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AMMONIUM HEPTAMOLYBDATE, AN INHIBITOR OF PLANT INVERTASES

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Key Word Index—*Saccharum officinarum*; Gramineae; sugar cane; invertase; ammonium heptamolybdate; inhibition.

Abstract—Ammonium heptamolybdate was an inhibitor of plant invertases. The inhibition was a linear mixed type and the constants K_i and aK_i were determined. α- and β-glycerophosphate, 2,3-diphosphoglycerate, glucose-1-phosphate, phosphoenolpyruvate, pyruvate and malate suppressed the inhibition. The curves of enzyme recovery against the concentrations of these activators were sigmoid. UV spectrophotometry showed complex formation between inhibitor and each activator, and indicated that sucrose did not form a complex with the inhibitor. Consequently, heptamolybdate is postulated to act by a reversible binding to the enzyme.

INTRODUCTION

The inhibitory action of 'ammonium molybdate' on some glycosidases has been known since 1949 when the inhibition of the Q-enzyme was reported [1]. Later, papers on the inhibitory action of ammonium molybdate on R-enzyme [2, 3], yeast isoamylase [4] and limit dextrinase [3] were published but the nature of the inhibition has not been examined. Furthermore, the commercial 'ammonium molybdate' is in fact the product known as ammonium heptamolybdate or ammonium paramolybdate, whose formula is represented by $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$; this is probably the inhibitory substance used in the cited works.

The present paper reports, for the first time, the inhibitory action of ammonium heptamolybdate on plant invertases. Effects of inhibitor concentration, pH and substrate concentration were determined, together with the activating effect of some chemicals when added to the reaction mixture. Possible mechanisms for both inhibition and activation are given.

RESULTS

The inhibitory effect of increasing concentrations of ammonium heptamolybdate on the soluble leaf-sheath invertase from sugar cane is shown in Fig. 1. The inhibitory effect tends asymptotically to a minimum value with increasing concentrations of the inhibitor. The inhibition is not due to the ammonium ion since 5 mM $(NH_4)_2SO_4$ and 12 mM NH_4Cl did not show inhibitory effect. Possible influence of the ionic strength can be discarded since 0 to 300 mM NaCl did not show any effect on the reaction. Therefore, the inhibitory effect is specifically caused by the 'molybdate' and not by the ionic strength or ammonium ion.

Effect of pH on inhibition

The effect of pH on the inhibitory action of 1.5 mM ammonium heptamolybdate is shown in Fig. 2. The inhibitor produces a displacement of the optimum pH of leaf-sheath soluble invertase from 5 to 6 accompanied by a loss of activity. Higher inhibitor concentrations produce an increasing inhibition even at pH 6. The apparent shift of the optimum pH may be explained by the properties of the heptamolybdate whose structure changes according to the pH of the solution. Higher pHs favour the simplest molecular forms; lowering the pH induces the formation of more complex poly acids of molybdenum [9, 10]. Thus, the concentration of the inhibitor poly acid decreases with the pH increase, producing an apparent shift of the optimum pH of the enzyme.

Effect of addition of ammonium heptamolybdate on enzyme extract

When the concentrations of an enzyme extract containing a reversible inhibitor are varied in incubation mixtures, the plot of enzyme concentration against activity will be a curve [11]. Such a curve was obtained when the sugar cane invertase used contained 10 mM ammonium heptamolybdate (Fig. 3). This result would support the fact that heptamolybdate is a reversible inhibitor.

Kinetics of inhibitory effect

The effect of increasing substrate concentrations in the presence of various inhibitor concentrations is shown in Fig. 4. The inhibition corresponds to a linear mixed type case. The system is adequately described by the equation:

$$v = \frac{\{V/(1 + [I]/aK_i)\} + [S]}{K_m\{(1 + [I]/K_i)/(1 + [I]/aK_i)\} + [S]}$$

which is based on quasi-equilibrium assumptions [12], where [I] is the inhibitor concentration, [S] the sub-

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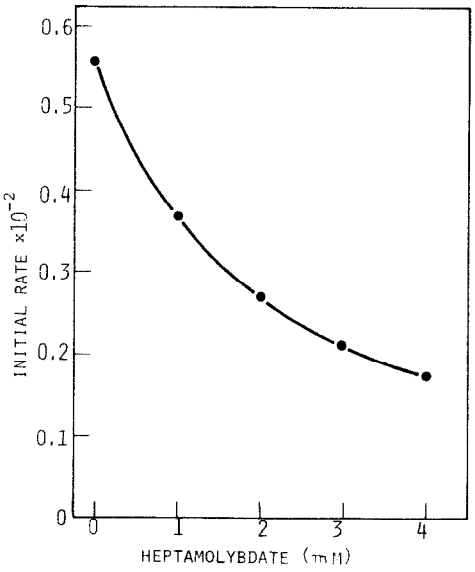

