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Investigations on Metaphosphate of High Molecular Weight Isolated from *Aspergillus niger*

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In some previous articles we have shown the occurrence of enzymes breaking down metaphosphate of high molecular weight [1-4]. Such enzymes were found in moulds, yeasts and the bacterium *Proteus vulgaris*. In these enzyme experiments synthetic metaphosphates were used as substrates having molecular weights of more than one million. Thus an inorganic colloid was broken down by enzymatic means. As these microorganisms have enzymes which break down metaphosphates of high molecular weights one might perhaps expect to find compounds of similar character in these organisms.

In the latter years of the 19:th century investigations indicating the occurrence of metaphosphate in yeast were performed by Liebermann [5-7], Kossel [8] and Ascoli [9]. These earlier investigations were forgotten for a long time, until in 1936 MacFarlane [10] isolated a nucleic acid preparation of high phosphorus content from yeast. Extracting this substance with hydrochloric acid MacFarlane obtained inorganic metaphosphate. Later Wiame [11] also isolated from yeast such substances containing 17 per cent P. During the last few years the occurrence of metaphosphate in yeast has been further confirmed [12-15]. The metaphosphate in yeast seems to be of two kinds, one acid soluble and one acid insoluble [13,14]. The two forms seem to differ in their metabolic function. Metaphosphate has also been found in different mutants of *Neurospora* [16]. Inorganic metaphosphate has also been isolated from the mould *Aspergillus niger* by Mann in 1944 by extracting the mycelium with trichloro acetic acid [17].

In the previous literature no molecular weight determinations on the metaphosphate found in microorganisms are described. During our studies on the enzymatic breakdown of synthetic metaphosphates of very high molecular weights we raised the question if not at least a fraction of the naturally occurring

metaphosphate, for instance that found in *A. niger*, could be of colloidal nature [1]. However, if the mould contains metaphosphate of high molecular weight, it must be difficult to isolate this substance in an undegraded and water soluble form. The mould *A. niger* has an enzyme which very quickly breaks down the metaphosphate within the pH-interval 4—7. Besides, both at low and high pH-values the colloidal metaphosphate is a labile substance because of the high rate of the spontaneous breakdown. Therefore, when trying to isolate metaphosphate of high molecular weight from *A. niger* one must work out a suitable method for removing the enzyme and other organic substances without breaking down the metaphosphate.

Applying the following technique non-dialyzable substances of high molecular weights and of high phosphorus content were isolated [18,19]. *A. niger* was extracted at a slightly alkaline pH. The extract was then filtered through large amounts of active carbon in order to remove the enzyme and other organic material. Finally the solution was dialyzed in cellophane bags, evaporated to a small volume *in vacuo*, filtered through active carbon, dialyzed again and dried in vacuo in the frozen state. One of the preparations, isolated as sodium salt, contained 25 per cent P. The ash content was 85 per cent. The characteristic absorption of nucleic acids in ultraviolet light could not be observed. The sedimentation constants were 2.2 and 2.3 Svedberg units for 1.5 and 0.5 per cent solutions. Another of these sodium salts of high molecular weight isolated from *A. niger* contained 29.3 per cent P. The ash content was more than 90 per cent. The sedimentation constant was 2.3 S for a 0.8 per cent solution.

PREPARATION METHODS

For the following experiments we have used the same culture of *Aspergillus niger* as before. (*A. niger* v. Tiegh. no. 594 from the National Collection of Type Cultures maintained in Britain by the Medical Research Council.) The mould was cultivated under sterile conditions in Petri dishes (diameter 20 cm) for one or two weeks at about 25° C on a medium of the following composition: 225 g pure saccharose, 75 g crude saccharose, 6 g K_2HPO_4, 12 g $NaNO_3$, 1 g $MgSO_4$, $7H_2O$ and 2700 ml water.

In the experiments reported in this article we have used the same preparation method as described in the two previous papers [18,19] and also some modifications of it. In the previous investigations blank determinations showed that the washed active carbon did not contain metaphosphate, but in order to be absolutely sure that no polymetaphosphate would be introduced in the preparations on the addition of active carbon, this was treated as follows:

The active carbon was kept in 0.3 *N* hydrochloric acid for some days, washed with distilled water and then with 5 per cent sodium or potassium carbonate solution and then with distilled water.

