Purification and Properties of α -D-Mannosidase from Rat Epididymis

By SYBIL M. SNAITH AND G. A. LEVY
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

(Received 14 March 1969)

1. α -D-Mannosidase from rat epididymis was purified 300-fold. β -N-Acetylglucosaminidase and β -galactosidase were removed from the preparation by treatment with pyridine. Zn^{2+} was added during the purification to stabilize the α -mannosidase. 2. Mammalian α -mannosidase is most stable at pH 6. At lower pH values it undergoes reversible spontaneous inactivation. The enzyme is also subject to irreversible inactivation, which is delayed by the addition of albumin. 3. Reversible inactivation of α -mannosidase is accelerated by EDTA and reversed or prevented by Zn^{2+} . Other cations, such as Co^{2+} , Cd^{2+} and Cu^{2+} , accelerate inactivation and the action of a toxic cation can be prevented by Zn^{2+} or by EDTA in suitable concentration. 4. The enzyme is stabilized by substrate and neither Zn^{2+} , EDTA nor a toxic cation has more than a small effect in the assay of an untreated preparation. The addition of Zn^{2+} is necessary, however, for a constant rate of hydrolysis during prolonged incubation of the enzyme with substrate. In an EDTA-treated preparation, Zn^{2+} reactivates the enzyme during the assay. 5. Evidence is presented that α -mannosidase is a dissociable Zn^{2+} -protein complex, in which Zn^{2+} is essential for enzyme activity.

α -D-Mannosidase (EC 3.2.1.24) is of considerable interest in the degradation of glycopeptides. The purification and properties of the enzyme from jack-bean meal were described by Snaith & Levvy (1968a). It was found that Zn^{2+} was essential for the catalytic activity of α -mannosidase. Certain other bivalent cations, such as Cd^{2+} , Co^{2+} and Cu^{2+} , including traces of such ions in the preparation, inactivated α -mannosidase by displacing Zn^{2+} . EDTA, on the other hand, inactivated the enzyme by sequestering Zn^{2+} . Addition of Zn^{2+} to an EDTA-treated preparation at once restored the enzyme activity, and no other cation had this property. Restoration of activity was slow when it demanded the displacement of a toxic ion by Zn^{2+} , without the intervention of EDTA.

The present paper describes a similar study of the α -mannosidase from rat epididymis. Although the mammalian enzyme is of much more general interest, especially with regard to the action of Zn^{2+} , rat epididymis is not quite such a rich source of α -mannosidase as jack-bean meal, and not nearly as readily available. Moreover, rat-epididymal α -mannosidase proved to be very unstable after purification. Its properties were compared with those of the jack-bean-meal enzyme, and further experiments were done with the latter when appropriate.

EXPERIMENTAL

α -D-Mannosidase assay. The hydrolysis of 6mm-*p*-nitrophenyl α -D-mannoside in 0.125M-acetic acid-NaOH buffer, pH 5.0, at 37° was measured as described by Levvy & Conchie (1966). The results are expressed in μg . of *p*-nitrophenol liberated in 1 hr.; 0.1mm- ZnSO_4 was included in the mixture for assaying the fractions during purification.

Other glycosidase assays. Preparations were examined for the presence of other glycosidases as described by Snaith & Levvy (1968a), and in addition for β -glucuronidase (Levy & Conchie, 1966). Results are expressed as μg . of aglycone liberated in 1 hr. at 37°.

Protein determination. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. The final product of the purification procedure (Table 1) had the same chromogenic value as albumin on an N basis. The crude aqueous extract (stage 2, Table 1) had an N content of 21% of the protein, measured as albumin, pointing to the presence of less chromogenic protein that was removed during purification (cf. Findlay & Levvy, 1960).

RESULTS

Purification procedure

General comments. Because of their potential action on glycopeptides, the removal of β -N-acetylglucosaminidase (EC 3.2.1.30) and β -galactosidase (EC 3.2.1.23) was considered to be as important as

Table 1. *Purification of α -mannosidase from rat epididymis*

The enzyme was diluted for the assay of α -mannosidase with 0.8 mM-ZnSO₄, giving a concentration of 0.1 mM in the incubation mixture. Specific activities are expressed as units/mg. of protein, with bovine serum albumin as protein standard.

