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EFFECT OF METAL AND HYDROGEN IONS ON THE ACTIVITY AND STABILITY OF ALLANTOICASE

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SUMMARY

1. The preparation of metal-free allantoicase (allantoate amidinohydrolase, EC 3.5.3.4) is described. Thirteen bivalent cations all restored catalytic activity although with differing effectiveness.

2. The pH–activity curves of nine metalloallantoicases were determined. Three groups of cations could be distinguished; the ratio of their optimal activities was 1:0.5:33. The alkaline side of the curves was determined by competitive inhibition of OH^-, forming metal–OH^- complexes. The acidic side was determined by non-competitive inhibition of H^+, which most probably protonates a group involved in the binding of metal ions to enzyme.

3. The acidic side of the pH–activity curve was shifted by the substrates and some competitive inhibitors (oxalate, phosphate, citrate).

4. The activity of metal-free enzyme is halved rapidly at 30°. The same is true for Mn^{2+}–allantoicase at pH values from 4 to 5. The halving process could be repeated by certain pH changes and was prevented by Mn^{2+} at pH above 6 and by the simultaneous presence of Mn^{2+} and allantoate at pH below 6.

5. Various cations (Mn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}), certain substrates and competitive inhibitors protect the enzyme against heat inactivation. The dissociation constant ($3 \cdot 10^{-5}$ M) of the Mn^{2+}–enzyme complex was determined from the protective effect of Mn^{2+} at different temperatures.

INTRODUCTION

Earlier studies[1–4] on allantoicase (allantoate amidinohydrolase, EC 3.5.3.4) from *Pseudomonas aeruginosa* suggested that the enzyme contains Mn^{2+} in the active site. Previous reports dealt with the purification of allantoicase[3], the non-enzymic hydrolysis of the substrates[5,6], the effect of metal ions on this hydrolysis, the specificity of binding subsites[4], and the effect of pH and temperature on some kinetic parameters[4]. The present study aims at a deeper insight into the mechanism of the enzymic reaction by an investigation of the effect of metal and H^+ on the stability and catalytic action of the enzyme.

MATERIALS AND METHODS

Native enzyme. All experiments were performed with the same enzyme preparation (specific activity, 418 units/mg protein) purified as described previously[3]. The activity was tested by measuring the amount of ureidoglycolate formed from allantoate[1]. The purified enzyme preparation is called native enzyme throughout this paper.

Metal-free enzyme. In order to remove metal ions the enzyme was pretreated at 30° and pH 5.85 in mixtures containing per ml, 14.6 μg protein, 10 μmoles Tris, 80 μmoles sodium acetate–acetic acid and 1.25 μmoles EDTA. After 24 h the solution was dialyzed at 4° against 3 changes of 0.1 M acetate buffer (pH 5.85), which contained $1.25 \cdot 10^{-3}$ M EDTA. Total dialysis time was 42 h. The metal-free enzyme obtained was stored in polyethylene tubes and EDTA was kept in it, since the enzymic activity was readily restored even by extremely low amounts of metal ions. The amount of EDTA present did not interfere in the determination of the catalytic activity, which was tested in the presence of an excess of metal ions. Extraction of all solutions with dithizone[7] yielded no better metal-free enzyme preparations and was therefore omitted. The efficiency of the removal of metal ions was measured in tests of the enzymic activity in the absence of added metal ions; this activity never exceeded 2.5% of the original activity and did not increase during storage at −20° in polyethylene tubes for at least 2 weeks. On addition of Mn^{2+} to the metal-free enzyme the activity was enhanced to 90% of the original value.

Half-life time. In studies on the stability of allantoicase, the original activity (a_0) of the enzyme preparation decreased to a lower level (a_1), which varied between 0 and 100% of the original activity. The velocity of inactivation is expressed in terms of the half-life time ($t_{\frac{1}{2}}$), which is the time needed to reach the activity $\frac{1}{2}(a_0 + a_1)$.

Photo-oxidation was performed at 30° in mixtures containing per ml, 14.6 μg protein, 10 μmoles Tris, 80 μmoles buffer of the desired pH and 0.2 mg methylene blue. Samples were illuminated by a 500-W light source focused through an f 2.5 lens; the distance between lens and sample was 9.5 cm. Control tubes with the same composition were kept in the dark for the duration of the experiment; no decrease in activity was observed in the control tubes.

All other materials and methods used were given in a preceding report[1].

RESULTS

Preparation of metal-free enzyme

The purified enzyme did not require the addition of a metal ion for catalytic activity under normal assay conditions and high concentrations of EDTA (up to 0.1 M) did not influence appreciably the enzymic activity during incubation for 1 h. However, enzyme material pretreated at pH 4.7 in the presence of $8 \cdot 10^{-2}$ M citrate was much more active in the presence of Mn^{2+} than in its absence. These results suggested that a metal ion, presumably Mn^{2+}, was tightly bound to the enzyme and could be removed only under special conditions.

The loss of enzymic activity on preincubation with a number of complexing substances was tested both at pH 5.85 and 7.8 (Table I). In the presence of these compounds the activity decreased more rapidly at pH 5.85 than at pH 7.8, whereas

TABLE I

EFFECT OF COMPLEXING SUBSTANCES ON THE CATALYTIC ACTIVITY OF ALLANTOICASE

Preincubation was performed in mixtures containing per ml, 14.6 μg protein, 80 μmoles sodium acetate–acetic acid buffer (pH 5.85) or triethanolamine–HCl buffer (pH 7.8) in the absence or presence of $1.25 \cdot 10^{-3}$ M complexing substance. At different time intervals 0.2 ml of the preincubated mixture was added to 1 ml 0.25 M triethanolamine–HCl buffer (pH 7.8) containing 254 μmoles sodium allantoate, and the enzyme activity was tested at 30°. The loss of activity followed first-order kinetics and $t_{\frac{1}{2}}$ represents the time interval in which 50% of the original activity was lost.

