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ACETOHYDROXAMATE, A COMPETITIVE INHIBITOR OF ALLANTOINASES OF *VIGNA RADIATA*

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Key Word Index—*Vigna radiata*; Leguminoseae; green gram; allantoinases; nodule; root and seedling enzymes; acetohydroxamate; competitive inhibition.

Abstract—Nodulated *Vigna radiata* plants contain allantoinases in nodules and in host tissue which have similar, although not identical, properties with regard to K_m, optimum pH and the lack of a metal ion requirement. Nodules, however, exhibit a two-fold higher allantoinase specific activity than host (root) tissue. Acetohydroxamate has been found, for the first time, to be a reversible, competitive inhibitor of root as well as nodule allantoinases with a K_i of *ca* 5 mM. It is suggested that acetohydroxamate is a general inhibitor of ureide-metabolizing enzymes, inhibition being in the order allantoinase < allantoate amidohydrolase < urease. The mechanism of the inhibition varies with the enzyme involved.

INTRODUCTION

The ureides (allantoin and allantoic acid) are important storage and translocatory forms of nitrogen in nodulated legumes. The first enzyme involved in allantoin metabolism is allantoinase, which converts allantoin to allantoic acid. Legumes synthesize very active allantoinases even in the absence of nodulation and the properties of allantoinases from several legume seedlings have been examined [1–3]. More recently, it has been found that in nodulated *Arachis hypogaea* the nodules produce an apparently different allantoinase from that present in host root tissue [4].

Specific inhibitors of ureide metabolizing enzymes have not been reported, with the exception of parabanic acid, which has been found to be a specific inhibitor of nodule but not of root allantoinase in *A. hypogaea* [4].

In this context, it may be mentioned that acetohydroxamate (AHM) had been considered a specific inhibitor of urease [5, 6]. Subsequently, Shelp and Ireland [7] have shown that AHM inhibits production of ammonia and carbon dioxide from the metabolism of allantoin as well as urea, presumably by an *in vivo* inhibition of urease. Winkler *et al.* [8] have found that AHM is more non-specific than believed and that it also inhibits allantoate amidohydrolase. Kleczkowski *et al.* [9] have reported that AHM also inhibits NADPH-glyoxylate reductase. We report herein, for the first time, that AHM is a competitive inhibitor of plant as well as nodule allantoinases in seedlings of *Vigna radiata* L.

RESULTS AND DISCUSSION

Allantoinases in nodulated V. radiata

Vigna radiata plants (45-day-old) nodulated with rhizobial strain M 10 were examined for the distribution of allantoinase activity in nodules and roots. Initial experiments established that the allantoinases of nodules and roots both had a pH optimum of 7.5 although the pH activity profiles were slightly different (Fig. 1). Neither allantoinase had a demonstrable cation requirement as indicated by experiments involving either addition of EDTA or dialysis against EDTA. However, the specific activities were different. The nodule allantoinase had a specific activity (μg allantoate formed/mg protein/30 min) of 45.3 as compared with 20.5 for the root enzyme; K_m values for allantoin were 20.7 and 20.8 mM respectively for the two allantoinases. Young, 72-hr-old, *V. radiata*

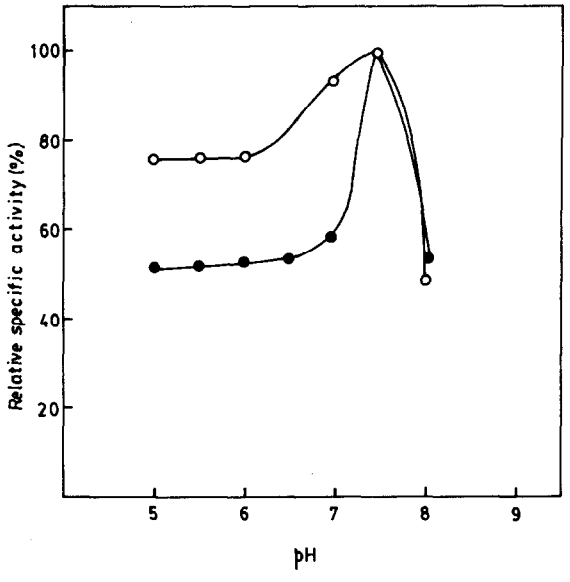

Fig. 1. Activity of *Vigna radiata* allantoinases from nodules (●—●) and roots (○—○) at various pH's. Values plotted relative to the specific activity at the optimal pH (7.5).

seedlings were also examined for their allantoinase content and the allantoinase was found to have properties similar to those of the root enzyme, except that the specific activity was 57.3, a value *ca* three times higher than that in roots of the older nodulated plants. With added metal ions, Cu^{2+} (1 mM) was found to be inhibitory to the nodule allantoinase (30% inhibition) but not to the root enzyme; the inhibition by Cu^{2+} could be fully reversed by thioglycollate (0.05–0.2 mM).