Stage	Description	Recovery (units/g. of moist tissue)			Specific activity of α -mannosidase
		α -Mannosidase	β -N-Acetylglucosaminidase	β -Galactosidase	
1	Homogenate	110 000	440 000	47 000	1800
2	Aqueous extract	93 000	380 000	39 000	3000
3(a)	0-20% Acetone	4400	9900	800	530
3(b)	20-40% Acetone				
	Wash	25	5400	680	29
	Extract	67 000	110 000	3500	83 000
	Residue	5200	4400	540	520
3(c)	40-60% Acetone	5500	210 000	34 000	300
4	Treatment with pyridine	38 000	3	0	320 000
5(a)	0-55%-satd. (NH ₄) ₂ SO ₄	6000	1	—	110 000
5(b)	55-70%-satd. (NH ₄) ₂ SO ₄	21 000	0	—	570 000
5(c)	70-80%-satd. (NH ₄) ₂ SO ₄	1600	0	—	360 000

increasing the specific activity in the purification of α -mannosidase. The α -mannosidase activity in homogenates of pooled rat epididymides varied from 70 000 to 160 000 units/g. of moist tissue. Extraction of the enzyme from the tissue by aqueous buffers was found to be improved by the addition of sodium chloride.

Results obtained by the final procedure with starting material of average activity are illustrated in Table 1. During precipitations water was added to correct for volume changes after the mixing of the reagents. Precipitates were allowed to settle at 0° for 30 min. before centrifugation at 35 000g for 20 min. at low temperature (below 10°). Centrifuging at low speed (1200g, stage 2) was done at room temperature. A.R. ammonium sulphate was used. From stage 3 onwards precipitates were taken up in 0.1M-acetic acid-sodium hydroxide buffer, pH 5.0, containing 0.1M-sodium chloride and 0.1mM-zinc sulphate. There was more than 300-fold purification of α -mannosidase with 19% recovery. Higher specific activities, up to 670 000, have been reached in other preparations.

Stage 1: homogenate. A mixture of white Wistar and hooded Lister adult male rats (discards from breeding stock) was used. From 100 rats, 104g. of epididymides was obtained. The tissue was cut into small pieces with scissors and suspended in water by using an Ultra-Turrax TP18/2N disintegrator. Acetic acid-sodium hydroxide buffer, pH 5.0, and sodium chloride solution were added to the suspension to give final concentrations of 0.1M of each and a total volume of 5 ml./g. of moist tissue. The homogenate was incubated for 1 hr. at 37° and stored at -20°. It retains its activity for a few weeks,

provided that it is not repeatedly thawed and frozen.

Stage 2: aqueous extract. A portion of the homogenate (200 ml., corresponding to 40g. of epididymis) was thawed and the solid material sedimented by spinning for 15 min. at 1200g. The supernatant liquid was decanted off through a plug of glass wool to retain floating fatty material. The sediment was washed on the centrifuge with its own volume of 0.1M-acetic acid-sodium hydroxide buffer, pH 5.0, containing 0.1M-sodium chloride, and the supernatant liquids were combined.

Stage 3: acetone fractionation. The aqueous extract (200 ml.) was cooled and ice-cold acetone was added to give in turn 20, 40 and 60% (v/v) concentration. The 0-20% and 40-60% acetone fractions were each suspended for assay in the Zn²⁺-containing buffer solution (20 ml.). The material that precipitated at 20-40% acetone was treated differently. It was washed on the centrifuge with 5-10 ml. of 0.1mM-zinc sulphate solution, in which α -mannosidase was virtually insoluble. It was then extracted thrice with the buffer solution. On each occasion, a glass homogenizer (Jencons Ltd., Hemel Hempstead, Herts.) was used to ensure complete suspension and the extract was separated on the centrifuge. The three extracts were combined (total volume 30 ml.) and residual insoluble material was suspended for assay in the buffer solution (20 ml.).

Stage 4: pyridine treatment. Pyridine (0.2 vol.) was added to the extract from stage 3 and the mixture was kept at 25° for 20 min. It was rapidly cooled and ice-cold acetone was added to give 40% (v/v) concentration. After being allowed to settle for 30 min.

at 0°, the precipitated enzyme was centrifuged down and washed on the centrifuge with aqueous 40% acetone. The enzyme was taken up in the buffer solution, and insoluble material was sedimented and washed on the centrifuge with buffer solution, to give a total of 15ml. of supernatant. The discarded debris retained less than 10% of the α -mannosidase activity that survived the treatment with pyridine.