Addition ($1.25 \cdot 10^{-3}$ *M*)	$t_{\frac{1}{2}}$ *(h)*	
	pH 5.85	*pH 7.8*
—	>1000	300
EDTA	4.0	10
1,10-Phenanthroline	4.0	7.5
α,α′-Dipyridyl	7.5	300
8-Hydroxychinoline	30	77
Cysteine	21	190
Dithiothreitol	5.4	300

in the absence of such compounds the reverse was true. EDTA and 1,10-phenanthroline were most effective. The loss of activity was reversible, since addition of Mn^{2+}, Cd^{2+} or Co^{2+} (all $1.2 \cdot 10^{-3}$ M final concentration) after preincubation for 24 h restored the activity to 90.5, 89.4 or 47% of the original value, respectively, when EDTA was used and to 86.4, 80.8 or 45.5%, respectively, when 1,10-phenanthroline was used.

Metal-free enzyme was prepared by pretreatment of native enzyme with EDTA for 24 h at 30° and pH 5.85 (see MATERIALS AND METHODS).

Metal specificity of the enzyme

Table II represents the effect of cations on the activity of metal-free enzyme at pH 7.8, which was in the optimal pH region of the native enzyme[1]. All cations tested enhanced the activity although with differing effectiveness. The metal ions could be divided into 2 classes: one group (Ba^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Sn^{2+}, Zn^{2+} and Fe^{3+}) was easily replaced by Mn^{2+}. If Mn^{2+} was added to incubation mixtures containing these metalloenzymes, which all demonstrate rather low activities, the enzymic activity rose almost instantly to the level of the Mn^{2+}–enzyme; the same metal ions did not protect the enzyme against heat inactivation. The second group (Ni^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}) could not be replaced by Mn^{2+} under these conditions, and all these ions and Mn^{2+} itself were able to protect the enzyme against heat inactivation.

Ag^{+} and Hg^{2+} inhibited the activity of purified enzyme measured for practical reasons at pH 6.5. In the presence of $2.4 \cdot 10^{-3}$ M, $2.4 \cdot 10^{-4}$ M, or $2.4 \cdot 10^{-5}$ M Hg^{2+}, the activity amounted to 2, 60 or 77% of the original value, respectively. Ag^{+} exerted about the same inhibition.

Fig. 1 shows the pH–activity curves of metal-free allantoicase reconstituted with various metal ions. Most allantoicases exposed a broad pH optimum and three levels of maximal activity were observed: 100% (Mn^{2+} and Cd^{2+}), about 50% (Co^{2+}

TABLE II

EFFECT OF CATIONS ON THE ACTIVITY OF THE METAL-FREE ENZYME AT pH 7.8

The incubation mixture at 30° contained per ml, 212 μmoles sodium allantoate, 208 μmoles triethanolamine–HCl buffer (pH 7.8), 2.4 μg metal-free enzyme, 0.2 μmole EDTA and the appropriate metal ion in a final concentration of $1.2 \cdot 10^{-3}$ M. The specific activity of native enzyme was 418 (MATERIALS AND METHODS).

Cation ($1.2 \cdot 10^{-3}$ *M*)	*Specific activity* (*Units/mg protein*)	*Increase of activity after addition of* Mn^{2+} *	*Protection against heat inactivation***
—	8	+	—
Be^{2+}	8.3	+	(+)
Ba^{2+}	19.7	+	—
Ca^{2+}	70	+	—
Fe^{2+}	76	+	—
Sn^{2+}	82.5	—	—
Ni^{2+}	106	—	+
Pb^{2+}	123	—	(+)
Zn^{2+}	123	+	—
Mg^{2+}	140	+	—
Co^{2+}	193	—	+
Cu^{2+}	322	—	(—)
Cd^{2+}	360	—	+
Mn^{2+}	378	—	+
Fe^{3+}	55.6	+	—

* $MnSO_4$ ($1.2 \cdot 10^{-3}$ M) was added after an incubation period of 25 min. In the cases indicated by +, the rate of substrate hydrolysis increased and became equal to that obtained in the presence of Mn^{2+}. In the absence of metal ions a subsequent addition of Mn^{2+} resulted in an increase to half of the activity of the Mn^{2+}–enzyme (*cf.* Fig. 2).

** Experimental conditions are given under *Heat stability of the enzyme.* — stands for no protection by the metal ion, (+) for a lower rate of heat inactivation, and + for protection by the metal ion.

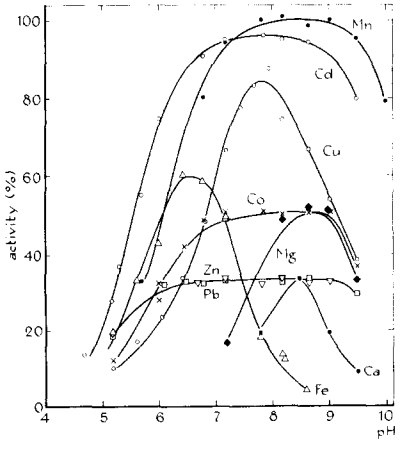

Fig. 1. pH–activity curves of metalloallantoicases. The incubation mixture at 30° contained per ml, 196 μmoles sodium allantoate, 115 μmoles buffer, 1.2 μmoles metal ions and 1.4 μg metal-free enzyme. Buffers used were: sodium acetate–acetic acid (pH 4–5.6), succinate–triethanolamine (pH 5.1–8.2), diethanolamine–HCl (pH 8.2–9.1) and carbonate–bicarbonate (pH 9.5–10). The specific activity of the reconstituted Mn^{2+}–enzyme (100%) amounts to 378 units/ml.