Four somewhat different preparation procedures have been used. (Preparations A—D.) A synthetic metaphosphate was also prepared in order to be used as a reference

substance. (Preparation E.) In the first preparation (A) the sodium salt was isolated as before; however, since sodium metaphosphate is a hygroscopic substance, in the later preparations (B—E) we have preferred to prepare instead potassium metaphosphate, less hygroscopic and easier to isolate.

Preparation A

1. Mould from 20—30 dishes cut in pieces.
2. Suspended in 900 ml 1 % sodium carbonate solution.
3. 15 min. at 80° C (in order to destroy the enzyme).
4. Ground in "Waring Blendor" with carbon and 300 ml 5 % sodium carbonate solution.
5. Filtered through filter paper.
6. Solution filtered through large amounts of active carbon.
7. pH of the filtrate adjusted to about 8.
8. Solution dialyzed in cellophane bags against distilled water for some days at 4° C.
9. Solution evaporated in vacuo at 30—40° C to about 50 ml.
10. Sodium chloride added to concentrated solution and solution filtered through active carbon.
11. pH of the filtrate adjusted to about 8 with sodium carbonate. Solution dialyzed against distilled water.
12. Solution dried *in vacuo* in the frozen state. Yield from 25 dishes: 50 mg, 29.4 % P. Theoretical value for $(NaPO_3)_n$, 30.4 % P.

Preparation B

The first twelve steps as described in A were used, but potassium salts were used instead of sodium salts.

Twelve crude preparations containing about 10—20 per cent P were taken together (in all 170 mg) and purified in the following way:

1. Crude products dissolved in 50 ml 0.2 % KCl solution.
2. pH adjusted to 8 with potassium carbonate.
3. Solution filtered through active carbon.
4. Filtrate dialyzed in nitrocellulose bag against distilled water at 4° C.
5. Solution dried *in vacuo* in the frozen state.

Yield from 309 dishes (Preparation B 2): 75 mg, 25.9 % P.
Theoretical value for $(KPO_3)_n$, 26.3 % P.
(Another more impure preparation (B 1) contained 24.4 % P.)

Preparation C

The first twelve steps as described in A, were used, but potassium salts were used instead of sodium salts and further the step no. 3 was changed from warming during 15 min. at 80° C to freezing at — 15° C for one day in order to destroy the cell walls.

Five crude preparations containing about 10—20 % P were taken together (in all 610 mg) and purified as follows:

1. Crude products dissolved in 15 ml water and pH adjusted to 9 with potassium carbonate.
2. 10 ml 30 % hydrogen peroxide added.

3. After 3 hours the solution was diluted to 50 ml with distilled water and filtered through active carbon.
4. Filtrate dialyzed in nitrocellulose bag against distilled water at + 4° C.
5. Solution dried *in vacuo* in the frozen state.
   Yield from 104 dishes: 190 mg, 26.6 % P.

Preparation D

1. Mould from 20—30 dishes cut in pieces.
2. Suspended in 900 ml water, pH adjusted to 9 with potassium carbonate.
3. 100 ml 30 % hydrogen peroxide added in portions.
4. After 1 hour the mixture was ground in "Waring Blendor".
5. 100 ml 30 % hydrogen peroxide added in portions.
6. After 1 day filtered through filter paper.
7. Solution filtered through large amounts of active carbon.
8. pH adjusted to 8—9 with potassium carbonate, 5 ml hydrogen peroxide added.
9. Solution dialyzed in nitrocellulose bags against distilled water for some days at 4° C.
10. Solution evaporated *in vacuo* at 30—40° C to about 50 ml.
11. Potassium chloride added to concentrated solution and solution filtered through active carbon.
12. pH adjusted to 8—9 with potassium carbonate.
13. Solution dialyzed against distilled water.
14. Solution dried *in vacuo* in the frozen state.
    Preparation D 1, yield from 17 dishes, 97 mg, 27.0 % P.