Stage 5: ammonium sulphate fractionation. Saturated ammonium sulphate solution, pH 5, was added to the enzyme solution to give 55, 70 and 80% saturation and at each step the precipitate was dissolved in a small volume of the buffer solution (3–4ml.). Preparation 5(b) (the 55–70%-saturated ammonium sulphate fraction) could not be dialysed, even in the presence of Zn^{2+} and albumin at 4°, without a rapid fall in activity. It could not be stored at 4° or –20° for more than a few days without a considerable loss in activity; this was least when 1% albumin was present.

Removal of other enzymes

Final procedure. It was shown by Snaith & Levvy (1968a) that incubation with 2M-pyridine at 37° freed α -mannosidase in jack-bean-meal preparations from the accompanying β -N-acetylglucosaminidase and β -galactosidase, and precipitated a great deal of inactive protein. As shown in Table 1, pyridine had a similar effect in rat epididymal preparations. Because α -mannosidase from rat epididymis was much less stable in pyridine solution, the temperature of incubation was lowered to 25° and exposure to pyridine was kept as brief as possible.

In addition to α -mannosidase, β -N-acetylglucosaminidase and β -galactosidase, the aqueous extract (stage 2, Table 1) showed the following activities in enzyme units/g. of moist tissue: α -L-fucosidase, 16000; β -glucuronidase, 1300; α -glucosidase, 890; α -galactosidase, 830; β -glucosidase, 610; β -mannosidase, 500; α -N-acetylglucosaminidase, 120. The preparation at stage 4 (Table 1) was free from these other glycosidase activities, except for some β -glucuronidase (500 units/g.), which was removed in the final stage of purification.

Rat epididymis is one of the richest mammalian sources of the enzyme that liberates N-acetylglucosamine from 2-acetamido-1-L- β -aspartamido-1,2-dideoxy- β -D-glucose, i.e. that destroys the glycosylamine linkage in glycopeptides (J. Conchie, personal communication). It was absent from our final product. Jack-bean meal, incidentally, is devoid of this enzyme, as well as of β -glucuronidase.

Other observations. The following observations were made during early attempts to free α -mannosidase in rat epididymal preparations from β -N-

Fig. 1. Effect on the activities of rat epididymal glycosidases of heating at 65° and pH 6.5 for various periods. The aqueous extract of the tissue was made without the addition of NaCl, and the enzymes were precipitated at 20–80% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in water and the solution was adjusted to pH 6.5 with NaOH. The activities (units/ml.) before heating were: α -mannosidase, 1600; β -N-acetylglucosaminidase, 4200; β -galactosidase, 1300. The results are expressed as percentages of the original activities. ●, α -Mannosidase activity; ○, β -N-acetylglucosaminidase activity; ▲, β -galactosidase activity.

acetylglucosaminidase and β -galactosidase. Zn^{2+} was not usually present in the preparations.

Following the observation by Conchie & Hay (1959) that β -galactosidase is less stable to heat than α -mannosidase in a rat epididymal preparation, the possibility was explored of freeing α -mannosidase from β -galactosidase and β -N-acetylglucosaminidase by selective heat inactivation. Fig. 1 shows that heating at 65° and pH 6.5 for 10 min. caused almost complete inactivation of β -galactosidase in a crude ammonium sulphate-precipitated preparation, with only about 10% loss in α -mannosidase activity. β -N-Acetylglucosaminidase was, however, only slightly less stable than α -mannosidase under these conditions.

Using a similar type of preparation, Findlay (1959) showed that 2 μM -mercuric chloride caused 25% inhibition of rat epididymal β -N-acetylglucosaminidase in the assay, rising to nearly 100% when the preparation was incubated under assay conditions for 30 min. before addition of the substrate. Again in the same kind of preparation, Conchie & Hay (1959) found that almost identical effects were produced on the β -galactosidase activity, but with less than 1 μM -mercuric chloride: α -mannosidase was inhibited to the extent of 45% by 5 μM -mercuric chloride in the assay and this value did not increase after 30 min. of preincubation.