and Mg^{2+}), and about 33% (Zn^{2+}, Pb^{2+} and Ca^{2+}). The maximal activity of the Cu^{2+}– and Fe^{2+}–enzymes was above 80 and 60%, respectively, but the exact location of the maximal levels could not be determined because of the sharpness of the curves. The pH–activity curves of all reconstituted metalloallantoicases appeared to be determined by two dissociation steps of the enzyme or *ES* complex; the p*K* value of each substrate was 3.3 (refs. 5 and 6). From previous studies[4] on the Mn^{2+}–enzyme it appeared that the lower p*K* value (pK_a) must be attributed to the *ES* complex. The pK_a values could be estimated from Fig. 1 and amounted to 5.0 (Zn^{2+} and Pb^{2+}), 5.6 (Cd^{2+}), 5.8 (Co^{2+}), 6.1 (Mn^{2+} and Fe^{2+}), 6.8 (Cu^{2+}) and 7.4 (Ca^{2+} and Mg^{2+}). Less definite information was gathered on the second p*K* value (pK_b). Mn^{2+}–allantoicase was inhibited competitively by OH^-, and it was assumed that OH^- acted by formation of an enzyme $-Mn^{2+}-OH^-$ complex[4].

Substrate and inhibitor specificity of some metalloallantoicases

The specific activity of the native enzyme against allantoate was about 5 times[1] that against (±)-ureidoglycolate. We found the ratio 4:1 for the two substrates in tests with metal-free enzyme reconstituted with Mn^{2+}, Co^{2+} or Zn^{2+}. Replacement of Mn^{2+} by other metal ions resulted in an almost similar susceptibility for competitive inhibitors (Table III).

TABLE III

EFFECT OF COMPETITIVE INHIBITORS ON SOME METALLOALLANTOICASES

The incubation mixtures at 30° contained per ml, 1.1 μg metal-free enzyme, 196 μmoles sodium allantoate, 226 μmoles triethanolamine–HCl buffer (pH 7.8), 1.2 μmoles metal ion and the indicated amount of inhibitor. The activity tested in the absence of inhibitor was in all cases taken as 100%. Activities are expressed in % of these values.

Competitive inhibitor	*Concentration tested (M)*	*Residual activity (%) Essential metal ion*		
		Mn^{2+}	*Co^{2+}*	*Zn^{2+}*
N-Carbamoyl-D-asparagine	$5 \cdot 10^{-3}$	42	56.8	48.4
N-Carbamoyl-DL-serine	$5 \cdot 10^{-3}$	45.2	59.6	63.8
D-Asparagine	$2 \cdot 10^{-2}$	64	34	51.9
Glycolate	$2 \cdot 10^{-2}$	32.6	20.8	37.8

Stability of the enzyme as a function of pH

The stability of the native enzyme as a function of pH is given in Fig. 2. The enzyme showed no loss of activity after pretreatment for 10 or 30 min at 30° and pH values between 5.6 and 12. A rapid inactivation took place at pH above 12 and below 3.8. At pH values between 3.8 and 5.6 only a part of the activity was lost within the first minutes, but a distinct amount of activity remained (Fig. 3). This amount was about half of the original activity at pH between 3.8 and 5.0. Pretreatment in the presence of citrate buffer or EDTA yielded an enzyme material which required Mn^{2+} for full catalytic activity. The rate of inactivation at pH 4.7 ($t_{\frac{1}{2}}$ = 3 min) was retarded in the presence of Mn^{2+} ($1.5 \cdot 10^{-3}$ M; $t_{\frac{1}{2}}$ = 5.6 min), allantoate (0.1 M; $t_{\frac{1}{2}}$ = 90 min), and glyoxylate and (±)-ureidoglycolate (0.05 M; $t_{\frac{1}{2}}$ = 10 min). The inactivation rate at pH 4.7 was retarded further if the substrates were tested in

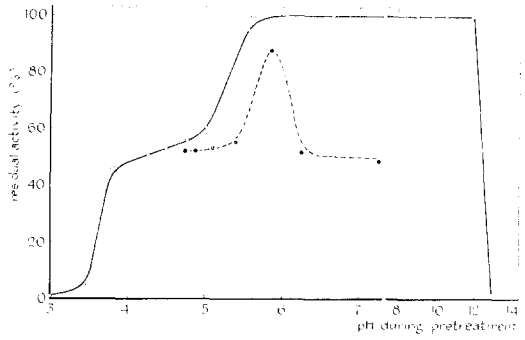

Fig. 2. Stability of native and metal-free enzyme as a function of pH. Preincubation was performed at 30° in mixtures containing per ml, 9.7 μg protein and 86.5 μmoles of the appropriate buffering substance. Buffers used were: formate (pH 3–3.8), acetate (pH 4–5.8), succinate (pH 6–6.7), triethanolamine (pH 7.5–7.8), diethanolamine (pH 8.5–9) and carbonate (pH 9.8–10.5); pH values above 11 were adjusted with NaOH. After 6 min (metal-free enzyme, ●—●) and 10 min (native enzyme, ○—○) 0.2 ml of the pretreated mixtures was added to 1 ml substrate solution which contained 254 μmoles sodium allantoate, 0.25 μmole $MnSO_4$ and 267 μmoles triethanolamine–HCl buffer (pH 7.8) and the activity was tested at 30°. Omission of $MnSO_4$ in the incubation mixtures had no effect on the enzymic activity of the native enzyme.