It would appear, therefore, that, unlike the situation in *A. hypogaea* [4], the allantoinases of the nodule and the host tissue are rather similar although the shape of the pH vs activity profiles and the differential effects of Cu^{2+} ions indicate some differences. The reason for these differences is not immediately apparent, although there are several likely possibilities, one of them being that several isozymes of allantoinase may be present in different plant tissues.

Effect of acetohydroxamate on allantoinases of V. radiata

In initial experiments, the effect of AHM on *V. radiata* nodule, root and seedling allantoinases was studied as a function of inhibitor concentration. The results (Fig. 2) show that there are quantitative differences in the concentration dependence between the nodule and the seedling allantoinases. Half maximal inhibition is attained at 2.5 mM AHM with the seedling allantoinase, whereas the corresponding figure is nearly three-fold higher (7.5 mM) for nodule allantoinase (although the total protein content is 3.75 and 14.25 mg/ml respectively for the nodule and seedling enzyme extracts tested). The concentration dependence of AHM inhibition in the case of root allantoinase is very similar to that of nodule allantoinase and has therefore not been indicated in Fig. 2.

The inhibition of allantoinase activity in all cases was eliminated by dialysis indicating that it is reversible. AHM inhibition could also be overcome by adjusting enzyme extracts incubated with this inhibitor to pH 4.2 followed by subsequently assaying such extracts after bringing them back to pH 7.5.

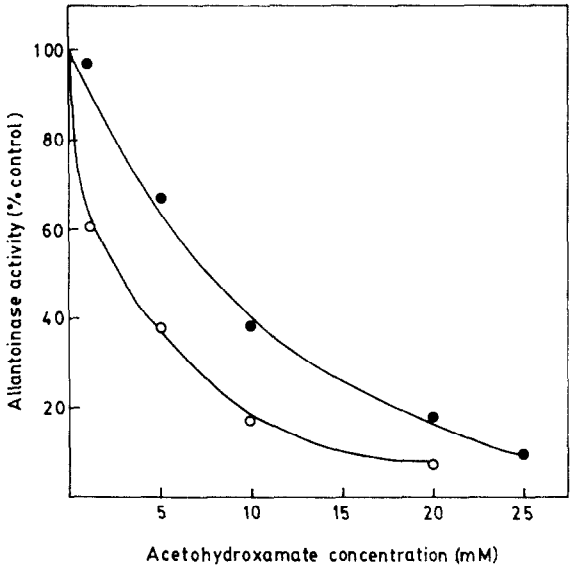

Fig. 2. Acetohydroxamate inhibition of the activity of allantoinase of nodules (●—●) and seedlings (○—○) of *V. radiata*.

Conventional double reciprocal plots of $1/V$ vs $1/S$ at different AHM concentrations in the inhibitory range showed that the inhibition was truely competitive. K_i values derived from such plots for the different allantoinases studied herein were between 5.2–5.8 mM. In the case of the nodule allantoinase, this was also the case when a partially purified (5-fold) enzyme was studied.

The most important finding from the present studies is that AHM, surprisingly, is a competitive inhibitor of plant as well as nodule allantoinases of *V. radiata*. It should be mentioned, however, that it is only moderately powerful as revealed by the K_i value of 5 mM for these enzymes whose K_m values are 20 mM. This is the first demonstration of competitive inhibition of an allantoinase by AHM.

It is of interest to compare the known features of AHM inhibition of the different enzymes considered to be involved in allantoin metabolism. Allantoin is converted to allantoate by allantoinase. For the subsequent metabolism of allantoate in plants, two different pathways have been reported [10–12]. The first of these involves allantoicase (allantoate amidinohydrolase) which cleaves allantoate to ureidoglycolate an urea. However, there is little direct evidence for allantoicase in higher plants. The alternative, (based on studies with soybeans) is an allantoate amidohydrolase-dependent breakdown of allantoate wherein urea is not an intermediate [11]. At present, it is not clear which of these is the most widespread in higher plants.

To return to the effect of AHM on allantoin metabolism, current evidence indicates that it does inhibit ammonia formation from allantoin in plants [7–9, 13]. Our data indicate that it is a competitive inhibitor of nodule as well as plant allantoinases in *V. radiata*. It also inhibits allantoate amidohydrolase with a K_i of 0.45 mM [9], and inhibits crystalline as well as crude urease from swordbean with a K_i of 2.2×10^{-7} M. On the other hand, a wide variety of enzymes of other pathways are not inhibited by AHM [5]. Taken together, the results imply that AHM specifically inhibits allantoin metabolism. At the same time, with regard to individual enzymes of the allantoin degradative pathway, the inhibition is not so specific in the sense of not being directed towards any single enzyme of the pathway. Nevertheless, it would seem to inhibit the enzymes in the order: allantoinase < allantoate amidohydrolase < urease.

