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Enzymatic Breakdown of Polymetaphosphate

IV. The Activation and the Inhibition of the Enzyme

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It has been known for several years that some phosphatases are activated by ions of bivalent metals. In 1927 Erdtman [1, 2] found that phosphatase from kidney was activated by magnesium ions. Quantitative measurements on the activation of this enzyme by Mg^{++} ions were carried out by Jenner and Kay [3] some years later, and in 1940 Bamann and Heumüller [4] published an investigation on the action of some bivalent cations on the alkaline phosphatase from liver using $Na_4P_2O_7$, $Na_3(PO_3)_3$, $Na_5P_3O_{10}$ and glycerophosphate as substrate. However, the activating effect of bivalent cations is not restricted to the enzymes from liver and kidney; the polymetaphosphate degrading enzymes from moulds and yeasts are also largely influenced. This paper will deal with the action of metal ions on these enzymes and with some experiments performed with compounds reacting specifically towards certain essential groups of enzymes.

The enzymes used in the experiments were extracted from *Aspergillus niger* and *Saccharomyces cerevisiae* (baker's yeast). For details of the enzyme preparation see Ingelman and Malmgren [5, 6]. In these experiments a high molecular potassium metaphosphate $(KPO_3)_n$ — designated as K 15 — was used as substrate, the molecular weight of which was more than one million. The polymetaphosphate is prepared by heating primary potassium orthophosphate.

$$n\,KH_2PO_4 \longrightarrow (KPO_3)_n + n\,H_2O$$

This polymetaphosphate is insoluble in water but soluble, for instance, in sodium salt solutions of suitable concentration. For particulars concerning the synthesis and properties of these substances see [7, 8]. The enzyme activity

was determined by means of viscosity measurements at 25° C. As a relative measure of the enzyme activity in comparison experiments (the same substrate and substrate concentration) a quantity z, defined by:

$$z = (\eta_{sp})_{t=0} \times \frac{\mathrm{d}\left(\frac{1}{\eta_{sp}}\right)}{\mathrm{d}t}$$

is used, where η_{sp} = specific viscosity and t = time. For a more detailed discussion of this method of calculation, see Ingelman and Malmgren [7].

INFLUENCE OF CATIONS ON THE ENZYME ACTIVITY

The enzymes in question are activated by some metal ions and inhibited by others. However, the measurements are complicated by the properties of the substrate, which is an electrolyte with colloidal properties due to the anion. The viscosity of a colloid of this type is not only dependent on the concentration of the substance but on the concentration and nature of the salts of low molecular weight which are also present. The effect of these salts is to cause a diminution of the solvation and of the charge on the anion; both of which factors contribute largely to the viscosity. One might expect the charge effect of the anion to be depressed in the presence of cations — the decrease being greater the higher the valency of the cations added. However, on reduction of the charge probably also the shape of the substrate is changed [8]. (A more detailed investigation on the physico-chemical properties of the substrate will shortly be published in *Acta Chem. Scand.*) Fig. 1 shows the variation of the intrinsic viscosity $(\eta_{sp}/c)_{c\to 0}$ as a function of the atomic quotient: bivalent metal/phosphorus (Me:P) for some metals. The measurements have been performed in acetate buffer of ionic strength 0.3 and pH 5.3.

As can be seen from Fig. 1 the values of the intrinsic viscosity do not differ very much from that of a metal-free solution as long as the quotient Me:P is not too great. Most bivalent metals from complexes of great stability with the polymetaphosphate and only a minor part of the metal is present in a free ionic state. In fact, the concentration of the Me^{++} *ions* in the solutions is very small. However, if the quotient Me:P exceeds a certain value the Me-polymetaphosphate is precipitated. For most metals, *e.g.* Mg and Ca a