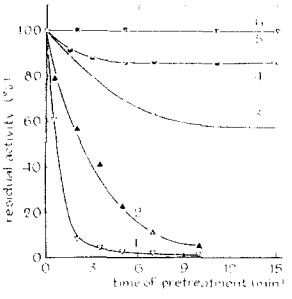

Fig. 3. Effect of the time of pretreatment on the enzymic activity. Pretreatments and activity tests were performed as given in Fig. 2. Buffers used were (1) formate, pH 3; (2) formate, pH 3.5; (3) acetate, pH 4.7; (4) acetate, pH 5.5; (5) succinate, pH 6.5; and (6) triethanolamine, pH 7.8.

the presence of Mn^{2+} ($1.5 \cdot 10^{-3}$ M) and $t_{\frac{1}{2}}$ amounted to 20 min for glyoxylate and (±)-ureidoglycolate and was larger than 1000 min for allantoate.

Native enzyme treated at pH 4.7 lost about 50% of the activity, and this enzyme material was stable at pH 7.8 both in the absence and presence of Mn^{2+}. However, a second pH shift back to 4.7 lowered the enzymic activity to about 25% of the original activity.

The stability of metal-free enzyme is also given in Fig. 2. The highest stability at 30° was observed at pH 5.85 ($t_{\frac{1}{2}}$ = 9 min), and both below and above this pH inactivation occurred rapidly ($t_{\frac{1}{2}}$ about 2 min). Again half of the total activity remained on prolonged treatment. The effect of repeated pH shifts was tested with metal-free enzyme at 30°. Enzyme material whose activity was halved by treatment for 10 min at pH 4.7 or 7.8, again lost half of the remaining activity (25% residual activity) on treatment for 10 min at pH 7.8 or 4.7, respectively. Metal-free enzyme was completely stable when stored for 2 weeks at −20° and pH 5.85.

Metal-free enzyme was stabilized by 0.1 M allantoate neither at pH 4.7 nor at pH 7.8. Therefore, the stabilization of native enzyme resulted apparently from the simultaneous presence of allantoate and small amounts of Mn^{2+}.

These studies on native and metal-free enzyme suggested that halving of the activity of native enzyme at pH 4.7 proceeded *via* formation of metal-free enzyme.

The rate of allantoate and (±)-ureidoglycolate hydrolysis was also 4:1 when measured with native enzyme which had lost half of the activity at pH 4.7.

Heat stability of the enzyme

Allantoicase demonstrated a rather high heat stability[2]. The apparent activation energy of heat denaturation[8] at 60–72° amounted to 51.1 kcal/mole. Allan-

TABLE IV

HEAT STABILITY OF ALLANTOICASE UNDER VARIOUS CONDITIONS

Mixtures containing per ml, 14.6 μg protein, 190 μmoles triethanolamine–HCl buffer (pH 7.8) and 100 μmoles of the indicated substance were heated for 3 min at 71°. The activity was tested as given in Table I after removal of the inhibitors by dialysis against 0.05 M triethanolamine–HCl buffer (pH 7.8).

Substance (0.1 M)	*Residual activity (%)*
—	5
Allantoin*	15
Allantoate	100
(±)-Ureidoglycolate	40
N-Carbamoyl-D-asparagine	48
N-Carbamoyl-L-asparagine	5
N-Carbamoyl-glycine	28.6
Glycolate	23.6
D(−)-Lactate	65.1
D-Asparagine	44.7
L-Asparagine	9.7

* Allantoin was present in a concentration of $4.1 \cdot 10^{-2}$ M.

toate, (±)-ureidoglycolate and some competitive inhibitors[4] protected the enzyme against heat inactivation (Table IV). The same optical specificity was observed in relation to protection against heat inactivation and to competitive inhibition. The substrates glyoxylate and urea and the inhibitors citrate, phosphate and oxalate failed to protect the enzyme. The latter inhibitors will be discussed in the next section.

The native enzyme was very stable in the presence of Mn^{2+} ($3.6 \cdot 10^{-4}$ M), and the decrease of activity followed first-order kinetics (Fig. 4a, $t_{\frac{1}{2}} = 325$ min). In the presence of Cd^{2+}, Co^{2+} or Ni^{2+} ($3.6 \cdot 10^{-4}$ M) the activity dropped rapidly at first ($t_{\frac{1}{2}} = 3$–4 min) but reached an almost constant value after about 20 min; thereafter, the rate of inactivation was equal to that obtained in the presence of Mn^{2+}. Cu^{2+} ($3.6 \cdot 10^{-4}$ M) protected the enzyme only partly and the rate of inactivation followed first-order kinetics ($t_{\frac{1}{2}} = 8$ min). Enhancement of the metal ion concentrations to

TABLE V

INTERCONVERSIONS OF Mn^{2+}–, Cd^{2+}–, Co^{2+}– AND Ni^{2+}–ALLANTOICASES

Metal ion	*Ratio of activity* (%)*	*Level obtained on heat inactivation** (%)*	*Relative recovery (%)*	*Activity of Mn^{2+}-reconstituted enzyme*** (%)*	*Relative recovery (%)*
Mn^{2+}	100	95	95	—	—
Cd^{2+}	95	65	68.4	70	102.5
Co^{2+}	51	48	94	96	102
Ni^{2+}	28	12.8	45.6	52	114

* Data from Table II.
** Data from Fig. 4a.
*** Data from Fig. 4b.