Table 2. *Effect of mercuric chloride on α -mannosidase, β -N-acetylglucosaminidase and β -galactosidase in a rat epididymal preparation*

The enzyme preparation was similar to that described in Fig. 1. It was incubated at 37° with 1mM-HgCl₂ in 0.125M-acetic acid-NaOH buffer, pH6, for the periods shown and diluted for assay in the usual way. When cysteine was included in the assay its concentration was 0.2mM. Inhibitions are expressed as percentages of the activities of controls incubated without HgCl₂.

Period of preincubation (hr.)	Inhibition (%)					
	α -Mannosidase		β -N-Acetylglucosaminidase		β -Galactosidase	
	- Cysteine	+ Cysteine	- Cysteine	+ Cysteine	- Cysteine	+ Cysteine
1	80	13	98	63	100	0
2	90	29	98	73	100	0

Attempts to exploit the action of Hg²⁺ to free α -mannosidase from the other two glycosidases by selective inactivation were unsuccessful. Much higher concentrations of mercuric chloride were required when the tissue concentration of the enzyme solution approached that encountered on the preparatory scale, and the effects were to some extent reversible, as could be shown by adding cysteine to the assay mixture (Table 2).

Gel chromatography on Sephadex G-100, as described for jack-bean-meal α -mannosidase (Snaith & Levvy, 1968a), gave no useful separation of α -mannosidase and β -N-acetylglucosaminidase in relatively impure preparations from rat epididymis. When the rat preparation at stage 4 (Table 1) was passed through Sephadex G-100, the recovery of α -mannosidase was very low.

Inactivation of α -mannosidase and the action of Zn²⁺

Reference is made below to two types of enzyme preparation, impure preparations at stage 2 (Table 1) and purified preparations made by an earlier variant of the procedure described for stage 5 (Table 1). The pyridine-treated enzyme was precipitated at 25–35% acetone concentration. This second acetone precipitation gave similar results to the precipitation with ammonium sulphate finally adopted, but it was less reliable in its limits. The properties of the final product were identical in the two cases. In the experiments described below the purified enzyme was freshly prepared and had a specific activity of 400 000–450 000. Although the preparation contained added Zn²⁺, its concentration was lowered by dilution to 1 μ M or less before changes in activity were examined. The Zn²⁺ content of adult rat epididymis is 100 p.p.m. moist weight (J. Quartermann, personal communication), which, if it all came through, would give a concentration of 300 μ M in the undiluted preparation at stage 2 (Table 1). In none of the following exper-

Fig. 2. Decay in α -mannosidase activity on incubation at 37° in the presence and the absence of Cu²⁺ and the restoration of activity by Zn²⁺. The purified preparation (see the text) was diluted 1:120 in 0.1M-acetic acid-NaOH buffer, pH5, containing 0.01% of albumin. After 2 hr. an equal volume of 2mM-ZnSO₄ in the buffered albumin solution was added to a portion of the enzyme solution and incubation was continued. ▲, Enzyme alone; △, enzyme+Zn²⁺; ●, enzyme+5 μ M-CuSO₄; ○, enzyme+Cu²⁺+Zn²⁺. Preparations were further diluted 1:5 before assay, and results are expressed as percentages of the original activity of the unincubated preparation.

iments was zinc sulphate included in the subsequent enzyme assay, unless it was essential for controls.

Reversible and irreversible inactivation. Like α -mannosidase from jack-bean meal (Snaith & Levvy, 1968a), the rat epididymal enzyme was unstable on incubation at 37° and pH5, and this effect increased progressively when the enzyme was purified. With a dilute purified enzyme preparation, it was necessary to add albumin to prevent

irreversible inactivation. For maximum stability of α -mannosidase in such a preparation, Zn^{2+} was required as well as albumin. The lowest concentration at which Zn^{2+} was completely effective was 0.1 mM , but at no concentration did it arrest inactivation in the absence of albumin. Albumin could be replaced by boiled extracts of epididymis.

No other cation was found that could replace Zn^{2+} in stabilizing the enzyme at $0.1\text{--}1\text{ mM}$, and $10\text{ }\mu\text{M}$ - Cu^{2+} , $-\text{Cd}^{2+}$, $-\text{Co}^{2+}$, $-\text{Hg}^{2+}$ and $-\text{Ag}^{+}$ accelerated inactivation. It was possible to demonstrate that Zn^{2+} could reverse inactivation, whether spontaneous or induced by toxic cations such as Cu^{2+} (Fig. 2), provided that irreversible inactivation was excluded.