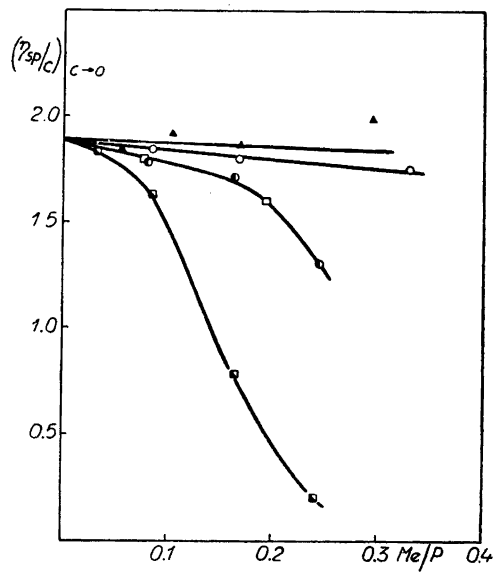

Fig. 1. $(\eta_{sp}/c)_{c\to 0}$ *as a function of Me/P for some metals* ▲ = *Zn*; ○ = *Mg*; □ = *Mn*; ◐ = *Ca*; ◹ = *Pb*

permanent precipitate is formed when Me:P $\sim$ $^1/_3$*. This value seems to vary a little depending on the metal added. The equilibrium:

bivalent metal ion + polymetaphosphate $\rightleftharpoons$ complex anion

is lagerly displaced to the right. (It should be noted that the monovalent silver ion forms complexes with the polymetaphosphate.)

The experiments have been performed as follows. The activity of the enzyme is determined when using a solution of K 15 of the following composition (50 mg K 15 + 10 ml buffer + 2 ml 0.15 *M* $NaNO_3$) as substrate. To 5 ml of this solution 1 ml of the enzyme solution is added and the deter-

* A rough estimation of the order of magnitude of the Me^{++} concentration may be made from the knowledge of the solubility products of some slightly soluble salts of the metal in question. Thus using the solubility products of the oxalates, the following values were found with respect to the substrate solutions

$$C_{Zn}^{++} < 10^{-7};\ 10^{-7} < C_{Ca}^{++} < 10^{-6};\ \text{at pH } 5.4$$

A review of the literature dealing with complex-formation between alkaline earths and heavy metals and a sodium polymetaphosphate called Graham's salt (in principle not differing very much from the substrate of this work though being of lower molecular weight) is given by Karbe and Jander [9].

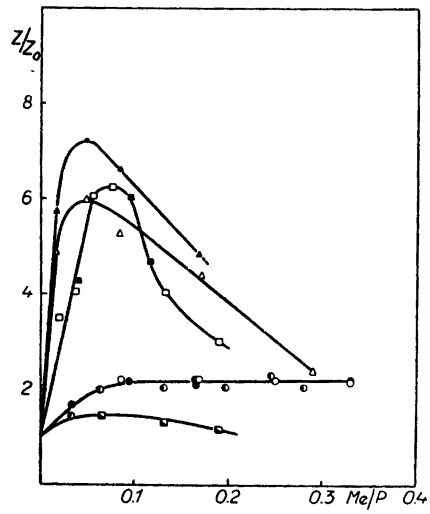

Fig. 2. z/z_0 as a function of Me/P for some metals (A. niger; pH = 5.4).
▲ = *Zn (serie I)*; ● = *Mg (serie I)*
△ = *Zn (serie II)*; ○ = *Mg (serie II)*
■ = *Mn (serie I)*; ◐ = *Ca*; ◹ = *Pb*;
□ = *Mn (serie II)*.

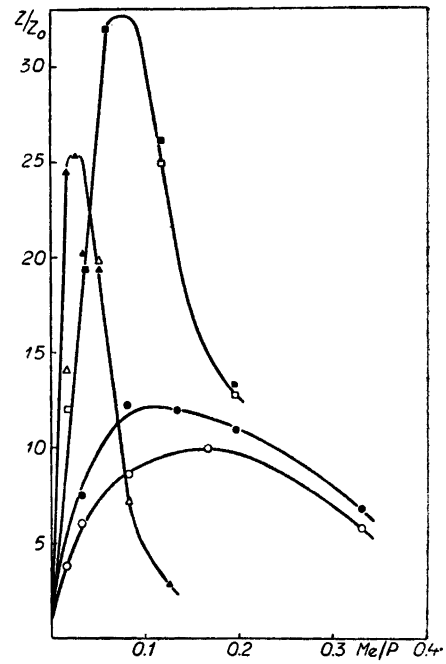

Fig. 3. z/z_0 as a function of Me/P for some metals (A. niger; pH = 6.6).
▲ = *Zn (serie I)* ■ = *Mn (serie I)*
△ = *Zn (serie II)* □ = *Mn (serie II)*
● = *Mg (serie I)*
○ = *Mg (serie II)*

mined activity is designated as z_0. Then the substrate solution is changed by substituting part of the sodium nitrate with 0.15 *M* $Me(NO_3)_2$ its composition being 50 mg K 15 + 10 ml buffer + *a* ml 0.15 *M* $Me(NO_3)_2$ + (2 — *a*) ml 0.15 *M* $NaNO_3$ (*a* changing from $\sim$ 0.05 — 1.00) and the enzyme activity is determined as usual. (The measurements of the activation of a certain metal were completed in a few hours to ensure that the z_0-value remained unchanged.) The influence of the following metals has been investigated Mg, Ca, Zn, Mn, Fe, Ba, Pb, Hg and the monovalent metals Tl and Ag.