Preparations E and K, synthetic reference substances

For comparision with the naturally occurring metaphosphate of high molecular weight in *A. niger* a metaphosphate, synthetically obtained by heating NaH_2PO_4, was prepared in the following way.

15 g of a commercial sodium metaphosphate was dissolved in 300 ml 0.8 *M* KCl solution. The solution was dialyzed for some days in a bag of nitrocellulose against 0.2 *M* KCl solution at 4° C. Then the solution was dialyzed for several days against distilled water. The solution was then evaporated *in vacuo* to about 50 ml and then dried in vacuo in the frozen state. The substance obtained (E) contained 26.6 % P.

Another potassium metaphosphate of very high molecular weight (more than one million) was obtained by heating KH_2PO_4 at a temperature of 500° C. This preparation (K) contained 26.3 % P.

CHEMICAL INVESTIGATIONS

In Table 1 the data of the chemical analyses of the obtained preparations are shown. The samples used for phosphorus analyses and determinations of ash content were previously dried at 120° C, whereas the substances used for physico-chemical measurements were kept over phosphorus pentoxide at room temperature as we did not want to heat the substances before these investigations.

Table 1. Some chemical analyses of the preparations.

Preparation	P %	Ash %	N %	Carbohydrate %	Ca/P max. value for soluble complex
A (Na salt)	29.4	96			
B2	25.9	(99)	0.2	Traces	
C	26.6	98.4			1/3.4
D1	27.0	97.2		0.6	1/3.3
E Synth. ref. subst.	26.6	99.5			1/3.2
K Synth. subst.	26.3				1/3.0
$(KPO_3)_n$ Theoretical values	26.3	100	0.0	0.0	

The phosphorus analyses of the crude preparations were performed colorimetrically after hydrolysis with sulphuric acid according to Lowry and Lopez [20]. The phosphorus contents of the final products were determined gravimetrically following the ammonium molybdate method as described in Treadwell [21].

The ash contents were determined by heating samples of the substances in a small platinum crucible at $\sim 700°$ C.

The nitrogen was determined according to the micro Kjeldahl method. Such determinations have been made only when substances have been available also for this purpose, as amounts of 50—70 mg were used in order to get accurate values. A solution of one of the preparations (C) was also investigated in the Beckman spectrophotometer but the characteristic absorption of nucleic acids in ultraviolet light could not be observed. The carbohydrate content was approximately estimated by the orcinol method [22] using the polysaccharide dextran for comparison.

A sensitive criterion on polymetaphosphates is their ability to form soluble complexes with bivalent metal ions, *e. g.* calcium, until a certain atomic ratio Me/P is exceeded, at which point a remaining precipitate is formed. (For

Table 2. Some physico-chemical data of the preparations.

Preparation	Conc. %	s	D_A	Molecular weight
A	1.7 0.6	2.2	 19	4000
B1	0.7	2.4		
B2	2.0 0.7	(1.5)	 29	(2000)
C	1.7 0.9	2.6	 13	7000
D1	2.1 0.8	2.6	 15	6000
E Synth. ref. subst.	1.0 0.7	2.4	 11	8000

further details reference is made to the review by Karbe and Jander [23].) This test was performed by adding 0.05 *M* calcium chloride solution to 50 ml of a neutral 0.08 per cent solution of the substance to be investigated until the formation of a remaining turbidity. The values obtained from the two preparations from *A. niger* are rather consistent with that of the reference substance.

Table 3. Electrophoretic mobility of the preparations at pH 6.7.

Preparation	Conc. %	Mobility 10^5 cm^2/volt sec.
B2	0.25	11.4
C	0.25	10.8
D1	0.25	10.3
E (synth. ref. subst.)	0.25 0.50	10.9 12.0

PHYSICO-CHEMICAL INVESTIGATIONS

Some physico-chemical investigations were also performed on these preparations. The results are given in Table 2 and 3.

