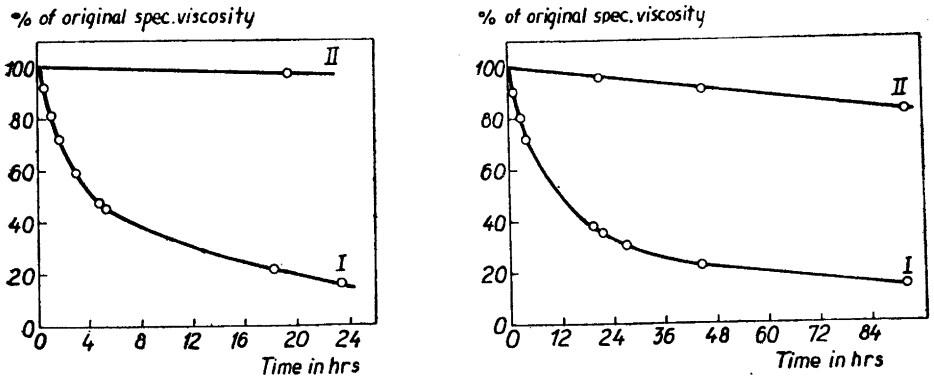
The experiment also shows that the »clarase» has a fair activity even when dialysed against distilled water. It thus appears that the enzyme does not readily loose any co-enzyme that may be necessary, or else, that it requires only very small quantities of such a co-enzyme.

Experiment with *Aspergillus niger* enzyme I

Aspergillus niger (from the Institute of Plant Physiology, Uppsala) was cultivated at 33° C on a medium of the following composition: 100 g sucrose, 4 g KH_2PO_4, 4 g K_2HPO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 2 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.2 g $CaCl_2$, 0.2 g enzymically degraded casein, a trace of a vitamin preparation (containing *inter alia* vitamin B_1), a trace of $FeSO_4$, 1 l tap water.

After 4 days growth the mould was ground up in the culture medium. The mixture was then filtered. The filtrate was neutralized with NaOH, and then dialysed against ice-water in a cellophane sac. After dialysing for 24 h some of the solution in the sac was taken out and used as enzyme solution for the experiment. Some more of the solution was kept at 100° C for 30 min for use as a blank.

A polymetaphosphate solution was prepared containing 0.5 % of K11 and 1.17 % NaCl (0.2 *M*) in a maleic acid-sodium maleate buffer (combined ionic strength of NaCl and buffer substances = 0.3, pH = 6.6). For the



Figs. 3 and 4. Breakdown of polymetaphosphate by A. niger enzym.

experimental series (no. I) 7 ml of this solution of K11 was mixed with 1 ml of the enzyme solution. The blank series (no. II) was made up in the same way, except that in this case the heat-inactivated enzyme solution was used. The alteration of viscosity with time for the two solutions is shown in fig. 3. When, after 23 h, the specific viscosity had fallen to 16 % of its original value, only traces of free orthophosphate-P had been formed (less than 0.5 % of the total quantity of P present).

Experiment with *Aspergillus niger* enzyme II

For this experiment the same enzyme solution as in the previous experiment was used, except that the dialysis had been continued for a further 48 h. A polymetaphosphate solution with the same composition as in the previous experiment was prepared. In series (no. I) 20 ml of this polymetaphosphate solution was taken and mixed with 2 ml of enzyme solution (pH 6.6). The solution for the blank series (no. II) was prepared in the same way, except that for this series an enzyme solution was used that had been inactivated by heating. In this case the breakdown was followed by viscosity determinations and phosphate determinations, and also by dialysis experiments and ultracentrifugations. The fall of the specific viscosity with time is shown in fig. 4. When the specific viscosity had sunk to 14 % of the original value, here again only traces of free orthophosphate-P had been formed (less than 0.5 % of the total P present).