Aspergillus niger. The enzyme from *A. niger* is completely inhibited by Ag^+ and Hg^{++}-ions * but activated by ions of the other metals mentioned above. In Figs. 2 and 3 the z/z_0-values, *i. e.* the activation, have been plotted as a

* The mecury-organic compound 'merthiolat' does not inhibit the enzyme.

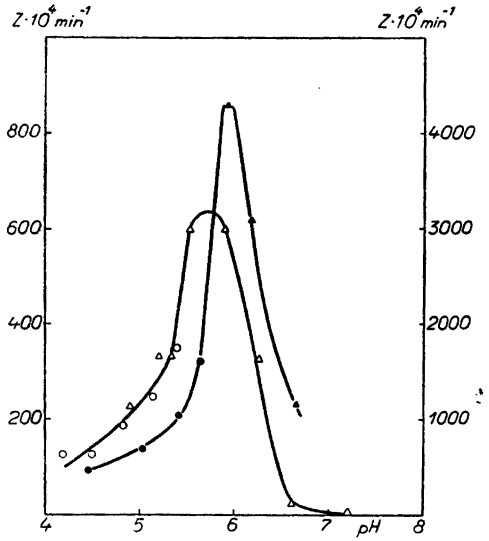

Fig. 4. z-values as a function of pH. Ordinata to the left: no activator; ○ = acetate buffer; △ = phosphate buffer. Ordinata to the right: activation by Mn^{++} ions; ● = acetate buffer; ▲ = phosphate buffer.

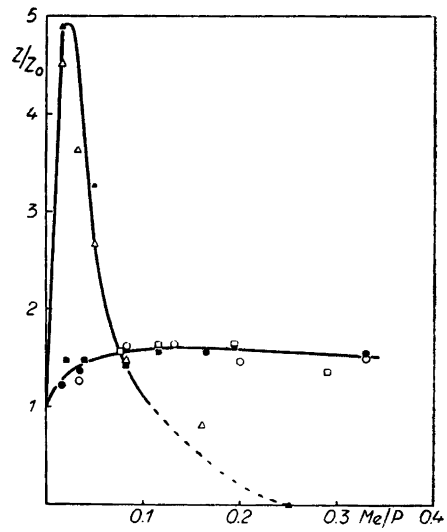

Fig. 5. z/z_0 as a function of Me/P for some metals (S. cerevisiae; pH = 6.6).
▲ = Zn (serie I); ■ = Mn (serie I);
△ = Zn (serie II); □ = Mn (serie II);
● = Mg (serie I);
○ = Mg (serie II).

function of the atomic quotient Me/P for some metals of biochemical importance, *viz.* Mg, Ca, Mn, Zn and Pb; the last mentioned metal being an activator instead of an inhibitor which was not expected. The medium used was acetate buffer of ionic strength 0.3 and pH 5.4 (Fig. 2), pH = 6.3 (Fig. 3).

The fact that zinc is a better activator than magnesium is concordant with the observation by Pett and Wynne[10] who state that zinc replaces magnesium as activator of phosphatases from bacteria. Under similar conditions the activation by zinc ions, for example, on the enzymes from *A. niger* and *Saccharomyces cerevisiae* is by no means identical; a fact justifying a suggestion that these enzymes are two different chemical entities. This is supported by the observed difference between the pH-optima[6].