In the impure preparation, the loss in α -mannosidase activity at 37° was slow, owing in part perhaps to the high Zn^{2+} content of rat epididymis. Addition

of Zn^{2+} was all that was required for complete stability.

Action of EDTA. The action of EDTA on rat epididymal α -mannosidase at 37° and $\text{pH } 5$ depended on the concentration of the chelating agent. At low concentrations ($1\text{--}100\text{ }\mu\text{M}$) it protected the enzyme against spontaneous inactivation, but at higher concentrations inactivation was accelerated. The purity of the preparation was not a factor in the action of EDTA, provided that irreversible inactivation was prevented by the addition of albumin. The protective action of EDTA in low concentration points to the presence of deleterious cations in the preparation as the cause of spontaneous inactivation (Fig. 2).

After inactivation by EDTA, no matter how extensive, α -mannosidase activity was instantaneously restored by the addition of excess of

Fig. 3. Effect on α -mannosidase of incubation for 3 hr. at 37° and $\text{pH } 5$ in 0.1 M -acetic acid- NaOH buffer- 0.01% albumin with different two-component mixtures. The concentration of each component was varied; residual activity with the fixed component alone is shown at the ordinate: (a) Zn^{2+} and Cd^{2+} ; the fixed component is at $10\text{ }\mu\text{M}$. (b) Zn^{2+} and Cu^{2+} ; the fixed component is at $10\text{ }\mu\text{M}$. (c) Zn^{2+} and EDTA; the fixed component is at $100\text{ }\mu\text{M}$. (d) Cu^{2+} and EDTA; EDTA is at $100\text{ }\mu\text{M}$, and inactivation by $1\text{ }\mu\text{M}$ - CuSO_4 and $10\text{ }\mu\text{M}$ - CuSO_4 in the absence of EDTA is shown by points (Δ) on the ordinate. $\bullet$, EDTA concentration varied; $\circ$, ZnSO_4 concentration varied; $\blacktriangle$, CdSO_4 or CuSO_4 concentration varied. Concentrations of the variable component are shown on a logarithmic scale. Results are shown as percentages of the initial activity in an untreated control; the value for this control after 3 hr. at 37° is shown by the broken line. The purified enzyme preparation (see the text) was incubated at $1:100$ dilution, making the initial ZnSO_4 concentration of the solution $1\text{ }\mu\text{M}$, and after incubation it was further diluted $1:5$ for assay.

Zn^{2+} . This is in contrast with the gradual restoration of activity by Zn^{2+} that is illustrated in Fig. 2. No other cation was found to cause any restoration of activity in an EDTA-treated preparation, suggesting that inactivation of α -mannosidase by EDTA is due to sequestration of Zn^{2+} . The order of mixing EDTA-treated enzyme, substrate and Zn^{2+} had no effect on the resultant α -mannosidase activity. Incubating the first two for 10 min. under assay conditions did not prevent the immediate restoration of activity when Zn^{2+} was added. This observation, which was also found to apply to the jack-bean-meal enzyme, supports the argument that the substrate does not combine with the metal-free enzyme (Snaith & Levvy, 1968a).

Neither β -*N*-acetylglucosaminidase nor β -galactosidase was affected by Zn^{2+} or EDTA in rat epididymal preparations.

Displacement curves. The action of toxic cations, such as Cu^{2+} and Cd^{2+} , on rat epididymal α -mannosidase can be explained if it is assumed that the active form of the enzyme is a complex of protein and Zn^{2+} , in which Zn^{2+} is obligatory for enzymic activity, but readily exchanges with other cations. The effects of EDTA are then explicable in terms of sequestration of the different cations. This view is supported by experiments in which Zn^{2+} , EDTA and a toxic cation (Cu^{2+} or Cd^{2+}) were titrated against each other. Two-component mixtures were incubated with the purified enzyme for 3 hr. at pH 5 in the presence of albumin (Fig. 3).