Fig. 1. Effect of ammonium heptamolybdate concentration on the activity of leaf-sheath invertase.

strate concentration, V the maximum rate of the reaction, K_i and aK_i the dissociation constants of the complex EI and EIS, respectively, according to the equilibrium:

$$\begin{array}{ccccc} E + S & \overset{K_m}{\rightleftharpoons} & ES & \overset{k_p}{\rightarrow} & P + E \\ + & & + & & \\ I & & I & & \\ \Big\downarrow\Big\uparrow K_i & & \Big\downarrow\Big\uparrow aK_i & & \\ EI + S & \overset{aK_m}{\rightleftharpoons} & EIS & & \end{array}$$

The value of $K_i = 1.1$ mM was calculated from the replot of slopes versus inhibitor concentration and

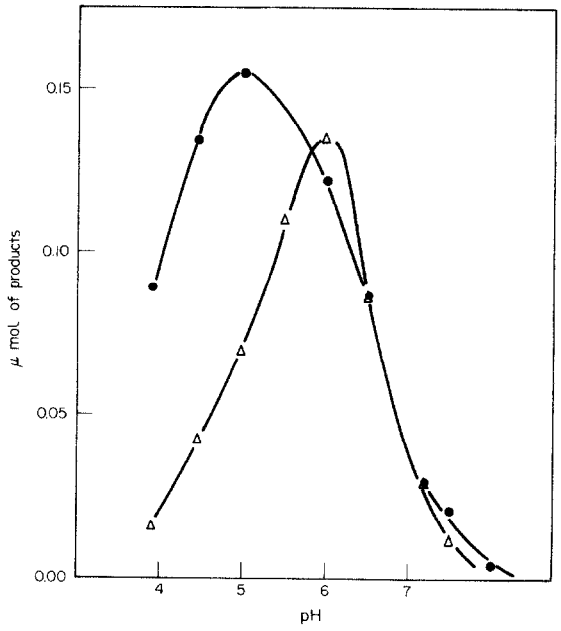

Fig. 2. pH dependence of invertase activity. ●——● Without ammonium heptamolybdate; △——△ with 1.5 mM ammonium heptamolybdate.

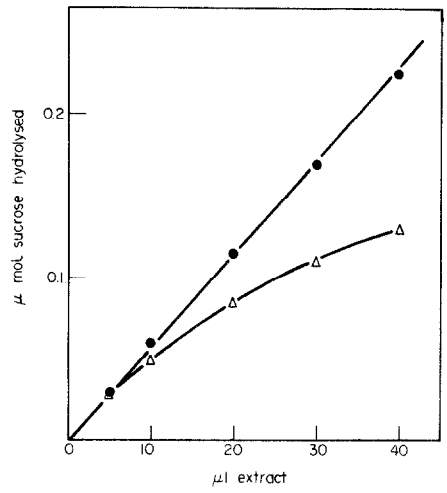

Fig. 3. Effect of dilutions of a mixture of 200 μl of enzyme and 100 μl 10 mM ammonium heptamolybdate. Control ●——●; mixture △——△.

$aK_i = 3.1$ mM from the replot of $1/V_i$, where V_i is the inhibited maximum rate, versus inhibitor concentration.

Effect of chemicals on inhibition by ammonium heptamolybdate

Different chemicals were added to incubation mixtures containing the inhibitor. Several of these chemicals produced an activity enhancement which was augmented by increasing concentrations of the chemicals in the reaction mixture. β-Glycerophosphate overcomes the inhibition by heptamolybdate after 10 min of enzyme incubation