The activating effect of a given metal ion seems to increase when the pH is changed towards the alkaline side, all other factors such as substrate and enzyme concentration, ionic strength, Me/P ratio etc. being constant. Thus, in the presence of a good activator *i.e.* Mn^{++} the pH-optimum is displaced a little towards the alkaline side. This is illustrated by Fig. 4 showing

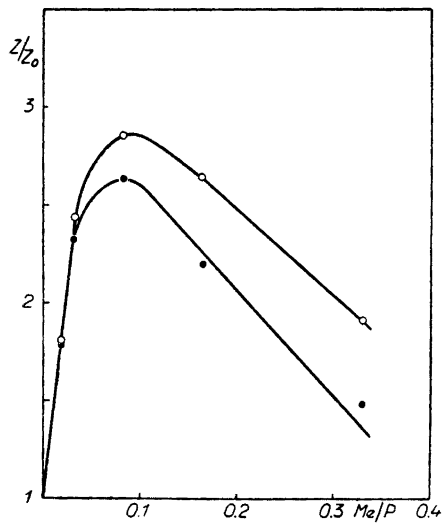

Fig. 6. z/z_0 as a function of Mg/P; (S. cerevisiae; pH = 7.3).
● = *serie I*
○ = *serie II*

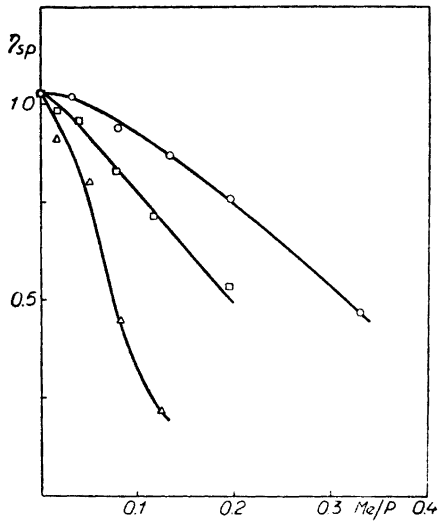

Fig. 7. η_{sp} as a function of Me/P for some metals. 0.42 % solutions in acetate buffer of pH = 5.4.
○ = *Mg*; □ = *Mn*; △ = *Zn*.

the pH-optima of *A. niger* enzyme in the presence and absence of Mn^{++} ions. The quotient Mn/P is 1 : 10.

Saccharomyces cerevisiae. The enzyme from *S. cerevisiae* behaves qualitatively like that from *A. niger* though under similar conditions (the same pH, the same ionic strength etc.) the activation of the *S. cerevisiae* enzyme seems to be smaller, see Figs. 5 and 6.

As seen from the figures the curves mostly show maxima. In some cases, however, the z/z_0-values are almost constant after a certain Me/P-ratio is exceeded. These Me/P-values are rather low, and at these low Me-concentrations probably the shape of the substrate molecule does not differ very much from that of Me-free polymetaphosphate. In Fig. 7 the $(\eta_{sp})_{t=0}$ values corresponding to the curves shown in Fig. 3 are plotted as a function of the Me/P-values. As long as the ratio Me/P does not exceed $\sim$ 0.05—0.10 the changes of the substrate are small if not negligible in comparison with the increase in the z/z_0-values.

The results of the activation experiments are given in Table 1.

Table 1. Data from activating experiments.

	pH	metal	z/z_0 (maximum value)
A. niger	5.4	Zn	6—7
		Mn	6
		Ca	2.2
		Mg	2.2
		Pb	1.5
	6.6	Mn	32
		Zn	25
		Mg	10—12
S. cerevisiae	6.6	Zn	5
		Mn	1.6
		Mg	1.6
	7.3	Mg	2.7

INFLUENCE OF ANIONS ON THE ENZYME ACTIVITY

Fluorine ions. According to Bauer [11] the activity of the pyrophosphatase of yeast (bottom yeast) is inhibited by NaF. However, the activity of the *A. niger* and *S. cerevisiae* enzyme is only slightly inhibited by NaF. See Table 2.

Cyanide-ions. Some enzymes, for instance part of the respiratory ones, are known to be inhibited by cyanide ions. With these polymetaphosphatases the inhibiting effect is rather small in the case of the enzyme from *A. niger*, but is almost complete with that from *S. cerevisiae*. See Table 2. The ionic strength of the buffers used in these experiments is 0.3. The buffer solutions were made 0.005 *M* with respect to NaCN, NaF and $NaNO_3$ (blank solution). Substrate concentration 0.5 %. 1 ml enzyme solution/5 ml substrate solution.

Table 2. Influence of fluorine and cyanide ions on the enzyme activity.