Although AHM is a metal chelator [14] and according to Winkler *et al.* [13] the inhibition of allantoate amidohydrolase (a Mn^{2+} requiring enzyme) by this inhibitor is possibly explicable on the basis of its metal chelation properties, in inhibition of *V. radiata* allantoinases is evidently quite different since these enzymes are not metal dependent as indicated by our present results. Whether the powerful inhibition of urease by this inhibitor is due to its great specificity for the nickel-dependent urease is not known, but should this be the case, the observed effectiveness of AHM inhibition of the different enzymes of allantoin metabolism may still be explicable on the basis that it inhibits the metal ion dependent enzymes of this pathway more powerfully (but in characteristic fashion dependent on the metal ions involved) and the metal ion independent ones less strongly, and possibly by an altogether different mechanism.

EXPERIMENTAL

Germination. Seeds of *Vigna radiata* were purchased locally and germinated under aseptic conditions as described earlier [1, 15] in 10 ml of dist. H_2O.

Plant material. V. radiata seeds were inoculated with a specific *Rhizobium* culture (*Rhizobium* sp., Cowpea strain M 10 from A.P. Agri. University, Hyderabad) and sown in June 1987. Field-grown plants were harvested around 45 days, the nodules dissected out, freed of root tissue, washed, and stored frozen in 20% glycerol. Root tissue freed of nodules was also stored frozen till required.

Preparation of enzyme extracts. Freshly germinated seedlings were freed of seedcoats and washed extensively with H_2O. seedlings were homogenized with an equal wt of acid-washed sand [3] and ice-cold diethanolamine–HCl (DEA) buffer (0.6 M, pH 7.5, 2–3 ml/g fr. wt) in a glass mortar. Essentially similar procedures were used for extraction of allantoinase from roots as well as nodules, the latter, after washing with cold water and blotting dry.

Tissue extracts were centrifuged at 20000 *g* for 20 min, the residue re-extracted and the pooled supernatants used as a source of enzyme.

Purification of nodule allantoinase. Allantoinase was extracted as described above. Extracts were subjected to $(NH_4)_2SO_4$ fractionation. The bulk of the enzyme activity, in each case, was found in the 60–80% ppt., which was dissolved in a minimum vol. of DEA buffer (0.6 M, pH 7.5) and dialysed exhaustively against DEA buffer (0.05 M, pH 7.5) at 2°. The dialysed extract was adsorbed onto a DEAE Sephadex A-50 column (0.7 × 17 cm), equilibriated with above buffer (0.05 M, pH 7.5). On stepwise column eluation with buffer containing 0.1 and 0.5 M NaCl, the bulk of the activity was recovered in 0.5 M NaCl. Allantoinase from nodules was thereby purified 5-fold with 75% recovery. Eluates were stored frozen. The enzyme was found to be stable for up to seven days.

Enzyme assay. Reaction mixtures for allantoinase assay in DEA buffer (0.6 M, pH 7.5) contained 0–0.5 ml of enzyme extract and allantoin (50 μmol) in a total vol of 1.5 ml (Incubation was at $30 \pm 1°$ for 30 min). The reaction was terminated by the addition of 0.2 ml TCA (50%). After centrifugation, aliquots were taken from the supernatants and allantoate formed estimated as described earlier [1, 16]. Activity has been expressed as μg of allantoate formed/mg protein/30 min under the assay conditions. When pH activity profiles were to be determined, enzyme assays were conducted in DEA buffer (0.6 M) adjusted to the different pH values as required.

Enzyme extracts were exhaustively dialysed with 1 mM EDTA against DEA buffer (0.6 M, pH 7.5), and then tested for activity as well as for possible metal ion requirement. Inhibition by metal ions was examined by inclusion of metal salts in the range 0–1 mM in the assay system. In some experiments, reversal of metal ion inhibition was studied with thioglycollate (0.05–0.2 mM).

Inhibition of allantoinase by AHM was examined by adding this inhibitor at desired concn to enzyme extracts, preincubating for 15 min and then adding allantoin. In studies concerned with possible reversal of AHM inhibition, assay mixtures, prior to addition of allantoin were: (i) adjusted to pH 4.2, held for 5 min, and back adjusted to pH 7.5, or (ii) dialysed exhaustively against DEA buffer (0.6 M, pH 7.5) and assayed as above. Appropriate controls were run as required.

Chemicals. Acetohydroxamate and DEAE Sephadex A-50 were from Sigma. All metal salts used were of analar grade.

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