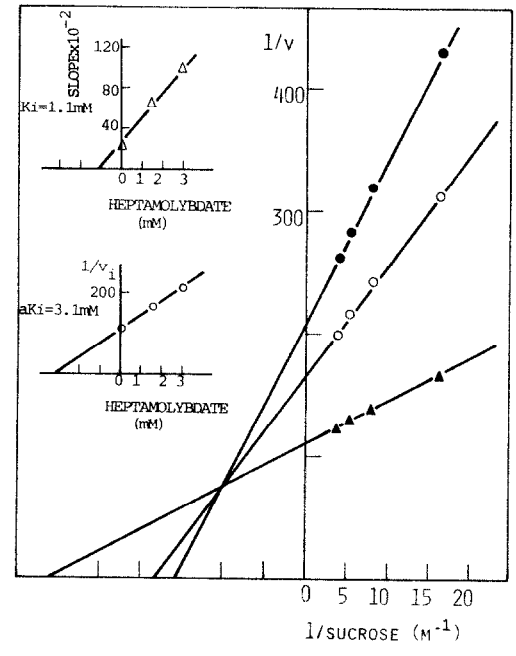

Fig. 4. Double reciprocal plot and replots of the inhibitory effect of ammonium heptamolybdate on leaf-sheath invertase from sugar cane. ▲——▲ Without inhibitor; ○——○ with 1.5 mM ammonium heptamolybdate; ●——● with 3 mM ammonium heptamolybdate.

with the inhibitor. This effect was also observed after 15 and 30 min of inhibition. Consequently, the activators appear to reverse the effect of the inhibitor after it is established. Sigmoid curves were found by plotting the per cent activation against concentration of sodium α- and β-glycerophosphate, sodium 2,3-diphosphoglycerate, glucose-1-phosphate, phosphoenolpyruvate and pyruvate. Even at pH 6 these chemicals suppressed the inhibition. Sodium tartrate and ATP produced a slight reinforcement of the inhibition by heptamolybdate, although tartrate by itself was not an inhibitor of the soluble invertase from sugar cane leaf-sheath. Finally, malate activated the inhibited enzyme. In general all of these substances were without effect in the absence of the inhibitor. Glycerol (10 mM) was without effect on the inhibited reaction. The term activation for this effect was used according to Reiner [13].

Complex formation between ammonium heptamolybdate and activating chemicals

The differences in the UV spectra of ammonium heptamolybdate and 'effector' mixtures, and of ammonium heptamolybdate alone were interpreted to be due to a complex formation. Maximal differences between both spectra were present at 270 nm, and these differences were used to quantify the complex formation at increasing effector concentrations. The plot of these differences against α-glycerophosphate concentrations are shown in Fig. 5. Sucrose did not change the ammonium heptamolybdate spectrum.

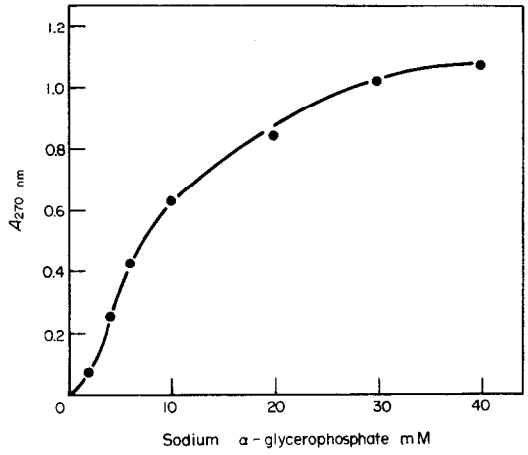

Fig. 5. Complex formation between ammonium heptamolybdate and increasing amounts of α-glycerophosphate.

Table 1. Inhibition of invertases by 2 mM ammonium heptamolybdate

Invertase source	pH of assay	Inhibition (%)
Yeast	5.5	30
Sugar cane leaf-sheath	5.5	54
Carrot roots	4.5	20
Turnip roots	6.0	26.2
Radish roots	6.0	14
Sweet potato	7.0	31

Effect of ammonium heptamolybdate on other plant invertases

Higher and lower plant invertases appear to be inhibited by ammonium heptamolybdate. Thus, acid invertases from yeast, sugar cane leaf-sheath and carrot, radish, turnip roots were inhibited by this substance. Alkaline (or neutral) invertase from sweet potato was also inhibited as shown in Table 1. Sugar cane invertase showed the maximal inhibition but the difference may probably be explained by the higher purification of this enzyme.

DISCUSSION

Ammonium heptamolybdate was shown to be an inhibitor for plant invertases. As the invertases from a monocotyledon, four dicotyledons and a lower plant were inhibited, it appears that heptamolybdate is a general inhibitor for plant β-fructofuranosidases.