	Substance added to the buffer	z/z_0 Expt. I	z/z_0 Expt. II
A. niger	$NaNO_3$ (blank) $z = z_0 =$ 360 (I), 459 (II)		
acetate buffer	NaF	0.98	0.99
pH = 5.4	NaCN	0.96	0.99
S. cerevisiae	$NaNO_3$ (blank) $z = z_0 =$ 77 (I), 80 (II)		
phosphate buffer	NaF	0.94	0.96
pH = 7.3	NaCN	0.08	0.04

INFLUENCE OF SOME SPECIFIC REAGENTS ON THE ENZYME ACTIVITY

Iodoacetic acid is an inhibitor of enzymes containing the sulphydryl radical -SH as essential group, but has no appreciable effect on the polymetaphosphate degrading enzymes in question.

Arsenite recognised as an inhibitor of some oxidizing enzymes has no influence on the enzyme from *A. niger*, but reduces the activity of the *S. cerevisiae* enzyme to about 30—40 %.

The bile acids generally inhibit the activity of phosphatases according to Uraki[12]. The effect of taurocholic acid on the polymetaphosphatases is, however, rather small. Owing to the difficulty in obtaining satisfactory solutions of the taurocholic acid under these experimental conditions the reproducibility is rather poor.

Formaldehyde in small concentration does not disturb the function of the polymetaphosphate degrading enzymes.

The substrate solution used in these experiments was 0.5 % with respect to K 15 in acetate- and phosphate buffers of ionic strength 0.3. As usual 1 ml of the enzyme solution was added to 5 ml of the substrate solution. The results of the measurements are collected in Table 3.

Table 3. Influence of some compounds on the enzyme activity.

	'Inhibitor' added to 5 ml substrate solution	z/z_0 Expt. I	Expt. II
	none (blank) $z = z_0 = 275$ (I), 427 (II)		
A. niger	5 mg CH_2ICOOH	0.99	0.93
acetate buffer	5 mg Na_3AsO_3	1.15	1.14
pH = 5.4	5 mg taurocholic acid	0.95	1.25
	0.1 ml 35 % formaldehyde	0.98	0.93
	none (blank) $z = z_0 = 83$ (I), 112 (II)		
S. cerevisiae	5 mg CH_2ICOOH	1.04	0.97
phosphate buffer	5 mg Na_3AsO_3	0.39	0.27
pH = 7.3	5 mg taurocholic acid	1.25	0.86
	0.1 ml 35 % formaldehyde	1.10	1.13

DISCUSSION

The mechanism of activating is not definitely known. E. Bauer [9] who worked on pyrophosphatase from yeast assumes the formation of a 'magnesium-bridge' between the substrate and the enzyme. Janner and Kay [3] report in their investigation on phosphatase from kidney that they have found a maximum activation by Mg^{++} ions at a certain concentration of those ions. Their curves resemble in principal those of Fig. 3 in this paper. They ascribe the decrease of the activity to the formation of a substrate-magnesium-enzyme complex with two magnesium atoms, which is formed when the amount of magnesium is greater than that required for the formation of the complex containing one atom of magnesium. However, one must not forget that there is another competitive reaction, *viz.* the complex formation between the Me^{++} ions and the polymetaphosphate. Perhaps the differences of activation of the metal ions in question may be due to differences in the equilibrium positions of these complex formations. Another possibility is that at a certain Me:P ratio the shape and charge or the substrate molecule is most suited to enzymatic breakdown without the Me-atom acting as a bridge between substrate and enzyme.

In a previous work [7] the sedimentation constant of the enzyme from *A. niger* has been determined as 3.2 Svedberg units, using a separation cell and calculating the *s*-value from the analysis of the activity of the original solution and that of the upper part of the cell after the run. The sedimentation constant of the substrate is $\sim$ 26 S in 0.1 % solution in acetate buffer of ionic strength 0.3. The sedimentation constant is largely dependent on the species and concentration of the low molecular salts present in the solution. However, if a complex substrate-enzyme is formed one may expect the sedimentation constant of the enzyme to increase in the presence of its high molecular weight substrate.

In order to decide whether such a complex is formed or not the following experiments were carried out. To 10 ml of a buffered polymetaphosphate solution (0.1 % with respect to K 15) was added 2 ml of an enzyme solution of high activity and the mixture was then immediately run in a separation cell, in the ultracentrifuge. During the run part of the mixture was kept at about the same temperature as that of the rotor chamber and after the run the activities were determined as described above. The data and the results of these runs are collected in Table 4.