The sedimentation constants were determined in the ultracentrifuge according to Svedberg [24]. The determinations were made in a buffer containing 0.025 *M* Na_2HPO_4, 0.025 *M* NaH_2PO_4 and 0.20 *M* NaCl. The values obtained for the sedimentation constants, *s*, are given in Svedberg units and refer to a temperature of 20.0° C and pure water as a solvent. The diffusion measurements were carried out in a cell according to Claesson [25] using Lamm's scale method [26]. The solvent was the same phosphate buffer as that used in the sedimentation measurements. The diffusion constants, D_A, calculated according to »the area method» [26], is given in units of 10^{-7} cm^2/sec. and referred to pure water of 20.0 °C. The sedimentation and diffusion experiments showed that the preparations obtained were of a polymolecular nature. The molecular weights were calculated with the aid of Svedbergs formula [24], $M = RTs/D(1-V\varrho)$. The partial specific volume of the solute, *V*, was assumed to be 0.32. This value was obtained for a synthetic potassium metaphosphate of high molecular weight dissolved in 0.4 *M* NaCl solution. (The value of *V* will be discussed in a future paper.) On account of the difficulty in determining low sedimentation constants with accuracy and on account of the polymolecularity of the substances, the molecular weights calculated give the orders of magnitude rather than accurate values. However, there is no doubt that the isolated substances are compounds of rather high molecular weights. It seems probable that the molecular weights are dependent on the preparation technique. The preparation methods A and B including heating at 80° C yielded substances of lower molecular weights, indicating a breakdown during the preparation.

The electrophoretic mobilities were determined in the Tiselius apparatus [27] at a temperature of 0.5° C. The experiments were performed in the same buffer (pH 6.7) as the sedimentation and diffusion measurements. As expected the substances moved toward the anode. The mobilities were calculated from the descending boundaries. At a concentration of 0.25 per cent the mobilities of the substances isolated from *A. niger* are consistent with that of the synthetic polymetaphosphate.

DISCUSSION

The analytical data for these non-dialyzable substances isolated from *A. niger* show that they are of inorganic character containing only traces of organic material and that the phosphorus content is the same as that of pure metaphosphate. Further, they form soluble complexes with calcium ions. These complexes are precipitated at the same atomic ratio Ca/P as the syn-

thetic metaphosphate of about the same molecular weight. It may also be mentioned that these substances give the same metachromatic staining with »toluidine blue» as the syntetic reference substance. (These staining experiments performed by B. Sylvén will be published later.) The substances have also the same electrophoretic mobility as the synthetic metaphosphate used for comparison. The ultracentrifuge and diffusion measurements show that the isolated substances have high molecular weights. Therefore, it can be concluded from the experiments reported here that at least a fraction of the metaphosphate occurring in *A. niger* is of high molecular weight.

The approximate molecular weights given in Table 2 are average values for the polymolecular substances. Therefore, metaphosphate molecules of higher weights than these average values are also present in the isolated substances. It may also be mentioned that it is possible that the native metaphosphate in *A. niger* has higher molecular weight than the isolated substances on account of some breakdown during the preparation.

It is not yet definitely established whether the metaphosphate itself or perhaps an organic complex containing metaphosphate represents the compound originally present in the cells of the mould.

Metaphosphate has yet only been found in certain microorganisms and never in higher animals. It seems rather unlikely that colloidal metaphosphates should occur in animals. Preliminary investigations which were reported showed that only metaphosphates of low molecular weights *e. g.* sodium trimetaphosphate are broken down by extracts of liver and other organs [3].

The biological significance of metaphosphate of high molecular weight in microorganisms is not known yet. It seems probable that due to the energy-rich phosphate bonds in metaphosphate this substance supplies energy to important processes in moulds, yeasts and certain bacteria when it is broken down by enzymes. Metaphosphate may also be a suitable substance for the storage of phosphate in these microorganisms. Wiame's investigations indicate that the substance in yeast cells called volutin which can be detected by the metachromatic staining technique, contains metaphosphate [28,29,30]. The role of volutin in yeast has been studied by *e. g.* Lindegren [31] who is of the opinion that in yeast this volutin is essential for chromosome division.

SUMMARY

From the mould *Aspergillus niger* a water soluble, nondialyzable substance has been isolated which has been shown to be metaphosphate with a molecular weight of the order of magnitude 6 000—7 000, *i. e.* an inorganic substance of high molecular weight.

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