It is shown in Figs. 3(a) and 3(b) that Zn^{2+} had to be present in considerable excess to protect α -mannosidase from inactivation by Cd^{2+} or Cu^{2+} . The effect of excess of EDTA in overcoming the stabilizing action of Zn^{2+} is shown in Fig. 3(c): Fig. 3(c) also illustrates the individual protective effects of EDTA and zinc sulphate at $100\text{ }\mu\text{M}$. Fig. 3(d) shows that $100\text{ }\mu\text{M}$ -EDTA protects the enzyme against $1\text{ }\mu\text{M}$ -, but not against $10\text{ }\mu\text{M}$ -copper sulphate.

The affinity of rat epididymal α -mannosidase for Cu^{2+} was even higher at pH 6. When the experiment in Fig. 3(b) was repeated at this pH, $10\text{ }\mu\text{M}$ -copper sulphate caused almost complete inactivation, and when 1 mM -zinc sulphate was also present, the residual activity did not reach 50%.

Effect of pH on stability. The effect of varying the pH on the stability of α -mannosidase in a dilute purified preparation is shown in Table 3. Before assay, the enzyme was incubated at 37° for 3 hr. in the presence of albumin. Optimum stability was displayed at pH 6, but the presence of Zn^{2+} was still essential. It appears, from the protection afforded by Zn^{2+} , that reversible inactivation became more pronounced at lower pH and irreversible inactivation at higher pH values.

Fig. 4 illustrates the effects of Zn^{2+} and EDTA on the activity of a purified preparation incubated

Table 3. *Effect on α -mannosidase activity in a purified preparation of incubation for 3 hr. at 37° and various pH values*

Addition	pH	Residual activity (%)	
		None	1 mM-ZnSO_4
...	4.5	45	80
...	5.0	61	92
...	5.5	69	90
...	6.0	70	91
...	6.5	58	76
...	7.0	37	33

Fig. 4. Effects of Zn^{2+} and EDTA on α -mannosidase activity on incubation for various periods at 37° at (a) pH 5 and (b) pH 6 (0.1M-acetic acid-NaOH buffer). ●, Enzyme alone; ○, enzyme + 1 mM-ZnSO_4 ; ▲, enzyme + 1 mM-EDTA . A purified preparation (see the text) was diluted 1:200 in 0.01% albumin. The solutions were further diluted 1:5 for assay and the results are expressed as percentages of the original activity of the unincubated enzyme preparation.

for various periods at pH 5 and 6. The difference in pH caused little change in the stability of the enzyme in the presence of Zn^{2+} , but spontaneous

Fig. 5. Hydrolysis at different pH values of 6 mM-*p*-nitrophenyl α -mannoside in 0.125M-acetic acid-NaOH buffer. The purified enzyme (see the text) was diluted 1:200 and incubated with substrate for 1 hr. in the presence (○) and absence (●) of 0.1 mM-ZnSO₄. The α -mannosidase activity is expressed as a percentage of the maximum value obtained in the presence of Zn²⁺.

Fig. 6. Hydrolysis of 6 mM-*p*-nitrophenyl α -mannoside for various periods at 37° in 0.125M-acetic acid-NaOH buffer, pH 5. ●, Enzyme alone; ○, enzyme + 100 μ M-ZnSO₄; Δ, enzyme + 5 μ M-CuSO₄; ■, enzyme + 100 μ M-EDTA. The purified enzyme preparation (see the text) was diluted 1:500 before addition to the incubation mixture.

inactivation or inactivation induced by EDTA was slower at the higher pH value.

As shown in Table 4, the impure enzyme preparation was much more resistant than the purified preparation to irreversible inactivation at pH 7. It was less dependent on added Zn²⁺ at lower pH values.

Table 4. Effect on α -mannosidase activity in an impure enzyme preparation of incubation for 3 hr. at 37° and various pH values

An impure enzyme preparation (stage 2, Table 1) was incubated at 1:20 dilution in 0.1M-acetic acid-NaOH buffer. Similar preparations were incubated in the presence of 1mM-ZnSO₄ and 1mM-EDTA. The solutions were further diluted 1:5 before assay. The results are expressed as percentages of the initial activity of the enzyme preparation.