As seen from the table there is an increase of the *s*-values at the pH-value neighbouring that of optimum activity. The *s*-values at more acid or alkaline pH-values show no appreciable deviation from 3.2. The *s*-values never amount

Table 4. Sedimentation constant of the enzyme in the presence of its high molecular weight substrate.

Activator	pH	s
none	4.3	4.1
	5.4	4.7
	6.6	6.4
	6.9	4.8
	7.3	3.7
	8.0	3.6
0.15 ml 0.07 *M* $Zn(NO_3)_2$/10 ml substrate solution	4.3	3.6
	6.6	5.3

to that of the substrate because of the breakdown of the polymetaphosphate during the centrifugation. In the presence of activating metal ions the breakdown of the substrate results in a lower s-value than in the absence of the same activator. However, the increase of the sedimentation constant in the experiments described above is not absolutely valid proof of a specific substrate-enzyme combination. It is well known, for instance by the works by Perlmann,[13] Hermann and Perlmann[14] and Briggs[15], that metaphosphoric acid forms compounds with proteins. However, if some essential groups of the enzyme protein are blocked by silver one may expect the enzyme to sediment with normal velocity, *i.e.* $s = 3.2$, even at the pH optimum and in the presence of the high molecular weight substrate, if these essential groups form the substrate-enzyme linkage. Now it is possible to inactivate the enzyme by addition of a silver salt and then revive the activity by removing the silver from the protein by KCN, which does not appreciably decrease the original enzyme activity.

To 5 ml of a 0.1 % K 15 solution in acetate buffer of pH = 5.4, 0.001 *M* with respect to $AgNO_3$, 2 ml of an enzyme solution *(A. niger)* was added. Part of the solution was run in the separation cell in the ultracentrifuge, and after the run the blank solution and centrifuged solution (upper cell) were made 0.015 *M* with respect to KCN. The activities were then measured as usual. Hitherto only three experiments have been carried out and the results $s = 6.6$, 7.9 and 7.3 respectively, seems to indicate that other groups than those blocked by silver atoms are involved in the formation of a substrate-enzyme complex. These investigations are beeing continued and will be extended to systems of an uncharged, non dissociating substrate.

ELECTRODIALYSIS

One could possibly assume that the activating action of some metal ions is due to the fact that the metal atoms play the role of a prosthetic group or part of such a group. However, if this assumption were true one might expect these metal atoms to be rather easily removable by electrodialysis, thus causing the enzymes to lose their activity.

The experiments were carried out in a cell of about 200 ml capacity; current $\sim$ 30 m A; current density $\sim$ 0.3 m A/cm^2, time 8 hours. Part of the enzyme solution was retained as a blank. During the experiment, the blank solution was stored in a test tube in the outer liquid of the electrodialysis cell in order to keep the blank solution at approx. the same temperature as the electrodialysed solution. The activity of both solutions was measured after the electrodialysis.

A. niger. The activity of an enzyme solution (in diluted acetate buffer) was decreased from $z = 865$ (blank solution) to 345 by the electrodialysis. (The activity measurements were carried out on 0.5 % K 15 solutions in acetate buffer of pH = 5.4.) However, if the substrate solution was made 0.01 *M* with respect to $Mg(NO_3)_2$ the activity was increased 2.21 times. Under similar conditions the enzyme blank was activated 2.26 times. Hence it is more likely that the activity decrease is due to denaturation, for instance at the membranes, than to removing of a prosthetic group.

S. cerevisiae. A solution of *S. cerevisiae* enzyme (in diluted phosphate buffer) lost all its activity after electrodialysis under the same experimental conditions as described above for *A. niger*. The activity could not be restored afterwards by addition of zinc or magnesium salt, and it appears that the *S. cerevisiae* enzyme is more easily denatured than that from *A. niger*.

SUMMARY

The enzymatic breakdown of a high molecular weight polymetaphosphate has been studied in the presence of some metal ions under various conditions. Among the metals investigated Zn and Mn are the best activators for the enzymes used (*A. niger* and *S. cerevisiae*); Ag and Hg completely inhibit the enzyme activity.

The formation of a substrate-enzyme complex has been shown by means of the ultracentrifuge using a separation cell. It is not likely that all the essential groups of the enzyme are involved in this complex formation.

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