The mechanism of this inhibition appears to consist of a reversible binding of the inhibitor to the enzyme molecule as is suggested by the lack of complex formation between substrate and ammonium heptamolybdate, the plot of Fig. 3 and the kinetics of the inhibition, which agrees with a case of inhibitor binding to the complex ES and to free enzyme. The activating effect of compounds such as β-glycerophosphate appears to be related to the complex forming capacity of ammonium heptamolybdate. Malate is a well known complexing agent for ammonium heptamolybdate [14], and is also able to activate the enzyme with a sigmoid response curve analogous to that of the β-glycerophosphate. Consequently, it appears that similar mechanisms are working in all cases. UV spectra of heptamolybdate in the presence of α- and β-glycerophosphate, 2,3-diphosphoglycerate, pyruvate and phosphoenolpyruvate confirm complex formation. All of these compounds have in common a group capable of protonization adjacent to a hydroxyl or to another protonizable group. Thus, the activation suggests an equilibrium between the inhibitor bound enzyme species and the free inhibitor, which is complexed by the enzyme activating agent with the corresponding enzyme liberation from the enzyme complexes containing the inhibitor.

EXPERIMENTAL

Enzymes. Yeast invertase was obtained by autolysis according to the procedure of ref. [5]. Extracts of 280 enzyme units/ml were obtained. Commercial carrot, turnip and radish roots (locally obtained) were peeled, cut into small pieces and treated as stated in a previous communication for sugar cane glycosidases [6]. Extracts containing *ca* 55–100 enzyme units/ml were used. Sweet potato invertase was prepared according to ref. [7] with some modifications: 160 g of cut roots were homogenized in 160 ml 10 mM K-Pi buffer, pH 7. The homogenate was squeezed through 2 layers of cheesecloth and centrifuged for 20 min at 15000 rpm. The supernatant was percolated through a column of Bio-Gel P6 (1.9 × 20 cm) pre-equilibrated with the homogenization buffer. The extract (free from low MW substances) contained 4 enzyme units/ml.

Sugar cane acid invertase. Leaf-sheaths (300 g) were cut into small pieces and homogenized in 300 ml 10 mM Na-Pi buffer, pH 7.5 with 1 mM mercaptoethanol and 5 μM $MnSO_4$. The homogenate was squeezed through 2 layers of cheesecloth and centrifuged for 10 min. The extract was satd with $(NH_4)_2SO_4$

and 10 min later centrifuged at 17000 rpm for 10 min. The ppt. was suspended in 2 ml 10 mM NaOAc buffer, pH 5.5 with 1 mM mercaptoethanol and dialysed against the same buffer for 2 hr. The dialysate was partially adsorbed on 0.081 mg calcium phosphate gel/mg of protein. The suspension was centrifuged and the supernatant was fractionated on a Pharmacia column K 25/45 filled with Sephadex G-150, preequilibrated with the same NaOAc buffer. The invertase which elutes between 80 and 130 ml was pooled and reconcentrated with solid $(NH_4)_2SO_4$ as previously stated. This enzyme was purified *ca* 20-fold with this procedure. The extract contained *ca* 19.6 mg of protein/ml and *ca* 170 enzyme units/ml. All procedures were carried out at 0–4°. All enzyme extracts were kept at −20°.

Proteins. Protein was estimated by *A* readings at 260–280 nm.

Enzyme units. 1 unit of enzyme was defined as the amount which hydrolyses 0.01 μmol of substrate/min at 37° at pH 5.5 or 7.

Enzyme assay. (a) *Sugar cane and carrot root invertases.* The reaction mixture for acid invertases consisted of 0.05 M NaOAc buffer, pH 5.5, 0.06 M sucrose, enzyme and H_2O in a final vol. of 0.01 ml. Ammonium heptamolybdate and activators were included up to the concn indicated in each expt and the pHs were adjusted to the indicated value. Ammonium heptamolybdate was the analytical reagent 'ammonium molybdate' from Mallinckrodt, lot. 1313. (b) *Yeast, turnip roots, radish roots and sweet potato invertases.* The reaction mixture consisted of 0.1 M NaOAc buffer, pH 5.5 or K-Pi buffer, pH 7, 0.045 M sucrose, enzyme and variable ammonium heptamolybdate amounts in a final vol. of 0.2 ml. In the case of sweet potato invertase, the inhibitor soln was adjusted to pH 7. All the incubations were performed at 37°. Reducing power release was followed with the method of ref. [8].

Chemicals. All chemicals were of analytical grade.

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