In parallel with this experiment a dialysis experiment was done at the same temperature (25° C) and employing the same solutions. 12 ml of each of

the polymetaphosphate solutions were transferred each to a separate cellophane sac, and the two sacs were then placed in separate cylinders, each containing 20 ml of the buffer solution. At suitable intervals phosphate was determined in the liquid outside the cellophane sacs, both directly and after hydrolysis with acid such as to break down to orthophosphate any pyrophosphate or polymetaphosphate of low molecular weight that had dialysed out. When, after 44 h, the specific viscosity of the solution had sunk to 23 % of its original value, only insignificant amounts of dialysable polymetaphosphate-P had dialysed out of the sac (less than 1 % of the total P present). After 92 h when the specific viscosity had sunk to 14 % of its original value, some few percent of the polymetaphosphate had been reduced to such small molecules as to have dialysed out. (Nevertheless, there were still only traces of orthophosphate.)

In parallel with these experiments 3 ultracentrifugation experiments were also carried out on solution no. I at different times, as well as an ultracentrifugation of the blank solution. The results are given in table 1. (The sedimentation constant, *s*, is given in Svedberg units.)

Table 1. Breakdown of polymetaphosphate by A. niger enzym.

Series no.	Time (h) after beginning of experiment	% of original specific viscosity	Sedimentation constant (*s*)
I	5	70	10.6
I	20	37	10.2
I	95	14	8.7
II	22	95	10.6

The sedimentation constant for solution no. I falls somewhat with time. As expected, however, it does not fall as markedly as the specific viscosity. (For thread-like molecules the sedimentation constant does not vary so sharply with the chain-length as does the specific viscosity.) Calculations were made, from the area under the »peak» of the centrifugation diagram, of the quantity of high-molecular polymetaphosphate recovered as sedimenting material. The same amount of material was observed in this way in both the first two centrifugations of solution I, and on centrifuging the solution II from the blank experiment. In the third centrifugation of solution I (after 95 h) there was also found practically the same amount of sedimenting substance. (The value found was a few percent lower, which would be in agreement with the dialysis experiment. However, the accuracy of quantity deter-

minations from centrifugation diagrams is not sufficient to permit of drawing quantitative conclusions from an alteration of only a few percent.) The centrifugation diagrams for solution I also showed that the substance becomes more and more polydisperse as the enzyme action proceeds. Since, therefore, practically all the polymetaphosphate seems to remain in a comparatively high-molecular form, it is seen that these centrifugation experiments support the conclusions as to the nature of the breakdown which we obtain from the viscosity and phosphate determinations. (Centrifugation experiments have also been done in connection with some of our other experiments; results of a similar character were obtained.)

Experiments with enzyme from *Penicillium expansum*

Several experiments have also been done with extracts from *P. expansum*, which showed that this micro-organism also produces a metaphosphatase with properties similar to those of the metaphosphatase from *A. niger*. We will confine ourselves to describing a few of the experiments with an extract from this organism.

P. expansum (no. 593 from the National Collection of Type Cultures maintained in Britain by the Medical Research Council) was cultivated at 25° C on a medium of the following composition: 40 g glucose, 0.8 g $(NH_4)_2SO_4$, 0.8 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.3 g enzymically degraded casein, traces of a vitamin preparation containing, *inter alia*, vitamin B_1, and 360 ml tap-water.

After 5 days growth the mould was ground up in the culture-medium with sand. The mixture was filtered, and the filtrate was neutralized with NaOH and dialysed against ice-water. A sample was removed after 4 h and was used as enzyme solution in some experiments, designed to ascertain in what pH region the optimum lay for this metaphosphatase.

Four different buffer solutions were prepared, containing 0.5 % polymetaphosphate (K11) and 1.17 % NaCl (0.2M). (Ionic strength of NaCl + buffer substances = 0.3). The pH of the various solutions was as follows:

A. Acetate buffer	pH 4.3
B. Acetate buffer	pH 5.4
C. Phosphate buffer	pH 6.9
D. Borax-soda buffer	pH 8.9

To 10 ml of each of these solutions 2 ml of enzyme solution was added, and the breakdown of the polymetaphosphate was followed viscosimetrically at

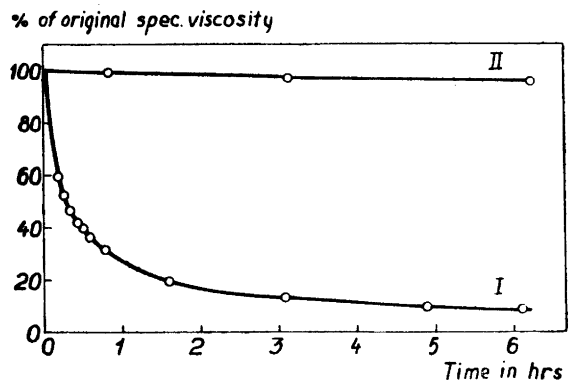


Fig. 5. Breakdown of polymetaphosphate by P. expansum enzym.