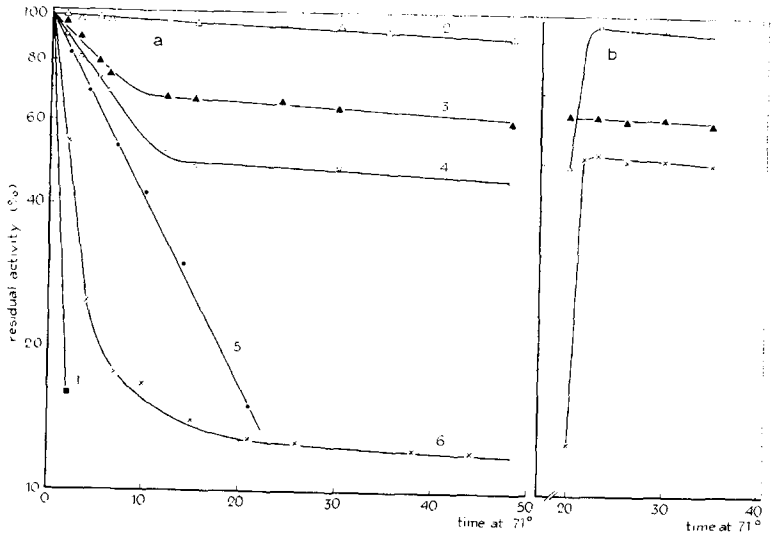

Fig. 4. a. Protection against heat inactivation by bivalent cations. Mixtures containing per ml, 20.5 μg protein and 190 μmoles triethanolamine–HCl buffer (pH 7.8) were heated at 71° in the absence (1) and presence of $3.6 \cdot 10^{-4}$M Mn^{2+} (2), Cd^{2+} (3), Co^{2+} (4), Cu^{2+} (5) and Ni^{2+} (6). At the indicated time intervals aliquots were removed and tested for activity. The incubation mixture contained per ml, 208 μmoles sodium allantoate, 246 μmoles triethanolamine–HCl buffer (pH 7.8) and 3.4 μg protein. b. Reconstitution of Mn^{2+}–allantoicase. Mixtures with the composition as given in Fig. 4a were heated for 20 min at 71° in the presence of $3.6 \cdot 10^{-4}$ M Cd^{2+}, Co^{2+} or Ni^{2+}. Then, the solutions were dialyzed for 24 h against 100 vol. 0.2 M triethanolamine–HCl buffer (pH 7.8) to remove excess Cd^{2+}, Co^{2+} or Ni^{2+}. Mn^{2+} was added to the dialyzed solutions in a final concentration of $1.4 \cdot 10^{-3}$ M and the mixtures were again heated at 71°. At the time intervals indicated aliquots were assayed for activity as given in Fig. 4a.

$1.4 \cdot 10^{-3}$ M did not affect the results obtained after 20 min heating. Be^{2+} and Pb^{2+} ($7 \cdot 10^{-4}$ M) protected the enzyme slightly: after 5 min at 70° in both cases 17% residual activity was found, whereas in the absence of these ions 11% were found. Ag^{+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Sn^{2+}, Ba^{2+}, Al^{3+}, Cr^{3+}, Fe^{3+}, La^{3+}, Ce^{3+} and Ce^{4+} (all $7 \cdot 10^{-4}$ M) did not show any protection.

The relative activities of the enzyme heated in the presence of Cd^{2+}, Co^{2+} and Ni^{2+} are about the same as those of metal-free enzyme reconstituted with these ions (Table V). Most probably, Cd^{2+}–, Co^{2+}– and Ni^{2+}–allantoicases were formed out of Mn^{2+}–allantoicase on heating in the presence of these ions. The exchange reaction with Cd^{2+} and Ni^{2+} was accompanied by partial heat inactivation of the enzyme. Mn^{2+}–allantoicase could be reconstituted from the Cd^{2+}–, Co^{2+}– and Ni^{2+}–enzymes (Fig. 4b). This process proceeded rapidly ($t_{\frac{1}{2}} < 1$ min) and the recovery was about 100%. Addition of Mn^{2+} ($2 \cdot 10^{-4}$ M) to heat-inactivated enzyme did not restore the activity. The rate of heat inactivation depended on the amount of Mn^{2+} present (Fig. 5). If Mn^{2+}–allantoicase (EMn^{2+}) is in equilibrium with Mn^{2+} and metal-free enzyme (E), the equilibrium constant will be $K_D = [E][Mn^{2+}]/[EMn^{2+}]$. The rate of inactivation followed first-order kinetics, $v = k_{obs}[E_T]$, in which E_T represents the total amount of enzyme. The results suggested that EMn^{2+} is stable and E is inactivated at a rate $v = k[E]$. The following equations will be valid

$$k_{obs} = k \frac{K_D}{[Mn^{2+}] + K_D} \quad \text{and} \quad t_{\frac{1}{2}} = \frac{[Mn^{2+}] + K_D}{k K_D} \ln 2$$

in which $t_{\frac{1}{2}}$ is the observed half-life time of heat inactivation. Fig. 5 represents $t_{\frac{1}{2}}$ as a function of the concentration of Mn^{2+}. The intercepts of the horizontal axis revealed K_D, which amounted to $3 \cdot 10^{-5}$ M and appeared to be independent of the temperature between 70 and 85°.

Preliminary experiments indicated that enzyme material whose activity was halved by pretreatment at pH 4.7 was about 10 times more sensitive to heat inactivation at 71°.