Addition	pH	Residual activity (%)		
		None	1mM-ZnSO ₄	1mM-EDTA
	4	10	13	0
	5	90	100	25
	6	96	99	85
	7	93	83	94

Effect of pH on activity. Fig. 5 shows pH optima for the hydrolysis of *p*-nitrophenyl α -mannoside by the purified enzyme in the presence and absence of Zn²⁺. At the lower pH values Zn²⁺ activated the enzyme, leading to a shift in the optimum from pH 5 to 4.5. With the impure enzyme the optimum remained at pH 4.5 whether Zn²⁺ was added or not: there was still about 10% higher activity in the presence of Zn²⁺ at this pH. For routine assay, pH 5 was adopted. If Zn²⁺ was included in the assay medium it caused up to 10% activation at all stages of purification: 0.1mM-zinc sulphate was found to be sufficient. Unlike jack-bean-meal α -mannosidase, the rat epididymal enzyme was not activated to any appreciable extent in the assay by the addition of albumin.

Effect of substrate. On incubation at pH 5 with *p*-nitrophenyl α -mannoside under normal assay conditions, rat epididymal α -mannosidase in the impure or purified preparation did not give a constant rate of hydrolysis for more than 1 hr. When Zn²⁺ was added, the rate of hydrolysis was linear for 4 hr. with the purified enzyme (Fig. 6), and virtually so with the impure preparation. At pH 6 added Zn²⁺ was not required for constant velocity over 4 hr., but the enzyme exhibited less than 50% of its maximum activity.

Fig. 6 also shows the effects of EDTA and of Cu²⁺ during the incubation of enzyme with substrate. From comparison of Fig. 6 with Figs. 2 and 4(a) it is evident that the substrate stabilized the enzyme under all conditions. The protective action of substrate with the enzyme at comparable dilutions is demonstrated in Table 5.

There remains to be mentioned one aspect of the action of substrate. In a preparation that had undergone reversible inactivation, as illustrated in Fig. 2, there was no restoration of activity on prolonged incubation with Zn²⁺ when substrate was

Table 5. *Effects of various agents on the incremental activity of α -mannosidase when incubated for various periods at 37° and pH 5*

The activities shown are difference measurements made at hourly intervals. In the cases where substrate is shown to have been absent from the original mixture, it was added for the final 1 hr. of incubation. Results are shown as percentages of the activity in the normal 1 hr. assay. A purified enzyme preparation (see the text) was used. In columns (1)–(4), it was diluted 1:500 before addition to the assay mixture (giving 1:4000 during incubation). In columns (5)–(8), it was diluted 1:200 (giving 1:1000 in the incubation without substrate, and 1:1600 in the assay). Concentrations in the incubation mixture were 100 μ M-ZnSO₄, 5 μ M-CuSO₄, and 100 μ M-EDTA.

Hourly interval	Addition	Activity (%)							
		Substrate present				Substrate absent			
		(1) None	(2) Zn ²⁺	(3) Cu ²⁺	(4) EDTA	(5) None	(6) Zn ²⁺	(7) Cu ²⁺	(8) EDTA
0–1		100	100	93	89	—	—	—	—
1–2		90	111	73	75	62	91	48	48
2–3		72	95	59	56	41	89	27	26
3–4		77	106	45	40	24	79	14	12
4–5		—	—	—	—	22	72	9	7

present. The rate of hydrolysis remained constant at the initial depressed value.

With the purified jack-bean-meal enzyme (Snaith & Levvy, 1968a), Zn²⁺ was not required to obtain a constant rate of hydrolysis during the first 4 hr. of incubation with substrate at pH 5. If a less pure preparation was employed, or if 5 μ M-Cu²⁺ was added to the purified enzyme, excess of Zn²⁺ was essential for constant velocity.

Action of o-phenanthroline and nitrilotriacetic acid. It was suggested above that inactivation of α -mannosidase by EDTA is due to sequestration of Zn²⁺. Had the chelating agent entered into a ternary complex with the Zn²⁺ and protein, other bivalent cations should have been capable of restoring the enzyme activity. A few experiments were carried out with two other chelating agents, o-phenanthroline and nitrilotriacetic acid, which have lower affinities for the cations than EDTA, to test whether there was any evidence for formation of a ternary complex. Because the other two chelating agents had not been tested against jack-bean-meal α -mannosidase (Snaith & Levvy, 1968a), their effects on this were also examined.