20.3° C. This showed that the breakdown occurred rapidly at low pH, but only slowly at pH 6.9 and over. Table 2 illustrates the result:

Table 2. Enzymatic breakdown at different pH values.

pH	Time from beginning of experiment	% of original specific viscosity
4.3	45 min	20
5.4	45 min	32
6.9	18 h	85
8.9	18 h	95

Like the metaphosphatase found by Mann in *A. niger* this metaphosphatase also has its optimum at an acid pH.

In the course of breakdown of polymetaphosphate by this enzyme it is likewise the case that at first no measureable quantities of orthophosphate are liberated. This is demonstrated by the following experiment: The same enzyme solution was used as in the preceeding experiment, except that it had been dialysed for a further 48 h. Some of the enzyme solution was inactivated by heating for use as a blank. A solution was made up as in the previous experiment with 0.5 % KI1 and 1.17 % NaCl in acetate buffer (pH 5.4). In the experimental series (I) 9 ml of this solution was treated with 2 ml enzyme solution. For the blank series (II) heat-inactivated enzyme solution was used instead. The fall of specific viscosity with time is shown by fig. 5 (Temperature 20.3° C).

When, after 24 h, the specific viscosity of mixture I had fallen to 4 % of its original value, only traces of free orthophosphate-P had yet been formed (less than 0.5 % of total P present).

Experiment with enzyme from *Schizosaccharomyces pombe*

A single experiment with an extract from *Schizosaccharomyces pombe* showed a weak effect at pH 5.6.

Attempts to isolate metaphosphate from *A. niger*

Preliminary experiments having the object of isolating the metaphosphate found by Mann in *A. niger* have shown that it is perhaps not impossible that a fraction of the naturally occurring metaphosphate may possess a relatively high molecular weight. We intend to publish these experiments at a later date.

DISCUSSION

These experiments confirm the observations of Kitasato and Mann that metaphosphatase occurs in *A. oryzae* and *A. niger*. It has also been possible to demonstrate activity in extracts from *P. expansum*. This phosphatase has its optimum at a rather low pH. All the metaphosphatases studied have possessed the power of breaking down polymetaphosphate of very high molecular weight. It is not always evident what was the molecular weight of the metaphosphate used in the experiments with metaphosphatase in the previous literature but it seems that low molecular substances have been used. (Kitasato says that he used hexa-metaphosphate $(NaPO_3)_6$ and Mann says that he used metaphosphate and hexa-metaphosphate. Neuberg and Fischer used triphosphate $Na_5P_3O_{10}$.) In our experiments we have used preparations of polymetaphosphate having molecular weights of more than a million. Thus in these experiments a colloid of purely inorganic character has been degraded by enzymatic means. The polymetaphosphate has been broken down by metaphosphatase through hydrolysis of -P-O-P- links at scattered points along the chains, so that comparatively large fragments are at first formed; the hydrolysis does not take the form of a splitting off of one or a few phosphoric acid residues at a time from the ends of the chains.

The experiments that have been done show that the well-defined polymetaphosphates of extremely high molecular weight, that have previously been studied in this laboratory, are well suited for use as substrates even in

the study of metaphosphatase preparations of low activity, since the breakdown is easily followed by viscosimetric means.

It has not yet proved possible to answer the question whether some micro-organisms may perhaps be capable of synthesizing and storing a polymetaphosphate of comparatively high molecular weight. Experiments have been initiated with the object of finding out whether or not this is so.

SUMMARY

The enzymatic breakdown of polymetaphosphate of very high molecular weight with enzymes from micro-organisms has been studied by viscosity measurements, ultracentrifugations, dialysis experiments and orthophosphate determinations. It has been demonstrated that the breakdown proceeds through the scission of scattered -P-O-P- links in the polymetaphosphate chains, so that comparatively large fragments are at first formed; it is not the case that the hydrolysis occurs through the removal of one (or a few) orthophosphoric acid residues at a time from the ends of the polymetaphosphate chains.

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