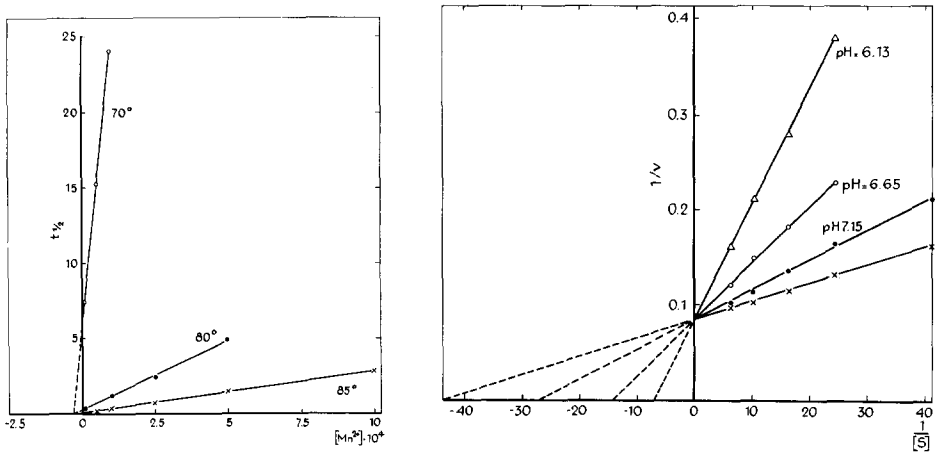

Fig. 5. Effect of the Mn^{2+} concentration on the heat inactivation at different temperatures. Mixtures containing per ml, 36.5 μg protein, 40 μmoles Tris–HCl buffer (pH 7.5) and different concentrations of $MnSO_4$ were heated at 70°, 80° and 85°. At different time intervals 0.2-ml aliquots were assayed for activity as given in Fig. 4a in the presence of $6 \cdot 10^{-4}$ M Mn^{2+}. From the rate of inactivation $t_{\frac{1}{2}}$ was determined.

Fig. 6. Double reciprocal plots of the rate of the allantoicase reaction as a function of the allantoate concentration. The effect of 0.33 M phosphate buffer at various pH values was tested. The experiments were performed as given previously[4]. × — ×, the curve measured in the absence of inhibitor.

pH-dependent inhibition by some competitive inhibitors

Previously[4] the effect of 150 substances on the catalytic activity of allantoicase has been tested and requirements were derived which a substance must fulfill to be a competitive inhibitor. Also citrate, phosphate and oxalate were competitive inhibitors, but these compounds showed hardly any structural similarity with the substrates and other competitive inhibitors. The inhibiting effect depended strongly on the pH of the incubation mixture (Fig. 6). The apparent $*K_i$ values were measured as a function of pH (Fig. 7). The observed phenomena best suited the assumption that the enzyme–inhibitor complex is protonated on lowering the pH and in this way more enzyme is withdrawn from catalytic action. The catalytic process[4] and the inhibiting effect are represented by the equations:

$$E + S \rightleftarrows ES \rightleftarrows EP \rightleftarrows E + P$$

$$E + I \rightleftarrows EI \qquad EI + H^+ \rightleftarrows EIH^+$$

If E_t, E, S, ES, I, EI, H^+ and EIH^+ represent the concentrations of the total amount of enzyme, free enzyme, substrate, enzyme–substrate complex, inhibitor,

enzyme–inhibitor complex, hydrogen ions and protonated enzyme–inhibitor complex, respectively; and if $K_m = E \times S/ES$, $K_i = E \times I/EI$ and $K_a = EI \times H^+/EIH^+$; and if v_0 and v_i are the initial velocities of the enzymic reaction at high substrate concentrations in the absence and presence of inhibitor, respectively, then the following equations are valid:
in the absence of inhibitor:

$$E_t = ES\left(\frac{K_m}{S} + 1\right)$$

in the presence of inhibitor:

$$E_t = ES\left[\frac{K_m}{S} + 1 + \frac{IK_m}{SK_i}\left(1 + \frac{H^+}{K_a}\right)\right]$$

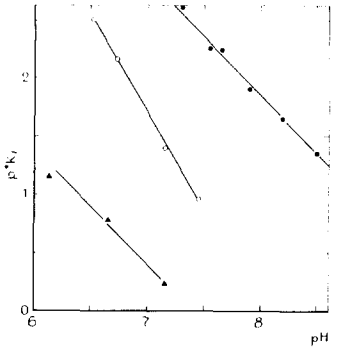

Fig. 7. Negative logarithms of the apparent $*K_i$ values ($p*K_i$) as a function of pH. Phosphate (▲), citrate (○) and oxalate (●) were tested.

The apparent $*K_i$ given in Fig. 7 will be

$$*K_i = \frac{K_a}{K_a + H^+} \cdot K_i$$

Moreover,

$$\frac{v_0}{v_i} = 1 + \frac{IK_m(K_a + H^+)}{K_iK_a(K_m + S)} \qquad (1)$$

or

$$\frac{v_0 - v_i}{v_i} = \frac{IK_m(K_a + H^+)}{K_iK_a(K_m + S)} \qquad (2)$$

Fig. 8 shows $\log v_i/(v_0 - v_i)$ as a function of pH. K_a is small compared to $[H^+]$ in that part of the graphs where the slopes are $+1$. Moreover, the effect of pH on the enzymic activity in the absence of inhibitors is shown. It appeared from previous experiments[4] that H^+ acts as a noncompetitive inhibitor of the enzyme. Since K_m appeared constant in the pH region around 5, the K_a values of E and ES must be about equal. In a manner similar to that given above the following relation between