When allowance was made for their lower affinities for Zn²⁺, o-phenanthroline and nitrilotriacetic acid followed the same general pattern of behaviour as EDTA towards the enzyme from either source. At low concentration they gave some protection against spontaneous inactivation of the enzyme, but at higher concentration they accelerated inactivation. Like inactivation by EDTA, the latter effect became less marked at pH values above 5. The enzyme was reactivated by subsequent addition of Zn²⁺, but not by other bivalent cations. However, reactivation by Zn²⁺ was never

as complete after o-phenanthroline treatment as after treatment with nitrilotriacetic acid or EDTA. Only very small effects were observed with o-phenanthroline or nitrilotriacetic acid in the usual enzyme assay.

DISCUSSION

Although the preparations were similar in specific activity, rat epididymal α -mannosidase was much less stable in the purified state than the jack-bean-meal enzyme (Snaith & Levvy, 1968a), owing perhaps to the readier dissociation of the protein-Zn²⁺ complex. Without added Zn²⁺, jack-bean-meal α -mannosidase was stable at pH 7 and, if substrate was present, at pH 5. Apart from this quantitative difference, the mammalian enzyme was very similar to the plant enzyme, in particular in its inactivation by EDTA and immediate reactivation by Zn²⁺. This pattern of behaviour with EDTA and Zn²⁺ has been found to extend to α -mannosidase from two other sources, almond emulsin and the limpet *Patella vulgata* (S. M. Snaith & G. A. Levvy, unpublished work).

The catalytic activity of the enzyme can be decreased to less than 10% (and that of jack-bean-meal α -mannosidase virtually to zero) by EDTA, and still exhibit immediate and almost complete reactivation by Zn²⁺. EDTA and Zn²⁺, as shown by the decay and displacement curves, act directly on the enzyme protein, and not on the velocity of hydrolysis of substrate. In its properties α -mannosidase therefore behaves as a dissociable enzyme-metal ion complex, as defined by Vallee (Vallee, 1955; Vallee & Coleman, 1964), and one that is specific for Zn²⁺.

The distinction between enzyme-metal ion complexes and metalloenzymes (Vallee, 1955; Vallee & Coleman, 1964) is an arbitrary one, since it depends on relative dissociation constants and the pH of action. The activating cation can be reversibly detached from several well-authenticated metalloenzymes, such as carboxypeptidase, by working at a pH more acid than usual. Jack-bean-meal α -mannosidase, on the other hand, is highly resistant to the action of EDTA at pH 7, although it is almost inactive enzymically at this pH. If the enzyme could be purified at pH 7, without loss of catalytic activity, it would be possible to determine its Zn^{2+} content, and thus to settle its status as a Zn^{2+} -dependent enzyme.

With regard to the action of rat epididymal α -mannosidase on glycopeptides (Snaith & Levvy, 1968*b*; Pierce & Goetinck, 1964), it is evident that this enzyme will never be a useful structural reagent until it has been obtained in a stabler condition than we have yet achieved. Even then it is unlikely to vie with the enzyme from richer or more readily available sources, such as jack-bean meal, for routine purposes. Nevertheless, the action of the mammalian enzyme in the catabolism of glyco-

proteins must command attention in a number of different connexions.

We are indebted to Mr A. J. Hay and Miss A. E. Robertson for skilled assistance. S.M.S. is in receipt of a grant from the Medical Research Council.

REFERENCES

- Conchie, J. & Hay, A. J. (1959). *Biochem. J.* **73**, 327.
Findlay, J. M. (1959). Ph.D. Thesis: University of Aberdeen.
Findlay, J. & Levvy, G. A. (1960). *Biochem. J.* **77**, 170.
Levy, G. A. & Conchie, J. (1966). In *Methods in Enzymology*, vol. 8, p. 571. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Pierce, J. G. & Goetinck, P. F. (1964). *Abstr. Pap. Amer. Chem. Soc., 148th Meet., Chicago*, no. 124, p. 61c.
Snaith, S. M. & Levvy, G. A. (1968*a*). *Biochem. J.* **110**, 663.
Snaith, S. M. & Levvy, G. A. (1968*b*). *Nature, Lond.*, **218**, 91.
Vallee, B. L. (1955). *Advanc. Protein Chem.* **10**, 317.
Vallee, B. L. & Coleman, J. E. (1964). In *Comprehensive Biochemistry*, vol. 12, p. 165. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.