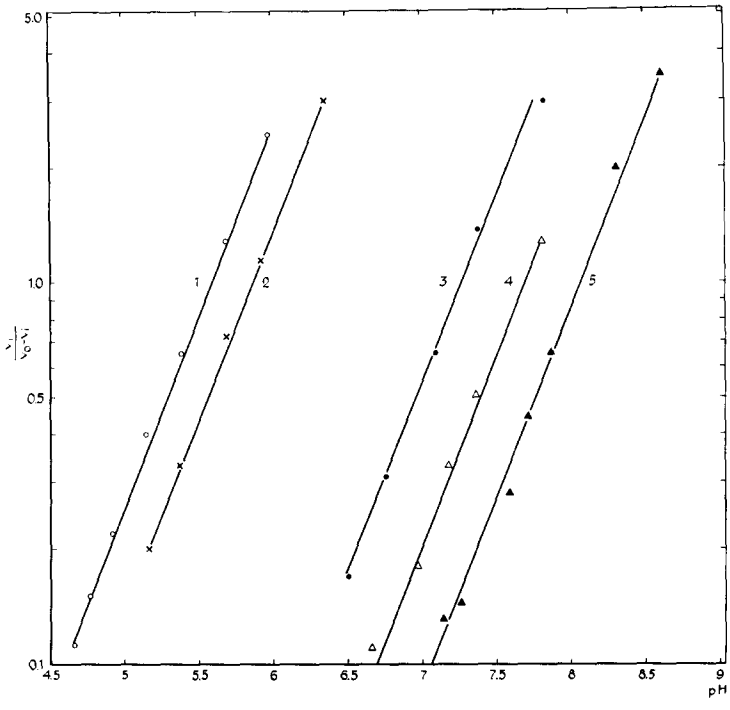

Fig. 8. pH-dependent inhibition by competitive inhibitors. Incubation was performed at 30° in mixtures containing per ml, 193 μmoles sodium allantoate or sodium (±)-ureidoglycolate, 0.23 μmole $MnSO_4$, 2.2 μg protein and 76 μmoles buffering substance containing the desired concentration of inhibitor. Curves 1 and 2 were measured in the absence of inhibitors with allantoate and (±)-ureidoglycolate as substrates, respectively. Curves 3–5 were measured, with allantoate as substrate, in the presence of 0.8 M citrate, 0.04 M oxalate and 0.08 M oxalate, respectively. Buffers used (acetate, succinate–triethanolamine, Tris, triethanolamine and diethanolamine) did not affect the catalytic activity. Activity was tested by measuring the amount of ureidoglycolate (Curves 1, 3–5) or glyoxylate formed (Curve 2).

v_0 and v_i is found, in which v_i represents the velocity of the reaction inhibited by H^+:

$$\frac{v_0 - v_i}{v_i} = \frac{H^+}{K_a} \qquad (3)$$

The K_a and K_iK_a values determined from Figs. 7 and 8 are given in Table VI. The pK_a values of the phosphate–, citrate– and oxalate–enzyme complexes could not be determined, but the values of K_iK_a and the course of the graphs of Figs. 7 and 8 indicated that the pK_a values are larger than 7.0, 7.3 and 8.5, respectively. The presence of EDTA ($8 \cdot 10^{-2}$ M) did not affect the pK_a value measured in the absence of inhibitors, nor did a variation in the allantoate concentration (0.025–1 M). The inaccuracy of the results is partly due to the fact that K_a was considered negligible as compared to the concentration of H^+. Moreover, the inhibiting effect of $H_2PO_4^-$ appeared to be about 3 times larger than that of HPO_4^{2-}. The results obtained with citrate are more complex than considered so far, since the slope of the plot for citrate is not −1 but −1.68 (Fig. 7). This indicates that more than one H^+ is involved in the protonation or that the dianionic form is a much stronger inhibitor than the trianionic form.

TABLE VI

EFFECT OF SOME pH-DEPENDENT, COMPETITIVE INHIBITORS OF ALLANTOICASE

Experiments were performed as given in Figs. 6, 7 and 8 in the presence of the indicated amount of inhibitors.

Substrate	*Inhibitor*	*Concentration of inhibitor (M)*	*pH at which* $2v_i = v_o$	K_iK_a	*Equation used*
(±)-Ureidoglycolate	—		5.85	$1.41 \cdot 10^{-6}$*	3
Allantoate	—		5.55	$2.82 \cdot 10^{-6}$*	3
	Phosphate	$8 \cdot 10^{-2}$	5.7	$1.9 \cdot 10^{-8}$	2
		$3.2 \cdot 10^{-1}$	6.05	$3.3 \cdot 10^{-8}$	2
		$3.3 \cdot 10^{-1}$	—	$4.2 \cdot 10^{-8}$	1
	Citrate	$8 \cdot 10^{-2}$	6.9	$1.2 \cdot 10^{-9}$	2
		$8 \cdot 10^{-1}$	7.25	$5.3 \cdot 10^{-9}$	2
		$2-10 \cdot 10^{-2}$	—	$2 \cdot 10^{-9}$	1
	Oxalate	$8 \cdot 10^{-3}$	6.95	$1 \cdot 10^{-10}$	2
		$4 \cdot 10^{-2}$	7.7	$9.4 \cdot 10^{-11}$	2
		$8 \cdot 10^{-2}$	8.05	$8.4 \cdot 10^{-11}$	2
		$1.8 \cdot 10^{-2}$	—	$1.4 \cdot 10^{-10}$	1

* These values represent K_a and not K_iK_a.

Photo-oxidation of the enzyme

The effect of pH during pretreatment (Fig. 2) and incubation (Fig. 8) led us to consider the possibility that a histidine residue was involved in the catalytic action of allantoicase. Therefore, the effect of photo-oxidation on the activity was studied. The susceptibility of the enzyme to photo-inactivation was independent of pH in the region 5.6–8. Photo-inactivation followed first-order kinetics ($k = 0.13$ min^{-1}) during the illumination time measured (30 min). In the presence of methylene blue the photo-oxidation of histidine, free or bound, is markedly dependent on pH[9,10]; tryptophan, tyrosine and methionine do not show this pH-dependence[9]. However, photo-inactivation of rabbit aldolase was independent of pH between 5.5 and 8, although loss of activity paralleled destruction of histidine residues[11]. Therefore, we cannot conclude that histidine plays a part in the catalytic action of the enzyme. A final answer may arise from the study of the amino acid composition of native and photo-oxidized enzyme.

DISCUSSION

Thirteen bivalent cations were tested for their effect on the enzymic reaction of allantoicase. All appeared to enhance the activity although with differing effectiveness (Table II).

The pH–activity curves of nine of the metalloallantoicases were studied extensively (Fig. 1). At the alkaline side of the graph, OH^- behaves as a competitive inhibitor, and the pK value of the group involved depends on the nature of the metal bound to the enzyme.

At the acidic side of the graph H^+ protonates a group with a dissociation constant K_a and behaves as a noncompetitive inhibitor. The pK_a values appear to

depend on the presence of competitive inhibitors and on the nature of the substrate and of the metal bound to the enzyme. The competitive inhibitors affecting the pK_a value are complexing substances, *viz.* oxalate, phosphate and citrate, and they expose a strongly pH-dependent inhibition. However, not all complexing substances exhibited this effect; for instance, 0.1 M EDTA did not inhibit the enzymic reaction at pH 7.8. In tests with allantoate as substrate, the K_a value observed is 2 times higher than in tests with (±)-ureidoglycolate as substrate. In the sequence Zn^{2+}–, Pb^{2+}–, Cd^{2+}–, Co^{2+}–, Mn^{2+}–, Fe^{2+}–, Cu^{2+}–, Ca^{2+}– and Mg^{2+}–allantoicases, pK_a increases. This sequence shows hardly any relationship to the stability constants of typical Me^{2+}–N or Me^{2+}–S complexes[12] (where Me^{2+} is a bivalent metal ion) but follows fairly well the stability constants of Me^{2+}–O complexes, like metal–lactate and metal–glycerate complexes[12]. This would suggest the involvement of a Me^{2+}–O group in the catalytic mechanism.

If all K_a values measured refer to the same group, this group must be part of the enzyme–metal complex, since the pK values of the substrates and the competitive inhibitor oxalate are much smaller than 6. The group is involved most probably in the binding of the metal ion, since K_a depends on the nature of the metal ion. Protonation of the group does not affect the affinity of the enzyme for the substrates, since K_m is invariant with changing pH from 4.5 to 8.5 (ref. 4), but the activity of the enzyme is strongly influenced. The results suggested that the group is an amino acid residue which plays a part in metal binding and has an essential role in the reaction mechanism of the enzyme.

All bivalent cations tested enhance the activity of the metal-free enzyme. Three groups of metals could be distinguished with 100%, 50% and 33% relative optimal activity, respectively. The various metalloallantoicases are about equally sensitive to certain competitive inhibitors and exhibit the same ratio of activities against allantoate or (±)-ureidoglycolate as substrate.

Besides its effect on the activity, metal ions strongly affected the stability of the enzyme. Ni^{2+}, Co^{2+}, Cd^{2+} and Mn^{2+} and to some extent also Pb^{2+}, Cu^{2+} and Be^{2+} protected the enzyme against heat inactivation. These metal ions are rather strongly bound since replacement by Mn^{2+} was negligible at 30° but occurred rapidly at 71° in the case of Ni^{2+}, Co^{2+} and Cd^{2+}. Other bivalent cations tested do not protect against heat inactivation and are readily replaced by Mn^{2+} at room temperature.

The dissociation constant of the enzyme–Mn^{2+} complex was determined from the protecting effect exerted by Mn^{2+} against heat inactivation. The constant was invariant with changing temperature between 70 and 85° and amounted to $3 \cdot 10^{-5}$ M; the complex constants of several other Mn^{2+}–ligand complexes (*e.g.* oxalate, thioglycolate and glycine) are almost independent of temperature[12]. Other enzymes binding Mn^{2+} have dissociation constants which amount to $4 \cdot 10^{-7}$ M for alkaline phosphatase[13], $2 \cdot 10^{-5}$ M for glutamine synthetase[14], $6.3 \cdot 10^{-5}$ M for pyruvate kinase[15] and $3.9 \cdot 10^{-5}$ M for phosphoenolpyruvate carboxykinase[16]. The enzyme is protected against heat inactivation by allantoate, (±)-ureidoglycolate and some competitive inhibitors. A similar effect is observed for other enzymes[17].

At pH above 12 and below 3.8 allantoicase is inactivated rapidly. The results observed between these pH values are noteworthy. At pH above 5.6 native enzyme is stable most probably due to the presence of Mn^{2+}. At pH between 5.6 and 3.8 the activity of native enzyme drops rapidly to half of the original value; the same holds

for metal-free enzyme at pH values from 3.8 to above 7.6. The residual activity is again halved when the pH is shifted to pH values above 6 and then again to values below 5.6 in the case of native enzyme, and when the pH region of 6 is passed in the upward or downward direction in the case of metal-free enzyme.

These data are too scanty to allow the elucidation of the mechanism of the halving process. A starting point may lie in a recent suggestion of HANSON[18] on the structure of enzymes. If an enzyme is composed of constitutionally equivalent subunits, which may adopt two alternative conformations according to their location in a sandwich structure, then the enzyme would be expected to have two sets of equivalent active sites. In the extreme case one set would be completely inoperative. Inactivation of the operative sites followed by dissociation and reassociation of the subunits should statistically restore 50% of the original activity.

The suggestion of HANSON[18] is supported by recent results obtained with some enzymes. O'LEARY AND WESTHEIMER[19] suggested that half of the active sites of acetoacetate decarboxylase are inactive or hidden and inactivation of metal-free glutamine synthetase at alkaline pH values results in a loss of about half of the original activity[14]. In this connection it is noteworthy that in some metalloenzymes only half of the metal ions participate in the enzymic reaction[13].

According to this view the halving process observed in the case of allantoicase would be a result of an inactivation process of part of the active sites accompanied by dissociation and reassociation of subunits. Mn^{2+} prevents the inactivation at pH values above 5.6. Allantoate stabilizes the enzyme also at low pH values in the presence of Mn^{2+}. It is known that substrate molecules can strongly influence dissociation reactions[20].

Preliminary results[3] indicate the existence of a subunit structure of allantoicase, and work is in progress to study the quaternary structure in more detail.

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